β-Adrenergic Signaling Inhibits Gq-Dependent Protein Kinase D Activation by Preventing Protein Kinase D Translocation

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Rationale: Both β-adrenergic receptor (β-AR) and Gq-coupled receptor (GqR) agonist–driven signaling play key roles in the events, leading up to and during cardiac dysfunction. How these stimuli interact at the level of protein kinase D (PKD), a nodal point in cardiac hypertrophic signaling, remains unclear.

Objective: To assess the spatiotemporal dynamics of PKD activation in response to β-AR signaling alone and on co-activation with GqR-agonists. This will test our hypothesis that compartmentalized PKD signaling reconciles disparate findings of PKA facilitation and inhibition of PKD activation.

Methods and Results: We report on the spatial and temporal profiles of PKD activation using green fluorescent protein-tagged PKD (wildtype or mutant S427E) and targeted fluorescence resonance energy transfer–based biosensors (D-kinase activity reporters) in adult cardiomyocytes. We find that β-AR/PKA signaling drives local nuclear activation of PKD, without preceding sarcolemmal translocation. We also discover pronounced interference of β-AR/cAMP/PKA signaling on GqR-induced translocation and activation of PKD throughout the cardiomyocyte. We attribute these effects to direct, PKA-dependent phosphorylation of PKD-S427. We also show that phosphomimetic substitution of S427 likewise impedes GqR-induced PKD translocation and activation. In neonatal myocytes, S427E inhibits GqR-evoked cell growth and expression of hypertrophic markers. Finally, we show altered S427 phosphorylation in transverse aortic constriction–induced hypertrophy.

Conclusions: β-AR signaling triggers local nuclear signaling and inhibits GqR-mediated PKD activation by preventing its intracellular translocation. PKA-dependent phosphorylation of PKD-S427 fine-tunes the PKD responsiveness to GqR-agonists, serving as a key integration point for β-adrenergic and Gq-coupled stimuli. (Circ Res. 2014;114:1398-1409.)

Key Words: cyclic AMP-dependent protein kinases ▪ GTP-binding proteins ▪ myocytes, cardiac ▪ protein kinase D ▪ receptors, adrenergic

Cardiac remodeling occurs after various types of insults to the heart, including inflammation and pressure and volume overload.1,2 Associated molecular, cellular, and interstitial changes, such as re-expression of fetal genes, myocyte hypertrophy, cell death, and fibrosis, eventually become maladaptive leading to cardiac dysfunction and failure. Therapeutic strategies, therefore, often target the signaling pathways that underlie the cardiac remodeling processes. In recent years, protein kinase D (PKD) has emerged as a key signaling nodal point affecting excitation–contraction coupling (via myofilament-binding protein C, cardiac troponin I, and L-type Ca2+ channels),3–6 gene expression (via histone deacetylases7–8 and cAMP-response element-binding protein),9 cell survival,10–12 and energy substrate use.13–15

In vivo, cardiac-specific expression of constitutively active PKD1 caused pronounced dilated cardiomyopathy,7 whereas cardiac-specific PKD1 knockout mice proved remarkably resistant to cardiac hypertrophy and fibrosis in response to pressure overload or chronic administration of adrenergic or angiotensin receptor agonists.16 Conversely, PKD inhibition decreased the tolerance to ischemia/reperfusion injury.17 PKD expression and activity are also increased in failing rat,7 rabbit, and human myocardium.18 These findings indicate the importance of PKD in modulating cardiac

1398

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pathophysiology and its potential as a therapeutic target for cardiovascular disease.

Three highly homologous PKD isoforms have been identified (PKD1–3, with PKD1 predominant in the heart).27,28 PKDs possess substrate specificity similar to calmodulin-dependent protein kinase and structural features reminiscent of PKCs. Indeed the modular structure of PKD consists of a C-terminal catalytic domain and an N-terminal region with autoinhibitory, regulatory domains, such as the diacylglycerol-binding domain and the pleckstrin homology domain.20,21 Several regulatory mechanisms (eg,22 for comprehensive review) can modulate PKD1 activity, including autoinhibition, phosphorylation, proteolytic degradation, subcellular localization, and protein interactions, resulting in complex, context-specific PKD signaling mechanisms.

The typical signaling cascade to PKD activation involves G-protein–coupled receptor–dependent activation of phospholipase C and production of diacylglycerols. The latter stimulate PKD directly and indirectly via coincident activation of PKCs, while recruiting PKD near its upstream kinase. The activation is largely dependent on phosphorylation of the activation loop residues (S744/S748 in murine numbering) by both PKC-dependent and PKC-independent mechanisms.23–26 The activation is largely dependent on phosphorylation of the activation loop residues (S744/S748 in murine numbering) by both PKC-dependent and PKC-independent mechanisms.23–26

Despite this general consensus on global PKD activation for 2 Gq-coupled agonists, phenylephrine and endothelin-1 (ET1), in adult cardiomyocytes. Although both agonists triggered comparable global PKD1 activation, phenylephrine induced fleeting sarcolemmal PKD1 translocation and activation followed by nuclear translocation and ET1 prompted persistent sarcolemmal recruitment and activity. The ability to alter the location and signal generation of PKD1 dynamically is clearly an integral part of achieving PKD signal specificity, yet this highly contextual and microdomain aspect of PKD signaling remains poorly understood.

Surprisingly, little is known about the effect of β-adrenergic receptor (β-AR)/PKA signaling on PKD function and activation in cardiomyocytes and the few existing reports are conflicting. Initial reports from the Olson group saw no effect of β-AR or PKA stimulation on PKD phosphorylation or activation.27 Carnegie et al28–31 later found that A-kinase anchoring protein (AKAP)-Lbc functions as a scaffold for PKA and PKC, facilitating PKD activation and the transduction of hypertrophic responses (via histone deacetylase 5 and MeT2 [myocyte enhancer factor-2]). Others, examining global PKD activity, report a counter modular role for PKA and PKC on PKD activation in adult myocytes,32–34 which is an inhibitory role for PKA.

In the present study, we resolve these conflicting findings by examining local PKD translocation and activation in response to PKA signaling alone and the crosstalk between these 2 signaling kinases in adult cardiac myocytes. We find that β-AR/PKA signaling drives nuclear activation of PKD without requiring a sarcolemmal recruitment phase. β-AR/PKA signaling also prevents Gq-coupled receptor (GqR)–induced translocation, activation, and function of PKD, and we show that these effects are mediated by PKA-dependent phosphorylation of PKD at S427. Moreover, initial observations in a transverse aortic constriction (TAC)–induced hypertrophy model indicate altered phosphorylation of this S427 site. Our study provides further evidence of compartmentalized PKD signaling and indicates how S427 could serve as a critical control point for modulation of the spatiotemporal dynamics of PKD activity and function during the pathogenesis of cardiac disease.

Methods

Cardiomyocyte Isolation and Culture

All animal and biohazard procedures were conducted in accordance with National Institutes of Health guidelines for animal research and with institutional approval. Biohazard procedures were performed in accordance with a University of California, Davis–approved Biological Use Authorization. Adult myocytes were isolated and cultured as previously described.27,28 Neonatal rat cardiomyocytes (NCMs) were cultured as instructed. Green fluorescent protein (GFP)-PKD-wildtype (WT) and GFP-PKD-S427E expressions were via adenoviral infection. Before experiments, myocytes were serum deprived for 24 hours by switching to DMEM with Nutridoma-SP.

Confocal, Fluorescence Resonance Energy Transfer, and Total Internal Reflection

Fluorescence Measurements

All confocal and total internal reflection fluorescence (TIRF) experiments were performed as previously reported.27 Myocytes were exposed to inhibitors for 15 to 20 minutes before and after the addition of agonists. Fluorescence resonance energy transfer was measured27 using both a ratiometric and an acceptor photobleach approach. Background-corrected fluorescence intensities at specific cellular locations were reported as a ratio of F_{CIFP}/F_{FYP} (normalized to initial ratio).

Immunoblotting

Cell lysates and subcellular fractions were obtained as previously described27,28 and probed with Cell Signaling PKD1, PKDpS916, phospho-PKA-substrate (RRXS/*T*+), phospho-Akt substrate (RXXS/*T*+), phospho-PKD-substrate and histone 3; Abcam GAPDH, PKDpS916, PKA, and a custom PKD pS427 antibody; Santa Cruz Biotechnology GFP and from Millipore NKA. Immunoreactive band intensities were quantified using ImageJ.

Immunoprecipitation

Immunoprecipitation was performed as before30 using anti-GFP, PKD, or PKARI1 (protein kinase A regulatory subunit II) antibody at 4°C overnight followed by incubation with protein A/G-coupled magnetic beads for 2 hours before resuspending in sample buffer.

Sequence Alignment

Sequence alignment and analysis were performed using Geneious version 6.0.4 by Biomatters (http://www.geneious.com/).

Cellular Hypertrophy

NCMs were treated as indicated before subjecting to standard immunocytochemistry and reverse transcriptase-polymerase chain reaction of hypertrophic markers. Fixed NCMs were stained for α-actinin (Sigma, 1:1000). Cell size measurements were made

<table>
<thead>
<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
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<tr>
<td>β-AR</td>
<td>β-adrenergic receptor</td>
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<tr>
<td>DKKAR</td>
<td>D-kinase activity reporter</td>
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<tr>
<td>ET1</td>
<td>endothelin-1</td>
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<tr>
<td>GqR</td>
<td>Gq-coupled receptor</td>
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<td>NCMs</td>
<td>neonatal rat cardiomyocytes</td>
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<td>TIRF</td>
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using ImageJ. Total RNA was isolated using TRIZOL Reagent and Nucleospin RNA II, followed by quantification using Nanodrop and then Quant-iT RiboGreen. cDNA was generated from RNA with RNA-to-cDNA-EcoDry-Premix and then amplified with GoTaq qPCR MasterMix. Custom primers (Illuema) were used for detection of each mRNA—control genes: Hmbs, Rpl13a, Hprt1, Myh6, Myh7, Acta1, Nppb, and Nppa. The threshold crossing value was noted for each transcript and normalized to internal control. The relative quantification of each was performed using the comparative Ct method.

TAC Surgery
All procedures were essentially as described.38 TAC was performed on 8-week-old male mice around a 27-gauge needle. Sham-operated mice were subjected to identical interventions except for aortic constriction. Cardiac dysfunction was measured 3 weeks later via echocardiography. The mice were euthanized, and hearts were harvested. For cardiac homogenates, the heart was powdered and resuspended in ice-cold buffer containing (in mmol/L): 10 Na2HPO4, 150 NaCl, 5 EGTA, 5 EDTA, 5 NaF, 1% orthovannadate with 1% TritonX-100, 1% Na deoxycholate, and protease and phosphatase inhibitors. After homogenization with polytron at 5000 rpm for 20 s and 5 strokes of a Dounce homogenizer, samples were centrifuged at 5000g. Supernatants were flash-frozen.

Statistical Analysis
Pooled data are expressed as mean±SEM. Statistical discriminations were performed with Student t test and 2-way ANOVA with P<0.05 considered significant. Expanded Methods can be found in the Online Data Supplement.

Results
β-AR/PKA Drives Noncanonical PKD Translocation
Initial experiments focused on establishing whether β-AR or PKA activation alone affects PKD signaling and localization. Adult cardiomyocytes expressing GFP-PKD1 (after adenovirus infection) were monitored via confocal microscopy after 100 mmol/L isoproterenol or 10 μmol/L forskolin exposure (Figure 1A). Quantification of the signal at the sarcolemma versus that in the cytosol (F_{nuc}/F_{cyto}) showed that neither isoproterenol nor forskolin induced sarcolemmal recruitment of PKD (Figure 1B). This is in contrast to the pronounced sarcolemmal recruitment of PKD, we previously observed in response to the GqR agonists: phenylephrine and ET1.28 Notably, even without a preceding sarcolemmal translocation phase, both isoproterenol and forskolin triggered nuclear import of PKD (after 60 minutes, F_{nuc}/F_{cyto} was increased by 12±2% and 13±2%, respectively; F_{nuc} contains both intranuclear and nuclear membrane fluorescence intensities). This nuclear import was also readily detectable in population studies of PKD distribution in response to isoproterenol (ie, unpaired measurements of F_{nuc}/F_{cyto}, Figure 1C).

To measure sarcolemmal recruitment of PKD more selectively, we also used TIRF microscopy to assess fluorescence only within ≈100 nm of the glass coverslip–solution interface (Figure 1D). As in the confocal experiments, neither the β-AR agonist isoproterenol nor the direct activation of adenyl cyclase by forskolin induced significant sarcolemmal recruitment of PKD. This suggests that β-AR/PKA activation triggers a translocation pattern that deviates from the classical PKD activation pattern of a membrane recruitment phase followed by translocation to subcellular targets, such as the nucleus.

The lack of an effect on global PKD autophosphorylation status may be because of the relatively minor contribution of nuclear PKD to that of the whole cell (estimated to be <3% of total PKD based on subcellular compartment estimates of total cell volume). So, fractionated mouse hearts (± isoproterenol) were analyzed for PKD activation (via pS916 signals) and phosphorylation of PKD targets (using a PKD consensus motif antibody). Although S916 and PKD target phosphorylation signals were slightly decreased in the cytosolic and sarcolemmal fractions, they were increased in the nuclear compartment (Online Figure I). These biochemical observations confirm that β-AR signaling stimulates local, nuclear PKD signaling.

Having established the effect on PKD of β-AR/PKA signaling alone, we set out to determine how this pathway affects signaling through GqR/PKD pathways (Figure 2B). We found that preactivation of PKA by forskolin prevented the increase in S916 phosphorylation induced by ET1 and phenylephrine. In contrast, forskolin pretreatment failed to prevent S916 autophosphorylation in response to phorbol 12,13-dibutyrate. These results indicate that PKA-dependent signaling suppresses global PKD activation in cardiomyocytes in response to isoproterenol or forskolin. Phosphorylation of the PKD-specific substrate sequence in this biosensor results in a decrease in fluorescence resonance energy transfer (ie, an increase in cyan fluorescent protein/yellow fluorescent protein ratio).39 Although the DKAR dynamic range is small (≈10%), they are able to detect spatiotemporal differences in PKD activation.28 We found that exposure of adult cardiac myocytes to isoproterenol or forskolin failed to elicit any PKD activation at the sarcolemma or cytosol, measured with DKAR-MyrP (poly-myristoylated) and DKAR-NES (nuclear exclusion signal), respectively (Figure 2A). However, both isoproterenol and forskolin activated nuclear PKD (Figure 2A). The effect was more potent for forskolin, reflecting the more robust PKA activation. We confirmed these results using the acceptor photobleach method (not shown). These observations support the notion that PKA activation induces nuclear translocation and activation of PKD without a preceding sarcolemmal recruitment and activation phase.

Using genetically encoded fluorescence resonance energy transfer–based D-kinase activity reporter (DKAR), we previously showed that the triggered PKD translocation parallels the evoked local kinase activity.28 Because of the possibility that PKD may be activated in the absence of previous sarcolemmal translocation, we used targeted DKARs (to nucleus, cytosol, or sarcolemma) to monitor the time course and magnitude of PKD activation selectively in response to isoproterenol or forskolin. Phosphorylation of the PKD-specific substrate sequence in this biosensor results in a decrease in fluorescence resonance energy transfer (ie, an increase in cyan fluorescent protein/yellow fluorescent protein ratio).39 Although the DKAR dynamic range is small (≈10%), they are able to detect spatiotemporal differences in PKD activation.28
response to G R-agonists, at least as sensed by S916 phosphorylation. Potent stimuli, such as the diacylglycerol analogue phorbol 12,13-dibutyrate, are still able to overcome this inhibition.

**β-AR/PKA Negatively Modulates Gq-Driven PKD Translocation**

Clearly, there are subcellular, compartmentalized responses to PKA- and G R-dependent activation of PKD. To assess whether micromdomain signaling plays a role in this PKA-dependent modulation of G q-coupled PKD activation, we examined PKD-GFP translocation in adult cardiomyocytes. Consistent with previous observations, confocal imaging revealed pronounced sarcolemmal translocation in response to phenylephrine and ET1 (stronger and sustained for ET1), followed by nuclear translocation, which is more rapid and prominent for phenylephrine. Isoproterenol pretreatment prevented both the ET1- and the phenylephrine-induced PKD translocation to the sarcolemma (Figure 3B, left). F nuc/F cyto at 60 minutes remained at baseline for isoproterenol-pretreated myocytes versus an increase of 9±1% and 31±4% for phenylephrine and ET1 alone. The G R-agonist–mediated nuclear import of PKD was also blocked by preincubation with isoproterenol (Figure 3B, right). The phenylephrine-triggered increase in F nuc/F cyto ratio after 60 minutes was only 1±2% in isoproterenol-pretreated myocytes (versus 18±2% for phenylephrine alone). Likewise, the increase in F nuc/F cyto ratio was only 2±1% after 60 minutes of ET1 in isoproterenol-pretreated cells (13±3%). Of note, the isoproterenol-induced nuclear import had already occurred within the 15-minute pretreatment period (an ≈10% increase). This higher, new baseline (reset to 1) was used to estimate the G R.

To corroborate our confocal findings, we used TIRF microscopy to dissect the isoproterenol-agonist signaling cascade, leading to alterations in phenylephrine/ET1-induced PKD translocation. Our TIRF measurements confirmed that β-AR activation with isoproterenol (15-minute pretreatment) prevents ET1-induced sarcolemmal recruitment of PKD in adult myocytes (Figure 3C, top). Elevating cAMP levels with forskolin to activate PKA more directly also blocked the ET1 effects. Consistent with these observations, preincubation with 8-Rp-Br-cAMP, a cell-permeable cAMP analogue, which specifically blocks PKA activation, prevented the isoproterenol- and forskolin-dependent inhibitory effects on ET1-driven PKD translocation (Figure 3C). Similar results were obtained for phenylephrine (Online Figure II), even though the phenylephrine-induced sarcolemma retention of PKD is lower than for ET1. Taken together, these data indicate that signaling through a β-AR/cAMP/PKA pathway negatively regulates G R-agonist–evoked PKD translocation.

**Figure 1.** β-Adrenergic receptor/protein kinase A (PKA) activation triggers nonclassical PKD translocation.

A, Confocal images of rabbit ventricular myocytes expressing green fluorescent protein (GFP)-tagged PKD1 before and after 20 minutes of 10 μmol/L forskolin (Forsk; left) or 100 nmol/L isoproterenol (ISO; right) treatment. B, PKD-GFP localization was analyzed as F nuc/F cyto and F sarcolemmal (SL) recruitment and nuclear import, respectively (n>13 myocytes; *P<0.001). C, Population analysis of ISO-triggered nuclear import of GFP-PKD1: confocal images (left) and quantification as F nuc/F cyto (right). D, Total internal reflection fluorescence (TIRF) analysis of agonist-dependent sarcolemmal recruitment of GFP-PKD1. Representative TIRF images in response to endothelin-1 (ET1) or ISO are on the left. Quantification of GFP-PKD1 TIRF signals is shown on the right (n>10 myocytes). Ctl indicates control.
β-AR Signaling Negatively Modulates Local PKD Activity

Targeted DKARs (Figure 3D) were used in parallel studies to test how GqR-agonists affect the spatiotemporal dynamics of PKD activation after β-AR stimulation. Although ET1 and phenylephrine triggered comparable sarcolemmal PKD activity, isoproterenol pretreatment blocked ET1- or phenylephrine-induced PKD activation at the sarcolemma (Figure 3D, top). Phenylephrine and ET1 also triggered nuclear PKD activation although to a much greater extent for phenylephrine. Isoproterenol pretreatment again effectively reduced PKD activation in the nuclear compartment in response to phenylephrine or ET1 (Figure 3D, bottom). Collectively, our data show that β-AR/cAMP/PKA signaling profoundly perturbs GqR-agonist–induced PKD activation and translocation in adult cardiomyocytes. Parallel subcellular fractionation experiments and probing for activated PKD (pS916) support these findings (Online Figure III). As shown for the sarcolemmal compartment, phenylephrine- and ET1-induced increase in signal is prevented by isoproterenol pretreatment.

PKA-Dependent Phosphorylation of PKD

To date, it was unclear whether the disruption of GqR-induced PKD signaling is mediated via direct PKA phosphorylation of PKD or other PKA-mediated effects. So we used 2 separate PKA-consensus-motif antibodies (pRRXS*/T* and pRXS*/T*) to test whether PKA can directly phosphorylate PKD. Western blots of purified PKD1 phosphorylated in vitro with PKA show increased immunoreactivity with both PKA-consensus-motif antibodies (Figure 4A). H89 inhibition of PKA strongly reduced the signals, whereas PKD inhibition with CID755673 had little effect. Thus, PKD is a PKA substrate. Moreover, PKD can be communoprecipitated with the PKA regulatory subunit (Online Figure IV) in myocyte lysates. So we tested whether PKD is a PKA target in vivo. Myocytes expressing GFP-PKD were treated with isoproterenol, forskolin, or vehicle before PKD immunoprecipitation using GFP antibody and protein A/G magnetic beads. Figure 4B shows a representative immunoprecipitation probed with pRRXS*/T* and GFP antibodies. The GFP panel shows that equivalent amounts of PKD were immunoprecipitated, and the control immunoprecipitations, using beads or control immunoglobulin G (Figure 4C), show the specificity of the pull-down. Isoproterenol and forskolin treatment increased the pRRXS*/T* signal in the myocyte lysate (preimmunoprecipitation lanes), indicating increased PKA phosphorylation. The pRRXS*/T* signal in immunoprecipitated PKD was also increased, indicating PKA phosphorylates PKD.

Consensus PKA sites were subsequently identified throughout PKD1 with the Scansite program (http://scansite.mit.edu) to further the notion of direct PKA phosphorylation of PKD. PKD contains several potential PKA substrate sequences (Figure 4D), including several known auto- and transphosphorylation sites (S916, S255, S748, and S203). Among these, the sequence surrounding S426 to S427 is of particular interest because either serine residue could serve as phosphoryceptor, the sequence is entirely conserved among vertebrate species. In addition, S427 was recently identified in vitro as a potential PKA target although no link to changes in PKD localization or catalytic activity was reported.40

PKD-S427E Mimics the β-AR Inhibition of GqR-Induced PKD Translocation

We speculated that direct phosphorylation of PKD by PKA at this site alters PKD translocation. We used confocal microscopy (Figure 5A) to test whether a phosphomimetic substitution at S427 (PKD-S427E) would similarly perturb GqR-induced PKD translocation. At baseline, this mutant had a slightly higher nuclear localization (Fnuc/Fcyto was 0.69±0.02 versus 0.62±0.02 for WT myocytes) but otherwise targeted like WT (Online Figure V). Our previous findings indicate a predominantly

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** β-Adrenergic receptor/protein kinase A (PKA) trigger nuclear activation of PKD. **A**, PKD activity measurements with targeted D-kinase activity reporter (DKAR) constructs in sarcolemmal, cytosol, and nuclear compartments (n=30 myocytes; *P*<0.01). **B**, Representative Western blot and quantification of S916 phosphorylation (=global PKD activity) in response to agonist treatment. GAPDH and PKD signals serve as loading controls (Ctl). ET1 indicates endothelin-1; FSK/FSK, forskolin; ISO, isoproterenol; MyrP, poly-myristoylated; NLS, nuclear localization signal; NES, nuclear exclusion signal; PKD, protein kinase D; PDBu, phorbol 12,13-dibutyrate.
Nichols et al  

β-AR Modulation of PKD Activation

sarcolemmal and nuclear response for ET1 and phenylephrine, respectively, so we focused our confocal analysis on the relevant compartment for each agonist. Adult myocytes expressing GFP-tagged PKD-S427E displayed severely blunted sarcolemmal recruitment in response to ET1 (versus PKD-WT; Figure 5A and 5B). ET1 application (for 60 minutes) increased \( F_{\text{SL}}/F_{\text{cyo}} \) by only 7±2% for the S427E mutant versus 31±4% for PKD-WT. Similarly, phenylephrine-triggered nuclear import was drastically reduced in PKD-S427E expressing myocytes versus WT. \( F_{\text{nuc}}/F_{\text{cyo}} \) after 60 minutes of phenylephrine was only 2±2% for PKD-S427E versus 18±2% for PKD-WT. Parallel TIRF experiments showed even more clearly that S427E substitution resulted in decreased membrane recruitment for both GqR-agonists (Figure 5C). Also of note, after isoproterenol treatment, \( F_{\text{cyo}}/F_{\text{cyo}} \) was increased (from 0.69±0.02 to 0.77±0.02, similar to WT; Online Figure V), indicating that the β-AR–induced nuclear import of PKD does not depend on S427. However, S427E substitution does recapitulate the PKA-mediated disruption of GqR-induced PKD translocation. Moreover, pS916 measurements show that like isoproterenol pretreatment (Figure 2B), S427E substitution renders PKD resistant to GqR stimuli but not to robust activation with phorbol 12,13-dibutyrate (Online Figure VI). Together, our data indicate that S427 mediates the PKA-dependent suppression of GqR-induced PKD activation and translocation.

**PKD-S427 Mediates the β-AR Effects on Downstream GqR-PKD Signaling**

We speculated that S427E substitution would also affect downstream GqR-induced PKD effects. So in light of the well-characterized role of PKD as a histone deacetylase kinase, we examined the effect of S427E substitution on cardiomyocyte hypertrophy. NCMs were infected with GFP-PKD-S427E, GFP-PKD-S427A, or GFP-PKD-WT. As shown in Figure 6A, the expression of PKD-S427E prevented sarcomere assembly and cell enlargement in response to phenylephrine or ET1, whereas S427A responded similar to WT. Cardiac hypertrophy is associated with reactivation of a pathological fetal gene program, such as genes encoding for atrial natriuretic peptide, brain natriuretic peptide, and atrial natriuretic peptide.

**A Confocal**

![Confocal images](image)

**B** Analysis of GFP-PKD1 localization shows ISO pretreatment blocks both phenylephrine (PE)-induced and ET1-induced sarcolemmal (SL) recruitment (left) and nuclear import (right; \( n=11–30; *P<0.05; \) SL recruitment for PE and ET1 was also significantly different). **C** Total internal reflection fluorescence (TIRF) measurements of PKD sarcolemmal recruitment in response to GqR agonists±pretreatment with ISO or forskolin (FSK; top). **Bottom** PKA inhibition with Rp-8Br-cAMP prevents the ISO and FSK effect (\( n>5 \) myocytes). **D** PKD activity measurements with targeted D-kinase activity reporter (DKAR) constructs in response to GqR agonists±pretreatment with ISO (\( n=30 \) myocytes; \( *P<0.001 \)). NLS indicates nuclear localization signal; WT, wildtype.
α-skeletal actin. Agonist-dependent elevation of hypertrophy markers can be assessed by reverse transcriptase-polymerase chain reaction. Figure 6B shows that isoproterenol blunts phenylephrine-dependent induction of α-skeletal actin expression (left) in NCMs expressing PKD-WT. α-skeletal actin transcript induction by phenylephrine was likewise markedly reduced in the presence of PKD-S427E, indicating that S427E substitution recapitulates the β-AR effects on downstream GqR-evoked PKD signaling. Similar results were obtained for atrial natriuretic peptide, brain natriuretic peptide, and myosin 7 (Figure 6B, right; Online Figure VII). In addition, isoproterenol was unable to suppress phenylephrine-dependent gene expression for the unphosphorylatable S427A mutant (Figure 6B).

Altered S427 Phosphorylation in Cardiac Disease

These data indicate that S427 is a key regulatory site on PKD, which can render the kinase resistant to GqR stimuli. Given the role of PKD in cardiac hypertrophy and heart failure development, it is therefore tempting to speculate that the phosphorylation state of this PKD control point would also change during the pathogenesis of heart failure. So we developed a custom antibody to monitor S427 phosphorylation. We verified antibody specificity using unphosphorylatable GFP-PKD1 constructs in human embryonic kidney cells. Figure 6C shows that immunoreactivity increased after forskolin treatment for WT and S426A but not for S427 and S5426/427AA, indicating this novel antibody specifically detects phosphorylation at S427. This antibody signal also increased in lysates from isoproterenol- or forskolin-treated myocytes but not in the presence of PKA inhibitors H89 or 8-Br-Rp-cAMP (Figure 6D). Subcellular fractionation experiments found that in response to isoproterenol, PKD pS427 signals were increased throughout the cell but particularly in the sarcolemmal compartment (Online Figure VIII). These findings confirm that isoproterenol triggers PKA-dependent phosphorylation of S427. We then probed S427 phosphorylation in cardiac homogenates from 3-week TAC mice versus their Sham controls. Echocardiography confirmed that banded mice had significant alterations in structural and functional cardiac parameters (Online Figure IX). Figure 6E shows that S427 phosphorylation, PKD1 expression, and S427-phosphorylated...
PKD1 were all increased in TAC versus Sham. These observations suggest that S427 phosphorylation could be modulated during the development of cardiac hypertrophy and heart failure and deserves further investigation.

**Discussion**

Here, we show for the first time that β-AR/PKA modulation triggers atypical PKD activation by inducing its nuclear activation without preceding sarcolemmal translocation. We also demonstrate that important crosstalk exists between GqR and β-AR signaling and uncover the effects of β-AR signaling on spatiotemporal regulation of PKD. Furthermore, we identify S427 as a novel regulatory phosphorylation site on PKD with a key role in tuning the spatiotemporal dynamics of PKD activity and downstream functional effects. Our study highlights the importance of local activation and regulation for a multifunctional kinase, such as PKD, to achieve its compartmentalized roles and substrate specificity.

Our data reconcile previous opposing findings of PKA-dependent facilitation and inhibition of PKD signaling. We find here that β-AR/PKA activation of PKD selectively drives nuclear PKD recruitment and activation without...
initial translocation to the sarcolemma (Figure 1). This differs from the stereotypical activation mechanism. This effect was not detectable with in vitro studies, examining global PKD activation via phosphorylation alone (pS916 measurements). This is consistent with previous work using immunoblotting that found no effect of β-AR/PKA stimulation alone on PKD activity. The critical advantage of our approach is that it permits in situ monitoring of subtle differences in PKD activity within specific subcellular domains, such as the nucleus. Our observation of local, nuclear PKD activation in response to β-AR/PKA signaling fits with reports from the Scott Laboratory that PKA activation amplifies PKD signaling. They proposed that AKAP-Lbc serves as a platform synchronizing PKA and PKC activities, thereby facilitating the release of activated PKD and downstream transcriptional activation. The increase in nuclear PKD (and its activity; Figure 7, left) could originate from this AKAP-Lbc–bound pool of PKD or from another perinuclear AKAP pool (eg, mAKAP [muscle A-kinase anchoring protein]). Increased PKA activity would trigger the release of activated PKD from this scaffold, permitting its movement to the nucleus without previous sarcolemmal recruitment (in this scenario PKA-dependent phosphorylation of S427 does not play a role). Therefore, specific stimuli could engage only a subset of PKD signaling pathway components that are spatially restricted to particular subcellular domains, allowing for precise fine-tuning of the functional outcome. The type of data we have obtained should lend valuable insight into the complexities of spatial and temporal regulation of PKD activity.

Aside from inducing local, nuclear PKD signaling, β-AR/PKA stimulation was also found to prevent GqR-agonist–induced...
translocation and subsequent PKD activation. Our working hypothesis is that these divergent effects are achieved by modulating distinct pools of PKD in the myocyte (Figure 7), highlighting the importance of precise spatial control of PKD activity. In addition, we provide strong evidence that PKA-dependent phosphorylation of PKD at S427 is critical to β-AR-dependent modulation of GqR-induced PKD activation and function. This is because the phosphomimetic substitution (PKD-S427E) dramatically reduces GqR-induced PKD translocation and cardiac hypertrophy (Figures 5 and 6). Therefore, our data resolve a long-standing question of how this striking β-AR inhibition of PKD activation by GqR-agonists occurs. Two other hypotheses had been advanced to date. First, β-AR-dependent modulation of phospholipase C could limit GqR-induced PKD activation (but see also Stangherlin et al42).33,43–45 Second, PKA stimulation of PP2a activity may abrogate PKD signaling (by terminating activating phosphorylations).46 We do not exclude that additional mechanisms (such as phosphatases) remain to be determined, but this site offers intriguing possibilities for modulation of PKD function and cellular phenotype during the pathogenesis of cardiac disease.

In conclusion, we used a fluorescence-centered approach to profile spatial and temporal changes in PKD activation in response to β-AR signaling. This allowed us to visualize local nuclear PKD signaling, undetectable in global PKD measurements. This approach also revealed that the hampered interactions and autoinhibitory effects. Indeed, a previous study found that PKA could directly phosphorylate S427 in vitro, but to date no functional consequences (on localization or activity) had been identified.40 Our experiments firmly establish the S427 site as a key regulatory site of PKD signaling, providing a mechanism to integrate inputs from diverse inciting stimuli: the β-AR and GqR signaling pathways. In this scenario, slight changes in PKA activity fine-tune the PKD responsiveness to other stimuli (and possibly which downstream targets are affected). Whether S427 phosphorylation is governed by other positive and negative regulators (kinases/phosphatases) remains to be determined, but this site offers intriguing possibilities for modulation of PKD function and cellular phenotype during the pathogenesis of cardiac disease.

Considering the effect of β-AR signaling in the fight-or-flight response and its maladaptive changes in heart failure, the PKA-dependent modulation of PKD signaling efficiency and specificity is likely to have a prominent pathophysiological role. Clearly, additional experiments are needed detailing the (β-AR dependent) modulation of the S427 site versus alterations in spatiotemporal regulation and function of PKD during different stages of heart failure development. β1-AR desensitization and attenuation of its downstream signaling could reverse the negative modulation of PKD, contributing to the enhanced PKD activity observed in heart failure.18 Other factors, such as AKAP expression, could influence the topography of PKD signaling (in time and space) in heart failure. Topographical analysis of PKD activity will be a key to unraveling the subcellular mechanisms that drive PKD dysfunction in cardiac disease.

In the present study demonstrates that PKA-mediated phosphorylation of PKD at S427 is the principal mechanism of β-AR/PKA inhibition of GqR/PKD signaling. Phosphoproteomics has identified a multitude of phosphorylation sites on PKD, but only a few have known functional consequences.22 Interestingly, the predicted PKA target sites on PKD (Figure 5) included known phosphosites, such as S748 and S916. However, phosphorylation at these sites would be expected to activate rather than to inhibit PKD activity, and we did not detect PKA-dependent phosphorylation at these sites in myocyte lysates (Figure 2B).33 Sites S205 and S255 were also less likely based on their known functional effects.11,47,48 The most interesting candidate was the S427 site just adjacent to the pleckstrin homology domain (ie, in the position to influence pleckstrin homology–mediated protein

Figure 7. Working hypothesis of β-Adrenergic receptor/protein kinase A (PKA) modulation of PKD signaling. For a first pool of PKD, β-AR/PKA activation triggers nuclear PKD activity by facilitating activation of PKD at an A-kinase anchoring protein (AKAP) scaffold (cyan circle indicates activated PKD). For a second pool of PKD, β-AR/PKA activation prevents Gα-coupled receptor (GqR)-dependent PKD activation and translocation primarily by PKA-dependent phosphorylation of PKD at S427E. We do not exclude that additional mechanisms (such as phospholipase C [PLC] inhibition or PP2A activation) could contribute to this inhibitory effect of PKA. AC indicates adenylyl cyclase; DAG, diacylglycerol; PE, phenylephrine; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; and PP2A, protein phosphatase 2.
G-R-induced PKD activation after β-AR stimulation is because of profound perturbations in PKD translocation. PKA-dependent phosphorylation of PKD-S427 was found to mediate the perturbation of G-R-induced PKD translocation and function. These findings emphasize the importance of stimuli integration and spatial segregation of PKD in achieving signaling specificity for such a multifunctional kinase. Future studies examining how desensitized β-AR signaling in heart failure contributes to PKD dysregulation will clarify the role of PKD in cardiac pathophysiology and its potential as a therapeutic target.

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Disclosures

None.

References

β-AR Modulation of PKD Activation

**Novelty and Significance**

**What Is Known?**

- Protein kinase D1 (PKD1) functions as a key transducer of stress stimuli in the events leading up to and during cardiac dysfunction.
- Hypertrophic, Gq-coupled receptor agonists (GqR), such as endothelin and phenylephrine, robustly activate PKD1.
- β-adrenergic (β-AR) signaling through the cAMP/PKA cascade leads to both cardiac protection and cardiac dysfunction.

**What New Information Does This Article Contribute?**

- β-AR signaling prevents GqR-driven PKD1 translocation and subsequent activation.
- PKA phosphorylates PKD1 at S427, which mediates the β-adrenergic suppressive effects on GqR-triggered PKD1 signaling.
- β-AR signaling also selectively induces local nuclear PKD signaling independent of PKD-S427 phosphorylation.
- PKD-S427 phosphorylation is altered during transverse aortic constriction-induced hypertrophy.

PKD1 is a stress-responsive kinase with a key role in pathological cardiac remodeling via phosphorylation of class II histone deacetylases and subsequent activation of MEF2 (myocyte enhancer factor-2) target genes. It is also well established that β-AR signaling, as the prime modulator of cardiac contractility and heart rate, is critical for normal and diseased heart function, and its dysregulation is a hallmark of heart failure. How β-AR signaling affects PKD1 is controversial. Using a unique fluorescence-based approach, our study reconciles these conflicting reports by identifying distinct microdomains of PKD1 signaling in response to β-AR. That is, β-AR triggered local nuclear PKD1 signaling (ie, activation) and suppressed GqR-induced PKD1 activation and signaling. The latter effects were mediated via PKA-dependent phosphorylation of PKD1 at S427. Our study identifies PKD1 as a key integration point of the GqR and β-AR signaling pathways. It also identifies S427 as an important regulatory site for rest-potentiation of twitches in rabbit and rat ventricular myocytes.

**References**

β-Adrenergic Signaling Inhibits G<sub>q</sub>-Dependent Protein Kinase D Activation by Preventing Protein Kinase D Translocation

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Correction to: β-Adrenergic Signaling Inhibits Gq-Dependent Protein Kinase D Activation by Preventing Protein Kinase D Translocation and Multimodal SHG-2PF Imaging of Microdomain Ca\(^{2+}\)-Contraction Coupling in Live Cardiac Myocytes


In both articles, the author Brittani Wood has been changed to Brent M. Wood.

These corrections have been made to the current online version of the articles, which are available at http://circres.ahajournals.org/content/114/9/1398 and http://circres.ahajournals.org/content/118/2/e19, respectively.
Supplemental Material

Detailed Materials and Methods

Construct Generation

The GFP tagged PKD1 mutant were generated using the QuickchangeXL Mutagenesis kit (Agilent) and the following primers (IDT): for S427E 5'-cacacgaagcggagcagctgtgtgaaggaagg-3' and 5'-ccttcctccacctatgctctccttcgccttg-3', for S427A 5'-cacacgaagcggagcagctgtgtgaaggaagg-3' and 5'-ttctccacctatgctctccttcgccttg-3', for S426A 5'-cacacgaagcggagcagctgtgtgaaggaagg-3' and 5'-ttcatcacagtgcgcttccttccttg-3' and for SS426/427AA 5'-cacacgaagcggagcagctgtgtgaaggaagg-3' and 5'-ttcatcacagtgcgcttccttccttg-3'. The mutations were verified by sequencing of the entire GFP-PKD1 construct before subcloning into pshuttleCMV vector and adenovirus generation (per the manufacturer's instruction, Adeasy system, Agilent).

Myocytes Isolation and culture

All animal procedures were conducted in compliance with the NIH guidelines for animal research and approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Biohazard procedures were performed in accordance with a UC Davis approved Biological Use Authorization. Rabbit ventricular myocytes were isolated from 3-4 month old adult male New Zealand white rabbit (Charles River Laboratories International, Inc., MA, USA) as previously described. All animal procedures were conducted in compliance with the NIH guidelines for animal research and approved by the Institutional Animal Care and Use Committee at the University of California, Davis. Biohazard procedures were performed in accordance with a UC Davis approved Biological Use Authorization. Rabbit ventricular myocytes were isolated from 3-4 month old adult male New Zealand white rabbit (Charles River Laboratories International, Inc., MA, USA) as previously described. The rabbits were euthanized with an overdose of pentobarbital solution (80 mg/kg, Euthasol, Virbac Corporation, TX, USA) and the heart removed. Collagenase B (Roche Diagnostics Corporation, Indiana, USA) was used for the retrograde Langendorff perfusion. Isolated ventricular myocytes were plated on laminin-coated culture inserts in PC-1 medium (Lonza, NJ, USA) supplemented with 5% penicillin-streptomycin. Myocytes were infected with recombinant replication-deficient adenovirus (MOI 10-50) expressing GFP-PKD1 (WT or S427E) or DKAR variants (DKAR-NLS, -NES, or –MyrP) with subsequent culture for 18-30 hr. GFP fluorescence (CFP/YFP for DKARs) indicated infection and localization.

All imaging experiments were performed at 23°C in Tyrode solution containing (in mmol/L) 140 NaCl, 4 KCl, 1 MgCl2, 1.8 CaCl2, 10 HEPES and 10 glucose at pH 7.4. Myocytes were treated for 1hr with ET1 (100 nmol/L), PE (10 µmol/L), PDBu (100 nmol/L), ISO (100 nmol/L) and forskolin (10 µmol/L). Pretreatments with ISO or Forsk were for 15-20min at the same concentration prior to PE or ET1 exposure. The PKA specific inhibitor 8- Bromoadenosine- 3', 5'- cyclic monophosphorothioate, Rp- isomer
(Rp-8-Br-cAMP) was used at 5 μmol/L, H-89 at 1 or 10 μmol/L. Experiments were performed on myocytes isolated from at least 3 rabbits.

Confocal and FRET measurements

GFP-PKD1 (WT or S427E) was measured by confocal microscopy using argon laser excitation at 488nm and emitted fluorescence (F) collected > 505 nm. Image J software (http://rsb.info.nih.gov/ij/) was used for analysis with the intensity of the regions of interest normalized to area (F_{nuc}, F_{cyto}, F_{SL}). Fluorescence intensities were also corrected for background signal. Nuclear import and sarcolemmal recruitment were assessed as F_{nuc}/F_{cyto} and F_{SL}/F_{cyto}, respectively.

FRET was measured 2 ways as previously described \(^2\) (1) a non-destructive, ratiometric approach to measure time-dependent FRET changes (here CFP and YFP emission are measured upon CFP excitation); and (2) acceptor photobleach method where FRET is measured quantitatively as the increase in CFP (donor) fluorescence upon YFP photobleaching (which eliminates energy transfer from CFP to YFP). For this study the ratiometric FRET measurements were performed using the MATL function of FV1000 Olympus confocal with CFP excitation using the 440 nm laser line and emitted fluorescence for CFP collected at 460-500nm (F_{CFP}) and for YFP at 520-600nm (F_{YFP}). Fluorescence intensities were corrected for background and DKAR FRET changes at specific cellular locations were reported as F_{CFP}/F_{YFP} normalized to the initial ratio.

**Total Internal Reflection Fluorescence Microscope (TIRF)** \(^2\)

GFP constructs were imaged on an inverted 60X oil objective lens with Olympus Cell^TIRF Illuminator using a 488 nm diode laser excitation and 515 nm longpass filter for emission (Olympus, USA). Image analysis of the evolution of the TIRF signal was performed using the Image Processing Toolbox in Matlab to select a region in the image corresponding to one cell at a time, and calculating the mean image intensity within that region for each frame of the image sequence. To compensate for a slight drift of the cell positions through the image sequence, each image of the sequence was translated back so that the cell was again in its original position, as determined by least-squares minimization of the difference, within the selected region, between the median-smoothed original image and the median-smoothed displaced image. The data is reported as an F/F\(_0\) time series.

**Immunoprecipitation (IP)** \(^3\)

The IP buffer (pH 7.6) is prepared with 0.1% TritonX-100 and in mmol/L: 10 Na\(_2\)HPO\(_4\), 150 NaCl, 1 EGTA, 1 EDTA, 5 NaF. Rabbit ventricular myocytes were infected with WT PKD-GFP adenovirus and cultured overnight. Cells were treated with ISO (100 nmol/L), forskolin (10 μmol/L) or vehicle for 1hr, before lysing in ice-cold lysis buffer containing
(in mmol/L, pH7.4): 10 Na₂HPO₄, 150 NaCl, 5 EGTA, 5 EDTA, 5 NaF, as well as 1% TritonX-100 and 0.5% sodium deoxycholate. The lysate was cleared by centrifugation at 5,000 rcf for 3 min before freezing in liquid nitrogen. Protein concentration in each lysate sample was determined using a BCA assay (Cat#23225, Thermo Fisher Scientific Inc. USA). Samples were diluted with IP buffer to a final concentration of 1 mg/mL. Immunoprecipitations were performed by end over end incubation with anti-GFP, anti-PKD or anti-PKA antibody at 4°C overnight, followed by 2 hour incubation with protein A/G-coupled magnetic beads (Millipore). The beads were then washed 3 times with 500 mL IP buffer followed by resuspension in sample loading buffer. A 4–20% precast polyacrylamide gel (#456-1093, Bio-Rad, Hercules, CA, USA) was loaded with 5% of the IP, and 30 µg of supernatant and lysate fractions. Proteins were transferred to 0.2 µm nitrocellulose (Cat# 162-0112, Bio-Rad, Hercules, CA, USA). Blots were blocked with 50% superblock (Cat# 37515, Thermo Fisher Scientific Inc. Rockford, IL USA) for 1 hr, then washed with TBS+0.05% tween (3x). Primary antibody incubation was overnight at 4˚C followed by several washes with TBS-T and secondary antibody incubation for 2 hours at room temperature. The blots were again washed in TBST before imaging on the Li-Cor’s Odyssey. The primary antibodies used were Phospho-PKA Substrate (RRXS*/T*) (100G7E) Rabbit mAb #9624, Phospho-Akt Substrate (RXXS*/T*) (110B7E) Rabbit mAb #9614 and anti-PKD1 #2052 (Cell Signaling Technology, Inc., Danvers, MA, USA, or cat#sc-638 and sc-935, , santa cruz biotechnology, inc., Santa Cruz, CA, USA), Anti-GFP (Cat# sc-9996, santa cruz biotechnology, inc., Santa Cruz, CA, USA), anti-PKA RII (Abcam, ab38949). Secondary antibodies were 926-32210 IRDye 800CW Goat anti-Mouse IgG (H + L) and 926-68071 IRDye® 680RD Goat anti-Rabbit IgG (H+L) (Li-Cor, Lincoln, NE USA) or light chain specific 211-652-171, Alexa Fluor 790 mouse anti-rabbit IgG (L) (Jackson Immunoresearch Laboratories, PA, USA). Immunosignals were quantified with ImageJ. All experiments were performed in triplicate

Immunoblotting

For figure 2B isolated myocytes were rinsed in PBS and treated as indicated before lysing in ice-cold buffer containing in mmol/L: 150 NaCl, 10 Tris (pH 7.4), 2 EGTA, 50 NaF, 0.2 NaVO₃, 1% Triton X-100, and protease and phosphatase inhibitor cocktail III (Calbiochem). Cell lysates were flash-frozen and stored at -80°C. Proteins were size-fractionated on 8% SDS-PAGE before transferring to a 0.2 micron nitrocellulose membrane. Immunoblots were blocked with 5% milk in Tris-buffered saline (TBS) Tween. The blots were then incubated overnight at 4°C with primary antibody: PKD, PKD-pS916 (1:1000; Cell Signaling). After incubation with the HRP–labeled secondary antibody, blots were developed using enhanced chemiluminescence (Pierce Supersignal). All signals were recorded using a UVP-EpichemII darkroom imaging system for quantification and captured on film for representation. Equal protein loading
was ensured by reprobing for GAPDH (1:5000; Abcam). All experiments were performed in triplicate.

For all other experiments myocytes were lysed in ice-cold buffer containing (in mmol/L): 10 Na2HPO4, 150 NaCl, 5 EGTA, 5 EDTA, 5 NaF, with 1% TritonX-100 and 1% Na Deoxycholate, Mini Complete protease inhibitor cocktail (Roche), orthovanadate 1 mM, okadaic acid 100 nM, and genistein 100 uM) for 5 minutes. The homogenate was made with a Dounce' homogenizer with 5 pulls, then spun at 10,000 X g to remove the particulates. The primary and the secondary antibodies used were as described for the IP, also with total PKD (PKD/PKCμ Antibody #2052, Cell Signaling Technology, 1:2000).

*Isolated Myocyte Fractionation*

Lysis and fractionation procedures were adapted from previously published. All procedures were performed continuously on wet ice or at 4°C. Freshly isolated myocytes were suspended in hypo-osmotic lysing solution (in mM: HEPES 10, MgCl2 3, Na+ pyrophosphate 20, DTT 1, EGTA 1, EDTA 1, Mini Complete protease inhibitor cocktail (Roche), orthovanadate 1 mM, okadaic acid 100 nM, and genistein 100 uM) for 5 minutes. Sucrose was added to the suspension to achieve 330 mM, and suspensions were disrupted by 10 gentle strokes in a 2 mL glass dounce homogenizer. Suspensions were passed through a 64 micron nylon mesh, centrifuged at 15,000 g for 10 minutes, and supe (S) and pellet (P1) fractions were separated. Fraction S was clarified by centrifugation at 80,000 g for 1 h, and the supernatant was saved for analysis of cytosolic proteins. P1 was resuspended in hypo-osmotic lysing solution with 300 mM KCl included and rested on ice for 5 minutes, to disrupt myofilaments. Suspensions were floated over 1.5 M sucrose (1:1 v/v) and centrifuged at 100,000 g for 1 h. The nuclear pellet (P2) was washed by suspension in hypo-osmotic lysis solution and centrifugation at 15,000 g for 10 minutes. The washed pellet was suspended in nuclear lysis buffer (in mM: NaCl 20, MgCl2 1.5, HEPES (pH7.9) 20, EDTA 0.2, plus 25% Glycerol, with protease / phosphatase inhibitors included as described above). The sucrose suspension removed from P2 was subsequently precipitated in 20% trichloroacetic acid, and centrifuged at 15,000 g for 10 minutes. The precipitate was gently washed in 100% ethanol (4°C), and then solubilized in RIPA buffer 3 for electrophoretic and immunoblot analysis. HDAC5 (Abcam, ab55403 dilution 1:1000) was used as nuclear marker, caveolin-3 (BD Transduction Laboratories, 610421,1:1000) and Na/K ATPase alpha 1 (Millipore, 1:2500) as sarcolemmal markers and GAPDH (1:10000; Abcam) as a cytosolic marker. PKD activation was probed with pS916 antibody from Abcam (Anti-PKC mu (phospho S916) ab136456, 1:2000).

*Mouse Heart Fractionation*
Procedure was as previously described. After excision of the heart, hearts were Langendorff perfused with Tyrode solution containing 100nM isoproterenol or not for 30 min. The ventricles were then flash-frozen. Hearts were pulverized and then homogenized with a Dounce glass tissue grinder before centrifugation for 10 min at 600g. The pellet was washed three times before resuspension in nuclear extract buffer which contains in mmol/L: 20 HEPES, 420 NaCl, 1.5 MgCl₂, 0.2 EDTA and 25% glycerol. The resulting supernatant is the nuclear fraction. The supernatant from the 1st centrifugation was centrifuged at 5000g to remove mitochondria, before ultracentrifugation at 100,000g to obtain the membrane fraction (mixed SR/sarcolemma). The supernatant is the cytosolic fraction. Both mitochondrial and SR/SL pellets were washed three times before resuspension in RIPA buffer (containing in mmol/L: 150 NaCl, 20 Tris, and 1% TritonX-100 and 0.1% SDS). All buffers also contained protease and phosphatase inhibitors (Roche).

**In vitro phosphorylation**

Recombinant PKD (100 ng protein, Life Technologies, PV3791) was phosphorylated using the purified catalytic subunit of PKA (2U, Calbiochem, 539482-50UG) in vitro. The reaction (total volume 30μl containing 50 mM Tris-HCl, 5 mM MgCl₂, pH 7.0) was initiated by the addition of 1mM MgATP (or vehicle for controls). Where indicated 1µM CID755673 or 10µM H-89 were added to inhibit PKD and PKA respectively. The samples were incubated at 30°C for 10 min before stopping the reaction with ice-cold 2x SB. Samples were then subjected to SDS-PAGE and Western blotting as described for the IP.

**Sequence alignment**

Sequence alignment and analysis were performed using Geneious version 6.0.4 created by Biomatters (http://www.geneious.com/).

**Culture NCMs.**

Neonatal rat cardiomyocytes were purchased from Lonza (R-CM-561, Allendale, NJ) and thawed as per instructions. The cells were plated at 4,000 per 2-chamber slide or 100,000 per 6 cm petri dish in Rat Cardiac Myocyte Growth Med Bul Kit (CC-4515, Lonza, Allendale, NJ). After 4 – 6 hours after plating the media was changes. After 48 hours, 1 uL of virus (1E+12 MOI) was added per chamber or 2 uL per 6-cm dish. After 2 hours the media was changed and the cells left in incubator at 37°C and 5% CO₂ overnight or up to 2 days. 24 hours prior to experiments the media was changed to 1% pent/strep in DMEM (Gibco) supplemented with Nutridoma-SP (11011375001, Roche, Indianapolis, IN). Treatment was started as stated in the text or figure legend. For mRNA measurement, six hours after treatment the media was removed and 2 mL RNALater (Invitrogen, Carlsbad, CA) was added to each 6-cm dish. The cells were then
scraped and placed into 2-mL Eppendorf microfuge tubes until further processing. The 2-chamber slides plated neonatal myocytes received 48 hours of treatment with fresh phenylephrine, isoproterenol, and endothelin given every 12 hours. After 48 hours the media was removed and ice cold 4% phosphate buffered paraformaldehyde was added for 10 min. The cells were blocked with 5% BSA in PBS for 40 min. The cells were then washed 3X PBS, and then incubated with primary antibody overnight in the cold room on a slow shaker. The cells were then washed 3X PBS followed by 2nd antibody incubation for 16 hours at 4°C. The cells were then washed 5X PBS, and DAPI was added to the PBS in which they were stored. The 2-chamber slides were then imaged on an Olympus Fluoview confocal microscope utilizing the following laser lines: 405nm for DAPI, 488nm for GFP, and 635nm for α-actinin. The antibodies and stains used were α-actinin (1:1000, Sigma-Aldrich), AlexaFluor goat anti-mouse (1:200, Invitrogen), and DAPI (Invitrogen, Carlsberg, NY). ImageJ (NIH software) was used to analyze the α-actinin staining and calculate cell size. No image adjustments were made prior to analysis. Images were adjusted for optimal presentation in the manuscript.

**RNA Extraction, cDNA Synthesis and qPCR**

Total RNA was isolated using the TRIZOL Reagent (Invitrogen, Carlsbad, CA) and NucleoSpin® RNA II (Clontech, Mountain View, CA) per the manufacturer’s instructions. The RNA concentration was determined first by Nanodrop (Thermo Fisher Scientific) followed by Quant-iT™ RiboGreen (Grand Island, NY). To generate cDNA, 1 µg of RNA was loaded in a total volume of 20 µL RNA to cDNA EcoDry Premix (Clontech, Mountain View, CA). From this mix, 2 µL of the cDNA was used with GoTaq® qPCR Master Mix (Promega, Madison, WI) and subjected to 95°C for 15 min, (95°C 30 sec, 58°C 20 sec, 72°C 60 sec)X35. The following custom primers (Illumina, San Diego, CA) were used for detection of each mRNA: Control genes; Hmbs FOR-(5’-TCCTGAAACTCTGCCTGCATTTGCT-3’) and REV-(5’-CAGTGATGGCTACTGGGGACT-3’); Rpl13a FOR-(5’-CTAGTATTGGATGGCCGGGCCC-3’) and REV-(5’-TACAACCACCTTTGCGGCCA-3’); Hprt1 FOR-(5’-GCCAGCTTCTCCTCAGACCGCTTTT-3’) and REV-(5’-TCATCAGTCGACAGTTGGAAGGG-3’); Myh6 FOR-(5’-ATAGGATTGTCCCTTTGCTACTGGGACT-3’); Acta1 FOR-(5’-TCTTGGTGCTCACCACCAAG-3’) and REV-(5’-ATACACTGGAGATCAGGACAGG-3’); Nppb FOR-(5’-GCCCTCGATGACATGAG-3’) and REV-(5’-GCCTGTGGTTCATTGAGC-3’); Nppa FOR-(5’-ATGGGCTCCTCCATCACCAGG-3’);
GATCTGTGTGGACACCAGCCTGTATACG-3'). The threshold crossing value was noted for each transcript and normalized to the internal control. The relative quantitation of each mRNA or miRNA was performed using the comparative Ct method. Experiments were performed using a Mastercycler® ep realplex (Eppendorf, Hauppauge, NY), and data processing was performed with Mastercycler® ep realplex software (Eppendorf, Hauppauge, NY).

**Transverse aortic constriction**

All procedures were approved by UC Davis Animal Care and Use Committee and were as previously described with minor changes. Briefly, eight-week-old adult mice (C57/BL6, Jackson Laboratory), weighing 18-22 g, were anesthetized with isoflurane (3% in 100% O2). After successful endotracheal intubation with 22-G Teflon catheter, the cannula was connected to a volume cycled rodent ventilator (Harvard Apparatus) on supplemental isoflurane/oxygen (1.5 – 2%/100%) with a tidal volume of 0.2 ml and respiratory rate of 110 per min. The chest cavity was entered in the second intercostal space at the left upper sternal border through a small incision, and aortic constriction (to 0.4 mm) was performed by tying a 6-0 nylon suture ligature against a 27-gauge needle after which the needle was removed. The pneumothorax was evacuated, and the animals were extubated and allowed to recover. Post-operative care included analgesic (buprenorphine, 0.5-0.1 mg/kg, SQ) and continued twice daily for the next 48 h. Three weeks later, echocardiograms (VisualSonics) were obtained to evaluate cardiac dysfunction. The mice were then euthanized so the hearts could be harvested. Cardiac homogenates were prepared by removal of the heart and wash in ice cold PBS. The hearts were snap frozen in liquid nitrogen. For the cardiac homogenates, the frozen heart was powderized on dry ice and resuspended in 2-mL of ice-cold buffer containing (in mmol/L): 10 Na2HPO4, 150 NaCl, 5 EGTA, 5 EDTA, 5 NaF, with 1% TritonX-100 and 1% Na Deoxycholate, Mini Complete protease inhibitor cocktail (Roche), orthovanadate 1 mM , okadaic acid 100 nM, and genistein 100 uM). The samples were then homogenized using a polytron at 5000 rpm for 20 sec and 5 strokes of a Dounce homogenizer with 5 pulls. This was followed by centrifugation at 5,000 X g to remove the particulates. The resulting supernatant was flash-frozen and stored at -80C.

**Statistical analysis**

Data are expressed as mean±SEM. Statiscal discriminations were performed with Student’s t test and 2-way ANOVA with p<0.05 considered significant.

**References**


Suppl Fig I: β-AR/PKA activation triggers nuclear PKD activity. (A) Representative Western blots of fractionated mouse hearts (±30 min 100 nM ISO perfusion, n=3). Blots were probed with a PKD consensus motif antibody (pPKD targets) to measure ISO-triggered phosphorylation of PKD target proteins and with pS916 to determine PKD activation state. NKA, histone and GAPDH signals were used as validation and loading controls for respectively the sarcolemmal, nuclear and cytosolic fractions. (B) Quantification of pS916 and pPKD targets signals (i.e. PKD activity) in response to ISO in the various subcellular compartments.
Suppl Fig II: TIRF measurements of PKD sarcolemmal recruitment in response to G_{q,R} agonists ± pretreatment with ISO or Forsk. Bottom panels show that PKA inhibition with Rp-8-Br-cAMP prevents the ISO and Forsk effect. (n>5 myocytes)
Suppl Fig III. Analysis of β-AR-GqR crosstalk in sarcolemmal fraction. (A) Validation of cytosolic (C), nuclear (N) and sarcolemmal (M) fractions using marker proteins for each compartment: GAPDH for the cytosol, Na/K-ATPase alpha 1 (NKAα1) and caveolin-3 for the sarcolemma. (B) Sarcolemmal fractions were obtained from freshly isolated myocytes treated for 15 min as indicated±15 min ISO pretreatment. The blots were probed for activated PKD with pS916 antibody. The findings confirm the stronger sarcolemmal effect of ET1 and that ISO pretreatment prevents GqR-dependent activation of PKD.
Suppl Fig IV. Endogenous PKA and PKD interact in adult myocytes. PKA and PKD interaction in adult myocytes was assessed via co-immunoprecipitation. (A) PKA-and PKD-mediated pull downs were probed for the presence of PKA (IP:ab124400) and PKD (IP:sc-638). A pull-down with beads only (or IgG (not shown)) serves as control (n=3). (B) Co-immunoprecipitation of PKA and PKD was confirmed using different PKD antibodies for the pull down: Santa Cruz’s sc-935 (PKD_{sc}) and Cell Signaling’s #2052 (PKD_{cs}). Blots were probed with PKA (ab124400) and PKD (Cell Signaling #2052).
Suppl Fig V: β-AR/PKA activation triggers nuclear import of S4207E PKD1. (A) Confocal images of rabbit ventricular myocytes expressing GFP-tagged PKD1-SE before and after 20min of 100nM ISO (including enlarged nucleus). Bar = 15 um. Quantification of $F_{\text{nuc}}/F_{\text{cyto}}$ in GFP-PKD1 WT vs. SE myocyte populations ± ISO treatment for 20 min (* P<0.05 vs. untreated, WT data same as Fig 1C).
Suppl Fig VI: S427E substitution recapitulates ISO-dependent inhibition of G_{q\text{R}}-triggered PKD activation. Myocytes expressing GFP-PKD1 WT or S427E were treated for 20 min with vehicle, 10 µM PE, 100 nM ET1 or 100nM PDBu. Representative western blots of the respective myocyte lysates are shown probed for pS916 (~PKD activity) and GFP (to verify WT and SE expression levels). The pS916 signals were quantified and normalized to the untreated samples (n=2).
Suppl Fig VII: PKD1-S427E mimics the effect of PKA stimulation on PKD downstream signaling. mRNA gene expression data in neonatal rat cardiomyocytes (NRCM) transfected with PKD1-WT-GFP or PKD1-S427E-GFP ando-virus. The cells were treated with Iso, PE, or Iso pre-treatment for 20 min, followed by Iso and PE treatment. Cells were harvested at 6 hours. N=4 different experiments. (A) Nppb = natriuretic peptide precursor B. (B) Myh7 = myosin, heavy chain 7, cardiac muscle, beta (*=P<0.05, **=P<0.001 SE vs. WT, #= P<0.05 SE treatment group vs. SE Ctl).
Suppl Fig VIII: S427 phosphorylation in response to ISO not compartment-specific.
Fractionated mouse hearts (see Suppl Fig1 for validation with compartment-specific markers) were probed for S427 phosphorylation in response to 30 min ISO perfusion.
Suppl Fig IX: echocardiographic and gravimetric analysis of SHAM vs. TAC hearts.

<table>
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<tr>
<th>Measurements</th>
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<th>SHAM (n=7, male)</th>
<th>TAC (n=6, male)</th>
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Correction

Correction to: β-Adrenergic Signaling Inhibits Gq-Dependent Protein Kinase D Activation by Preventing Protein Kinase D Translocation

and

Multimodal SHG-2PF Imaging of Microdomain Ca^{2+}-Contraction Coupling in Live Cardiac Myocytes

In the article by Nichols et al, “β-Adrenergic Signaling Inhibits Gq-Dependent Protein Kinase D Activation by Preventing Protein Kinase D Translocation,” which published in the April 25, 2014 issue of the journal (Circ Res. 2014;114:1398-1409. DOI: 10.1161/CIRCRESAHA.114.303870.), and in the article by Awasthi et al, “Multimodal SHG-2PF Imaging of Microdomain Ca^{2+}-Contraction Coupling in Live Cardiac Myocytes,” which published in the January 22, 2016 issue of the journal (Circ Res. 2016;118:e19-e28. DOI: 10.1161/CIRCRESAHA.115.307919.), corrections were needed.

In both articles, the author Brittani Wood has been changed to Brent M. Wood.

These corrections have been made to the current online version of the articles, which are available at http://circres.ahajournals.org/content/114/9/1398 and http://circres.ahajournals.org/content/118/2/e19, respectively.