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Authors

Nichols, C Blake
Chang, Chia-Wei
Ferrero, Maura
[et al.](#)

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β -Adrenergic Signaling Inhibits G_q -Dependent PKD Activation by Preventing PKD Translocation

C. Blake Nichols¹, Chia-Wei Chang^{1,2}, Maura Ferrero¹, Brittani M. Wood¹, Matthew L. Stein¹, Amanda J. Ferguson¹, Derrick Ha¹, Robert R. Rigor¹, Sven Bossuyt³, and Julie Bossuyt¹

¹Department of Pharmacology, University of California, Davis, CA ²Department of Physiology, Loyola University Chicago, Maywood, IL ³Aalto University School of Science and Technology, Helsinki, Finland

Abstract

Rationale—Both β -adrenergic (β -AR) and G_q -coupled agonist (G_qR) 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 upon co-activation with G_qR agonists. This will test our hypothesis that compartmentalized PKD signaling reconciles disparate findings of protein kinase A (PKA) facilitation and inhibition of PKD activation.

Methods and Results—We report on the spatial and temporal profiles of PKD activation using GFP-tagged PKD (wildtype or mutant S427E) and targeted FRET based biosensors (DKARs) 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 G_qR -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 G_qR -induced PKD translocation and activation. In neonatal myocytes, S427E inhibits G_qR -evoked cell growth and expression of hypertrophic markers. Lastly, we show altered S427 phosphorylation in TAC-induced hypertrophy.

Conclusions— β -AR signaling triggers local nuclear signaling and inhibits G_qR -mediated PKD activation by preventing its intracellular translocation. PKA-dependent phosphorylation of PKD S427 fine-tunes the PKD responsiveness to G_qR -agonists, serving as a key integration point for β -adrenergic and G_q -coupled stimuli.

Address correspondence to: Dr. Julie Bossuyt, Department of Pharmacology, University of California, Davis, 451 Health Science Dr. Genome Building 3511, Davis, CA 95616-8636, Tel: 530-754-7419, FAX: 530-752-7710, jbossuyt@ucdavis.edu. C.B.T. and C-W.C. contributed equally to this study.

DISCLOSURES

None.

Keywords

Protein kinase D; β -adrenergic signaling; protein kinase A; cardiac myocytes; adrenergic receptor; cardiac; G proteins

INTRODUCTION

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 Ca^{2+} channels),³⁻⁶ gene expression (via histone deacetylases (HDACs)^{7, 8} and cAMP-response element binding protein (CREB)⁹), cell survival¹⁰⁻¹² and energy substrate utilization.¹³⁻¹⁵

In vivo, cardiac-specific expression of constitutively active PKD1 caused pronounced dilated cardiomyopathy,⁷ while 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 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).^{2, 19} PKDs possess substrate specificity similar to CaMK and structural features reminiscent of PKCs. Indeed PKD's modular structure consists of a C-terminal catalytic domain and an N-terminal region with autoinhibitory, regulatory domains such as the diacylglycerol (DAG) binding domain and the pleckstrin homology domain.^{20, 21} Several regulatory mechanisms (e.g.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 GPCR-dependent activation of phospholipase C and production of DAGs. 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 (S744/748 in murine numbering) by both PKC-dependent and -independent mechanisms.²³⁻²⁶ Despite this general consensus on global PKD activation for GPCR agonists, our group^{27, 28} recently identified dramatic spatiotemporal differences in PKD1 activation for two G_q -coupled agonists, phenylephrine (PE) and endothelin-1 (ET1) in adult cardiomyocytes. While both agonists triggered comparable global PKD1 activation, PE induced fleeting sarcolemmal

PKD1 translocation and activation followed by nuclear translocation and ET1 prompted persistent sarcolemmal recruitment and activity. The ability to dynamically alter the location and signal generation of PKD1 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 of the effect of 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 activity.⁷ Carnegie and colleagues later found that AKAP-Lbc functions as a scaffold for PKA and PKC, facilitating PKD activation and the transduction of hypertrophic responses (via HDAC5 and Mef2).^{29–31} Others, examining global PKD activity, report a counter modulatory role for PKA and PKC on PKD activation in adult myocytes.^{32–34} That 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 two 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 G_qR-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 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 NIH guidelines for animal research and with institutional approval. Biohazard procedures were performed in accordance with a UC Davis approved Biological Use Authorization. Adult myocytes were isolated and cultured as previously described^{27, 35}. Neonatal rat cardiomyocytes (NCM) were cultured as instructed. GFP-PKD-WT and -S427E expression was via adenoviral infection. Prior to experiments myocytes were serum-deprived for 24 hours by switching to DMEM with Nutridoma-SP.

Confocal, FRET and Total Internal Reflection Fluorescence (TIRF) measurements

All confocal and TIRF experiments were performed as previously reported.²⁷ Myocytes were exposed to inhibitors for 15–20 minutes prior to and following addition of agonists. FRET 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_{CFP}/F_{YFP} (normalized to initial ratio).

Immunoblotting

Cell lysates and subcellular fractions were obtained as previously described^{27, 36} and probed with: from Cell Signaling PKD1, PKDpS916, phospho-PKA-Substrate (RRXS*/T*), phospho-Akt Substrate (RXXS*/T*), phospho-PKD-substrate and histone 3; from Abcam GAPDH, PKDpS916, PKA and a custom PKDpS427 antibody; from Santa Cruz Biotechnology GFP and from Millipore NKA α 1. Immunoreactive band intensities were quantified using ImageJ.

Immunoprecipitation (IP)

Immunoprecipitation was performed as before³⁷ using anti-GFP, PKD or PKARII antibody at 4°C overnight followed by incubation with proteinA/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 RT-PCR of hypertrophic markers. Fixed NCM were stained for α -actinin (Sigma, 1:1000). Cell size measurements were made using Image J. 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® qPCRMasterMix. Custom primers (Illumina) 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.

Transverse aortic constriction (TAC) surgery

All procedures were essentially as described³⁸. 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 sacrificed 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 orthovanadate with 1% TritonX-100, 1% Na Deoxycholate, and protease and phosphatase inhibitors. After homogenization with polytron at 5000rpm for 20sec and 5 strokes of a Dounce homogenizer, samples were centrifuged at 5,000g. Supernatants were flash-frozen.

Statistical analysis

Pooled data are expressed as mean \pm SEM. Statistical discriminations were performed with Student's t-test and 2-way-ANOVA with p<0.05 considered significant.

Expanded methods can be found in the online supplement.

RESULTS

β -AR/PKA drives non-canonical PKD translocation

Initial experiments focused on establishing whether β -AR or PKA activation alone affects PKD signaling and localization. Adult cardiomyocytes expressing GFP-PKD1 (following adenovirus infection), were monitored via confocal microscopy following 100nM isoproterenol (ISO) or 10 μ M forskolin (Forsk) exposure (Fig 1A). Quantification of the signal at the sarcolemma vs. that in the cytosol (F_{SL}/F_{cyto}) showed that neither ISO nor Forsk induced sarcolemmal recruitment of PKD (Fig 1B). This is in contrast to the pronounced sarcolemmal recruitment of PKD we previously observed in response to the G_qR agonists PE and ET1.²⁸ Notably, even without a preceding sarcolemmal translocation phase, both ISO and Forsk triggered nuclear import of PKD (after 60 min F_{nuc}/F_{cyto} was increased by 12 \pm 2% and 13 \pm 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 ISO (i.e. unpaired measurements of F_{nuc}/F_{cyto} , Fig 1C).

To more selectively measure sarcolemmal recruitment of PKD, we also used total internal reflection fluorescence (TIRF) microscopy to assess fluorescence only within ~100nm of the glass coverslip-solution interface (Fig 1D). As in the confocal experiments, neither the β -AR agonist ISO nor direct activation of adenylyl cyclase by Forsk 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.

cAMP-dependent signaling selectively activates nuclear PKD

Using genetically encoded FRET-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 prior sarcolemmal translocation, we used targeted DKARs (to nucleus, cytosol or sarcolemma) to selectively monitor the time course and magnitude of PKD activation in response to ISO or Forsk. Phosphorylation of the PKD-specific substrate sequence in this biosensor, results in a decrease in FRET (i.e. an increase in CFP/YFP 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 ISO or Forsk failed to elicit any PKD activation at the sarcolemma or cytosol, measured with DKAR-MyrP and DKAR-NES respectively (Fig 2A). However both ISO and Forsk activated nuclear PKD (Fig 2A). The effect was more potent for Forsk, 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.

We also used immunoblotting (Fig 2B) to assess global effects on PKD activity in response to β -AR or PKA. For these in vitro measurements, autophosphorylation at S916 was used as readout of PKD activity. Consistent with our previously published findings, myocyte exposure (for 20 min) to PDBu (a phorbol ester), PE or ET1 significantly increased PKD S916 autophosphorylation compared to untreated myocytes. In contrast, ISO or Forsk treatment did not alter baseline S916 phosphorylation. The lack of an effect on global PKD autophosphorylation status may be due to 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 (\pm ISO) were analyzed for PKD activation (via pS916 signals) and phosphorylation of PKD targets (using a PKD consensus motif antibody). While S916 and PKD target phosphorylation signals were slightly decreased in the cytosolic and sarcolemmal fractions, they were increased in the nuclear compartment (Supplemental Fig I). These biochemical observations confirm 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 G_q R/PKD pathways (Fig 2B). We found that pre-activation of PKA by Forsk prevented the increase in S916 phosphorylation induced by ET1 and PE. In contrast, Forsk pretreatment failed to prevent S916 autophosphorylation in response to PDBu. These results indicate that PKA dependent signaling suppresses global PKD activation in cardiomyocytes in response to G_q R agonists, at least as sensed by S916 phosphorylation. Potent stimuli, like the DAG analogue PDBu, are still able to overcome this inhibition.

β -AR/PKA negatively modulates G_q -driven PKD translocation

Clearly there are subcellular, compartmentalized responses to PKA- and G_q R-dependent activation of PKD. To assess whether microdomain 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 prior observations confocal imaging revealed pronounced sarcolemmal translocation in response to PE and ET1 (stronger and sustained for ET1), followed by nuclear translocation, which is more rapid and prominent for PE. ISO pretreatment prevented both the ET1- and PE-induced PKD translocation to the sarcolemma (Fig 3B, left). F_{SL}/F_{cyto} at 60 min remained at baseline for ISO-pretreated myocytes vs. an increase of $9\pm 1\%$ and $31\pm 4\%$ for PE and ET1 alone. The G_q R-agonist mediated nuclear import of PKD was also blocked by pre-incubation with ISO (Fig 3B, right). The PE-triggered increase in F_{nuc}/F_{cyto} ratio after 60 min was only $1\pm 2\%$ in ISO-pretreated myocytes (vs. $18\pm 2\%$ for PE alone). Likewise, the increase in F_{nuc}/F_{cyto} ratio was only $2\pm 1\%$ after 60 min of ET1 in ISO-pretreated cells (from $13\pm 3\%$). Of note, the ISO induced nuclear import had already occurred within the 15 min pretreatment period (an $\sim 10\%$ increase). This higher, new baseline (reset to 1) was used to estimate the G_q R.

To corroborate our confocal findings, we used TIRF microscopy to dissect the ISO-induced signaling cascade leading to alterations in PE/ET1-induced PKD translocation. Our TIRF measurements confirmed that β -AR activation with ISO (15min pretreatment) prevents ET1 induced sarcolemmal recruitment of PKD in adult myocytes (Fig 3C, top). Elevating cAMP

levels with Forsk to more directly activate PKA likewise blocked the ET1 effects. Consistent with these observations, pre-incubation with 8-Rp-Br-cAMP, a cell-permeable cAMP analogue, which very specifically blocks PKA activation, prevented the ISO- and Forsk-dependent inhibitory effects on ET1-driven PKD translocation (Fig 3C). Similar results were obtained for PE (Supplemental Fig II), even though the PE-induced SL 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_q R-agonist evoked PKD translocation.

β -AR signaling negatively modulates local PKD activity

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

PKA-dependent phosphorylation of PKD

Thus far, it was unclear whether the disruption of G_q -induced PKD signaling is mediated via direct PKA phosphorylation of PKD or other PKA-mediated effects. So we used two separate PKA-consensus-motif antibodies (pRRXS*/T* and pRXXS*/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 (Fig 4A). H-89 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 co-immunoprecipitated with the PKA regulatory subunit (Supplemental Fig IV) in myocyte lysates. So we tested whether PKD is a PKA target in vivo. Myocytes expressing GFP-PKD were treated with ISO, Forsk or vehicle prior to PKD immunoprecipitation (IP) using GFP antibody and protein A/G magnetic beads. Fig 4B shows a representative IP probed with pRRXS*/T* and GFP antibodies. The GFP panel shows that equivalent amounts of PKD were immunoprecipitated and the control IPs, using beads or control IgG (Fig 4C), show the specificity of the pull-down. ISO and Forsk treatment increased the pRRXS*/T* signal in the myocyte lysate (pre-IP lanes) indicating increased PKA phosphorylation. The pRRXS*/T* signal in immunoprecipitated PKD was likewise 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 (Fig 4D) including several known auto- and trans-phosphorylation sites (S916, S255, S748, S203). Among these, the

sequence surrounding S426–S427 is of particular interest since either serine residue could serve as phosphoacceptor, 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 G_qR-induced PKD translocation

We speculated that direct phosphorylation of PKD by PKA at this site, alters PKD translocation. We used confocal microscopy (Fig 5A) to test whether a phosphomimetic substitution at S427 (PKD-S427E) would similarly perturb G_qR-evoked PKD translocation. At baseline this mutant had a slightly higher nuclear localization ($F_{\text{nuc}}/F_{\text{cyto}}$ was 0.69 ± 0.02 vs. 0.62 ± 0.02 for WT myocytes) but otherwise targeted like WT (Supplemental Fig V). Our previous findings indicate a predominantly sarcolemmal and nuclear response for ET1 and PE, 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 (vs. PKD WT; Fig 5A&B). ET1 application (for 60min) increased $F_{\text{SL}}/F_{\text{cyto}}$ by only $7\pm 2\%$ for the S427E mutant vs. $31\pm 4\%$ for PKD-WT. Likewise, PE-triggered nuclear import was drastically reduced in PKD-S427E expressing myocytes vs. WT. $F_{\text{nuc}}/F_{\text{cyto}}$ after 60 min of PE was only $2\pm 2\%$ for PKD-S427E vs. $18\pm 2\%$ for PKD-WT. Parallel TIRF experiments showed even more clearly that S427E substitution resulted in decreased membrane recruitment for both G_qR-agonists (Fig 5C). Also of note, following ISO treatment $F_{\text{nuc}}/F_{\text{cyto}}$ was increased (from 0.69 ± 0.02 to 0.77 ± 0.02 , similar to WT, Supplemental Fig 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 G_qR-induced PKD translocation. Moreover, pS916 measurements show that like ISO pretreatment (Fig 2B), S427E substitution renders PKD resistant to G_qR stimuli but not to robust activation with PDBu (Supplemental Fig VI). Together, our data indicate that S427 mediates the PKA-dependent suppression of G_qR induced PKD activation and translocation.

PKD S427 mediates the β -AR effects on downstream G_qR-PKD signaling

We speculated that S427E substitution would also affect downstream G_qR-induced PKD effects. So in light of the well-characterized role of PKD as an HDAC kinase, we examined the effect of S427E substitution on cardiomyocyte hypertrophy. Neonatal cardiomyocytes (NCMs) were infected with GFP-PKD S427E, S427A or WT. As shown in Fig 6A, expression of PKD-S427E prevented sarcomere assembly and cell enlargement in response to PE or ET1, while 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 (ANP), brain natriuretic peptide (BNP), and α -skeletal actin. Agonist-dependent elevation of hypertrophy markers can be assessed by RT-PCR. Fig 6B shows that ISO blunts PE-dependent induction of α -skeletal actin expression (left) in NCMs expressing PKD-WT. α -skeletal actin transcript induction by PE was likewise markedly reduced in the presence of PKD-S427E, indicating that S427E substitution recapitulates the β -AR effects on downstream G_qR-evoked PKD signaling. Similar results were obtained for ANP, BNP and myosin7 (Fig 6B right and SVII). Additionally, ISO was unable to suppress PE-dependent gene expression for the unphosphorylatable S427A mutant (Fig 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 G_qR 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 HEK cells. Fig 6C shows that immunoreactivity increased following Forsk-treatment for WT and S426A, but not for S427 and SS426/427AA, indicating this novel antibody specifically detects phosphorylation at S427. This antibody signal also increased in lysates from ISO- or Forsk-treated myocytes, but not in the presence of PKA inhibitors H89 or 8-Br-Rp-cAMP (Fig 6D). Subcellular fractionation experiments found that in response to ISO PKD pS427 signals were increased throughout the cell, but particularly in the sarcolemmal compartment (Supplemental Fig VIII). These findings confirm ISO triggers PKA-dependent phosphorylation of S427. We then probed S427 phosphorylation in cardiac homogenates from 3 week TAC mice *vs.* their SHAM controls. Echocardiography confirmed banded mice had significant alterations in structural and functional cardiac parameters (Supplemental Fig IX). Fig 6E shows that S427 phosphorylation, PKD1 expression and S427-phosphorylated PKD1 were all increased in TAC *vs.* 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 G_qR 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^{29, 30} and inhibition of PKD signaling.^{32–34} We find here that β -AR/PKA activation of PKD selectively drives nuclear PKD recruitment and activation without initial translocation to the sarcolemma (Fig 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 prior work using immunoblotting that found no effect of β -AR/PKA stimulation alone on PKD activity.^{7, 32, 33, 41} 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 lab^{29, 30} 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) we see here (Fig 7 left) could originate from this AKAP-Lbc-bound pool of PKD or from another perinuclear AKAP pool (e.g. mAKAP⁴²). Increased PKA activity would trigger the release of activated PKD from this scaffold permitting its movement to the nucleus without prior 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 G_qR-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 (Fig 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 G_qR-induced PKD activation and function. This is because the phosphomimetic substitution (PKD-S427E) dramatically reduces G_qR-induced PKD translocation and cardiac hypertrophy (Fig 5&6). Therefore our data resolve a long standing question of how this striking β -AR inhibition of PKD activation by G_qR agonists occurs. Two other hypotheses had been advanced thus far. First, β -AR-dependent modulation of phospholipase C (PLC) could limit G_qR-induced PKD activation (but see also Stangherlin *et al.*).^{33, 43–45} Second, PKA stimulation of PP2a activity may abrogate PKD signaling (by terminating activating phosphorylations).⁴⁶ We do not exclude the possibility that these alternate mechanisms could contribute nor that additional PKA phospho-sites exist on PKD. However, the present study demonstrates that PKA-mediated phosphorylation of PKD at S427 is the principal mechanism of β -AR/PKA inhibition of G_qR/PKD signaling.

Phosphoproteomics has identified a multitude of phosphorylation sites on PKD, but only a limited few have known functional consequences.²² Interestingly the predicted PKA target sites on PKD (Fig 5) included known phosphosites such as S748 and S916. However phosphorylation at these sites would be expected to activate, rather than inhibit PKD activity and we did not detect PKA-dependent phosphorylation at these sites in myocyte lysates (Fig 2B).³³ Sites S205 and S255 were also less likely based on their known functional effects.^{11, 47, 48 47}. The most interesting candidate was the S427 site just adjacent to the PH domain, i.e. in the position to influence PH-mediated protein interactions and autoinhibitory effects. Indeed, a previous study found that PKA could directly phosphorylate S427 in vitro, but thus far 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 G_qR 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 impact 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, further experiments are needed detailing the (β -AR-dependent) modulation of the S427 site *vs.* alterations in spatiotemporal regulation and function of PKD during different stages of heart failure development. β 1-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. Topographic analysis of PKD activity will be key to unraveling the subcellular mechanisms that drive PKD dysfunction in 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 G_q R-induced PKD activation following β -AR stimulation, is due to profound perturbations in PKD translocation. PKA-dependent phosphorylation of PKD S427 was found to mediate the perturbation of G_q 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

| | |
|--------------|---------------|
| ISO | isoproterenol |
| Forsk | forskolin |
| ET1 | endothelin-1 |

| | |
|------------------------------|--|
| PE | phenylephrine |
| GPCR | G protein coupled receptor |
| β-AR | β -adrenergic receptor |
| G_qR | G _q -coupled receptor |
| FRET | Fluorescence resonance energy transfer |
| DAG | diacylglycerol |
| PH | pleckstrin homology |
| NCM | neonatal rat cardiomyocytes |
| PDBu | Phorbol 12,13-dibutyrate |
| ANP | Atrial natriuretic peptide |
| BNP | Brain natriuretic peptide |

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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, G_q-coupled receptor agonists (G_qR) 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 G_qR driven PKD1 translocation and subsequent activation.
- PKA phosphorylates PKD1 at S427, which mediates the β-adrenergic suppressive effects on G_qR-triggered PKD1 signaling.
- β-AR signaling also selectively induces local nuclear PKD signaling independent of PKDS427 phosphorylation.
- PKDS427 phosphorylation is altered during TAC-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 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 (i.e. activation) *and* suppressed G_qR-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 G_qR and β-AR signaling pathways. It also identifies S427 as an important regulatory site on PKD1, controlling the spatiotemporal dynamics of PKD activity and downstream functional effects on cardiac hypertrophy and gene expression. Our finding that PKA signaling renders PKD resistant to G_q stimuli provides fundamental insight into how β-AR desensitization in heart failure could contribute to PKD dysregulation and further cardiac dysfunction.

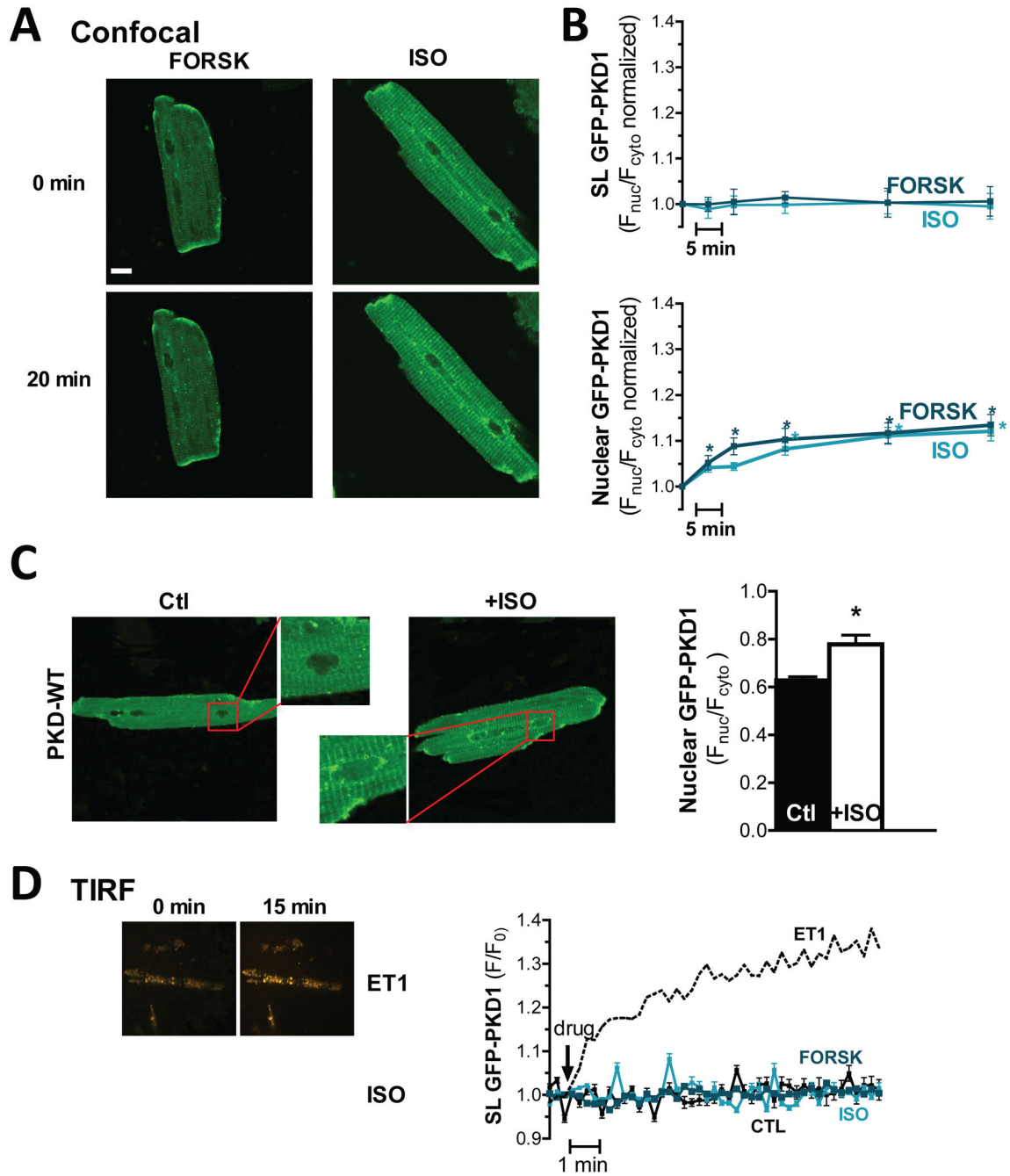


Figure 1. β -AR/PKA activation triggers non-classical PKD translocation

(A) Confocal images of rabbit ventricular myocytes expressing GFP-tagged PKD1 before and after 20min of 10 μ M Forsk treatment (left) or 100nM ISO (right). (B) PKD-GFP localization was analyzed as $F_{\text{SL}}/F_{\text{cyto}}$ and $F_{\text{Nuc}}/F_{\text{cyto}}$ for sarcolemmal 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 $F_{\text{Nuc}}/F_{\text{cyto}}$ (right). (D) TIRF analysis of agonist-dependent sarcolemmal recruitment of GFP-

PKD1. Representative TIRF images in response to ET1 or ISO are on the left. Quantification of GFP-PKD1 TIRF signals is shown on the right (n>10 myocytes).

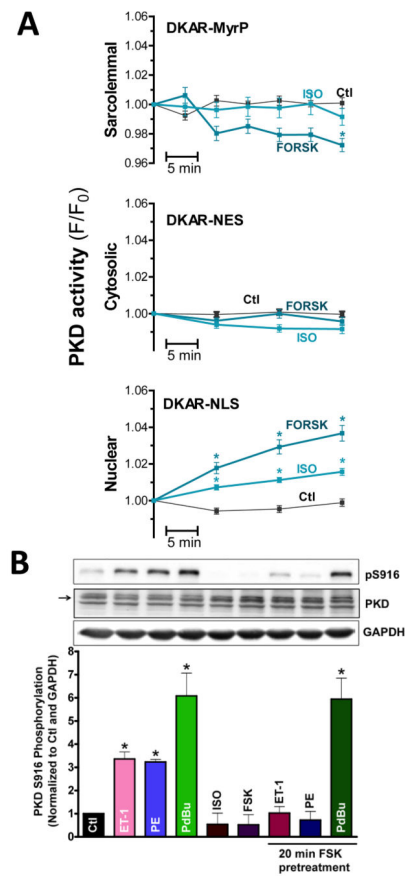


Figure 2. β -AR/PKA trigger nuclear activation of PKD

(A) PKD activity measurements with targeted DKAR constructs in sarcolemmal, cytosol and nuclear compartments ($n > 30$ myocytes, $*P < 0.01$). (B) Representative western blot and quantification of S916 phosphorylation (\sim global PKD activity) in response to agonist treatment. GAPDH and PKD signals serve as loading controls.

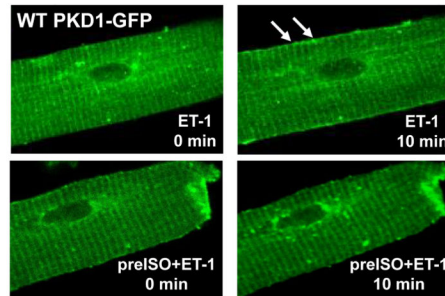
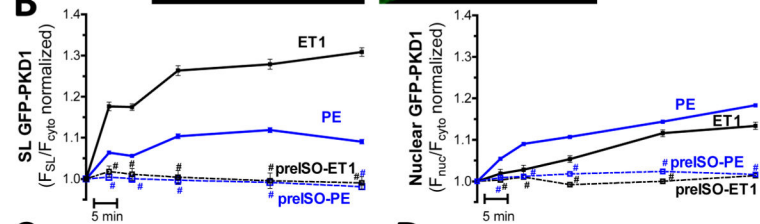
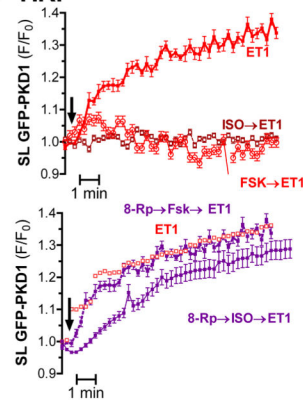
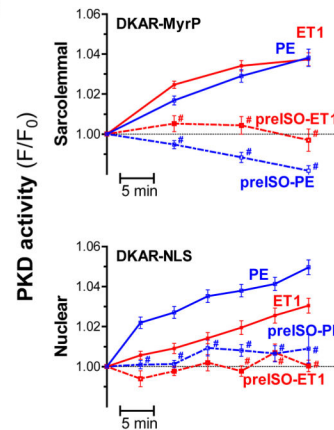
A Confocal**B****C TIRF****D**

Figure 3. β -AR/PKA signaling prevents G_qR -induced PKD translocation and activation
(A) Confocal images of GFP-PKD1 translocation in response to ET1 alone (top) or following ISO pretreatment (bottom). **(B)** Analysis of GFP-PKD1 localization shows ISO pretreatment blocks both PE- and ET1-induced sarcolemmal recruitment (left) and nuclear import (right) ($n=11-30$, $*P<0.05$, SL recruitment for PE and ET1 was also significantly different). **(C)** TIRF measurements of PKD sarcolemmal recruitment in response to G_qR -agonists \pm pretreatment with ISO or Forsk. Bottom panels show PKA inhibition with Rp-8-Br-cAMP prevents the ISO and Forsk effect. ($n>5$ myocytes) **(D)** PKD activity measurements with targeted DKAR constructs in response to G_qR agonists \pm pretreatment with ISO ($n>30$ myocytes, $*P<0.001$).

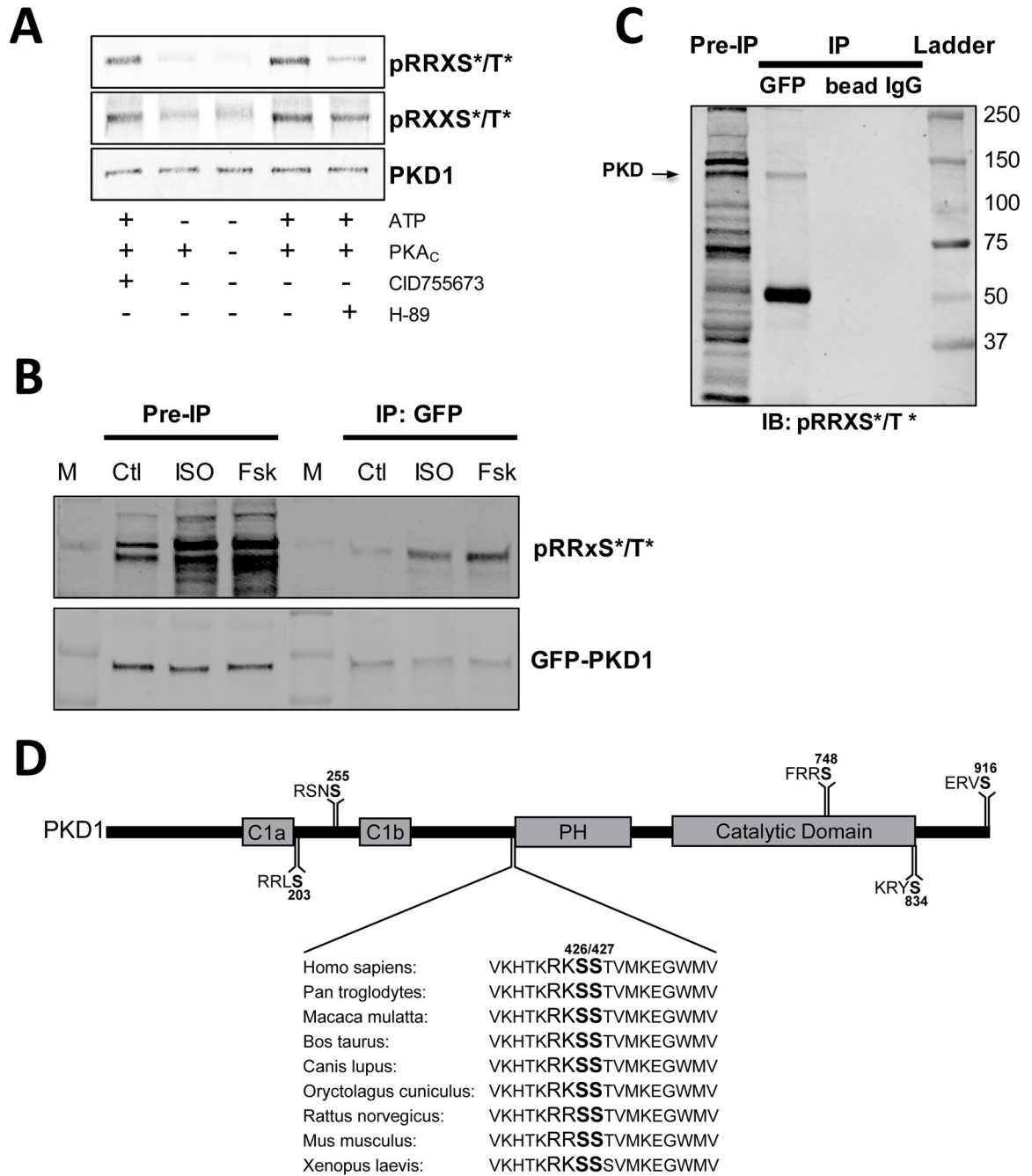


Figure 4. PKA-dependent phosphorylation of PKD

(A) In vitro phosphorylation of purified PKD with the catalytic subunit of PKA. Phosphorylation is measured with 2 different PKA consensus motif antibodies (RRXS*/T* top and RXXS*/T* bottom). (B) ISO- and Forsk-induced phosphorylation of immunoprecipitated GFP-PKD1 measured with a PKA consensus motif antibody (RRXS*/T*). Blots are probed with GFP for loading control. (C) Control immunoprecipitations (beads only and control IgG) demonstrate specificity of the PKD pull-down. (D) Potential PKA phosphorylation sites on mouse PKD1 sequence.

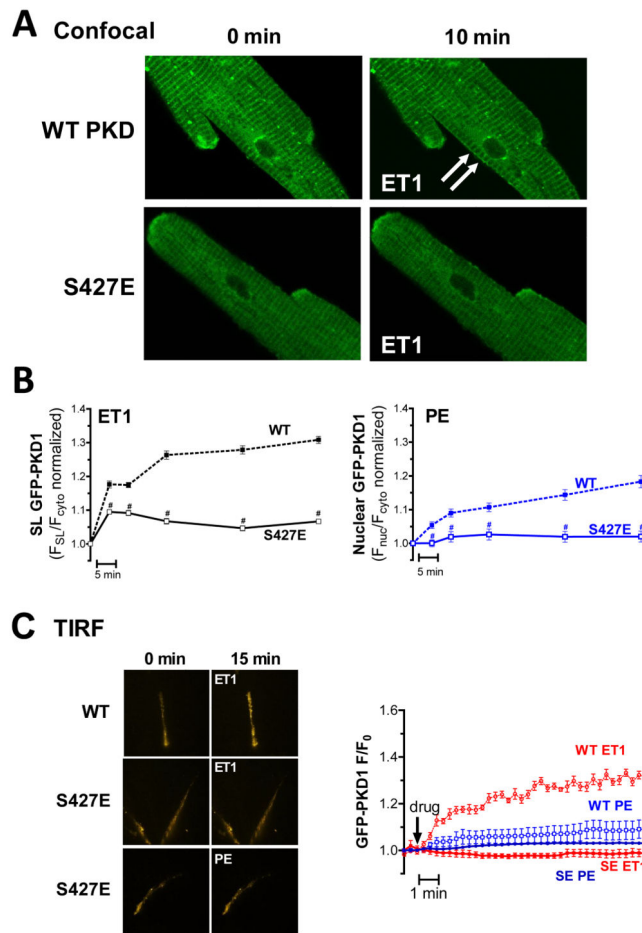


Figure 5. S427E substitution mimics β -AR effects on G_qR -induced PKD translocation
(A) Confocal images of GFP-PKD1 WT (top) and S427E (bottom) translocation in response to ET1. **(B)** Analysis of S427E vs. WT translocation profiles in response to G_qR -agonists: ET1-induced sarcolemmal recruitment (left) and PE-induced nuclear import (right) are shown (* $P < 0.001$). **(C)** TIRF analysis of GFP-PKD S427E vs. WT translocation in response to G_qR -agonists ($n > 6$). Representative images are shown on left.

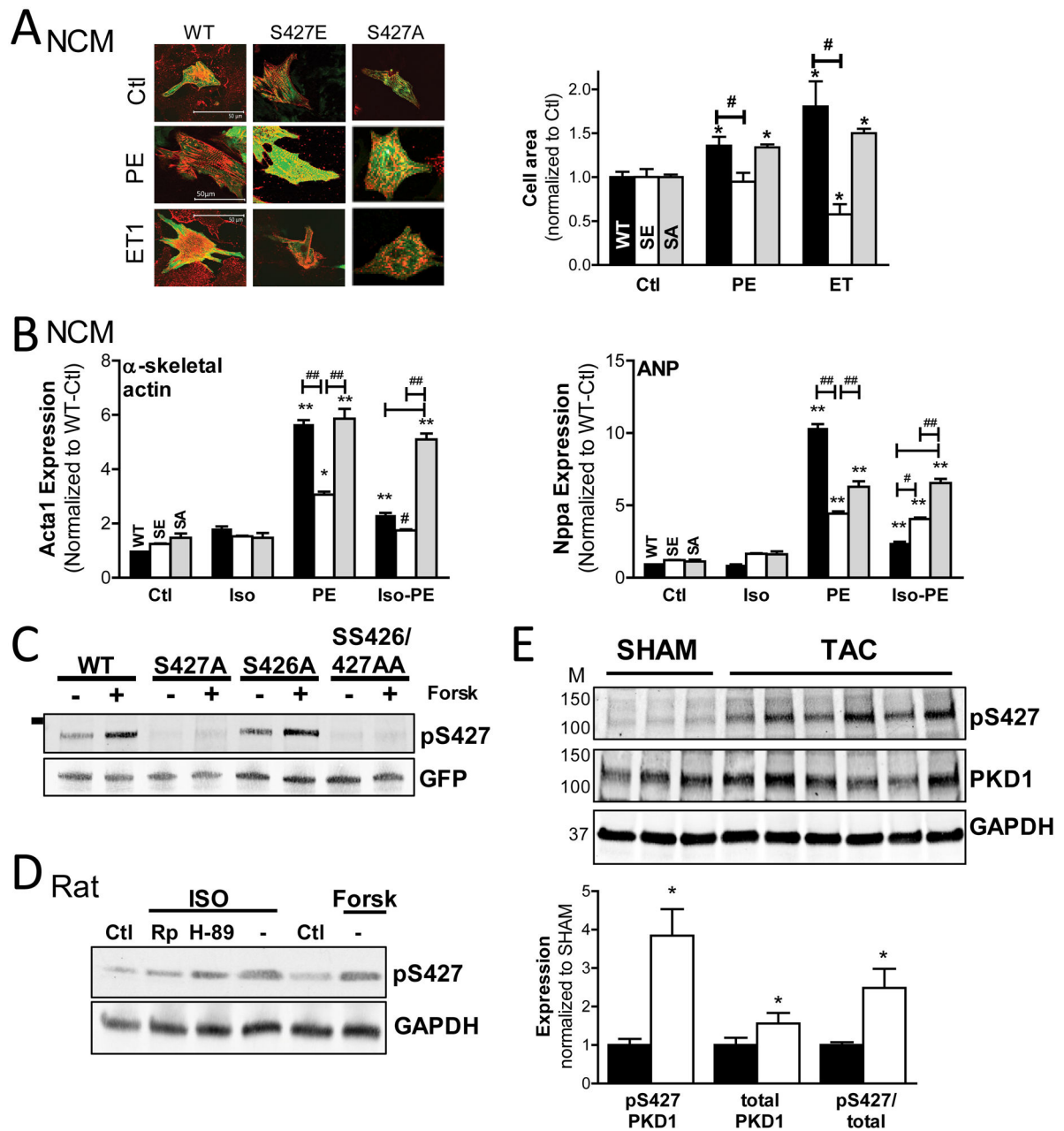


Figure 6. S427E substitution mimics PKA-dependent effects on G_q R-PKD signaling
(A) Confocal images (left) of effect of GFP-PKD1-WT, -S427E and -S427A expression (green) in NCMs on sarcomere assembly (β -actinin:red) and cell enlargement in response to G_q R-agonists (quantification right panel). ($n=50-100$ cells, $*P<0.05$) **(B)** RT-PCR analysis of S427E and S427A vs. WT effect on hypertrophic marker induction in NCMs in response to agonist. Results for α -skeletal actin (left) and ANP (right) are shown. ($*P<0.05$ vs. Ctl, # vs. WT). **(C)** Test of pS427 antibody using unphosphorylatable GFP-PKD1 variants expressed in HEK293 cells ($n=2$, GFP=loading control). **(D)** Measurement of PKD S427 phosphorylation in rat adult cardiomyocytes in response to Forsk or ISO \pm PKA inhibitors, H89 and Rp-8Br-cAMP ($n=2$). **(E)** Western blot analysis of S427 phosphorylation and

PKD1 expression in cardiac homogenates from 3 week TAC mice vs. their SHAM-operated controls. Representative blot is shown on the left and quantification of signals on the right.

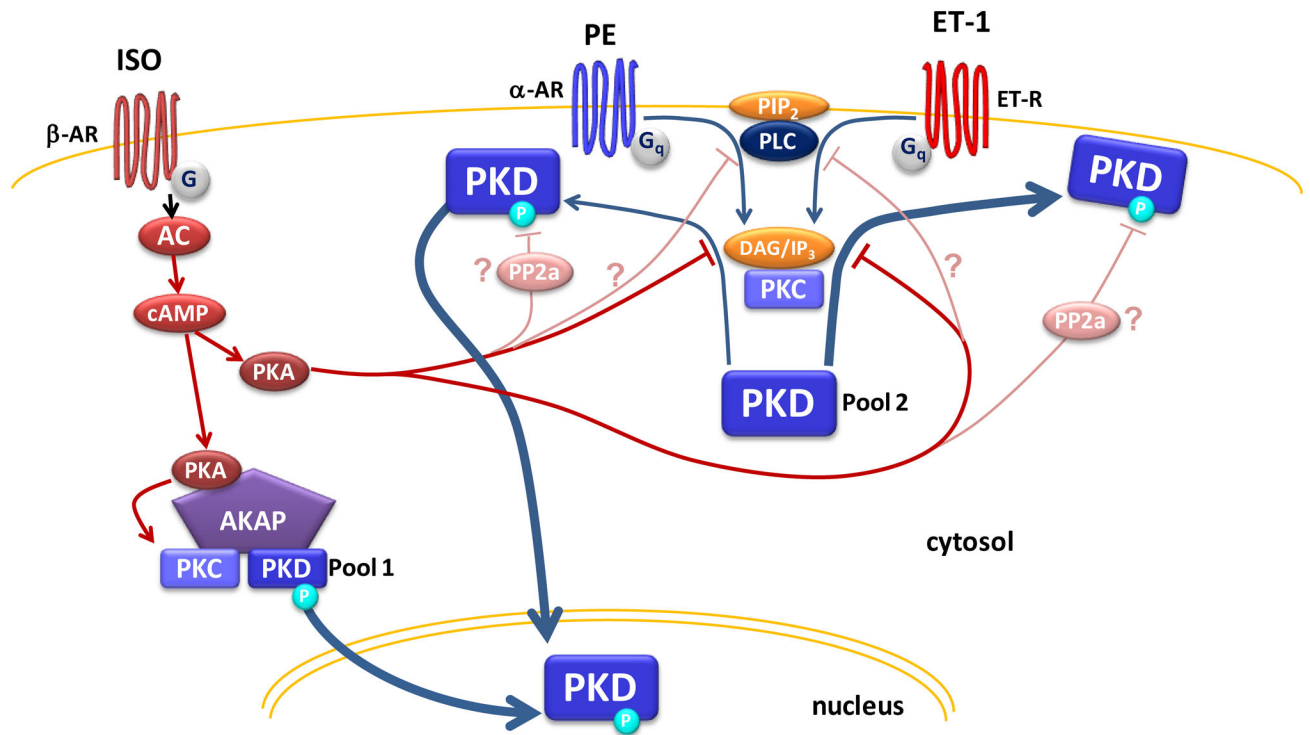


Figure 7. Working hypothesis of β -AR/PKA modulation of PKD signaling

For a 1st pool of PKD, β -AR/PKA activation triggers nuclear PKD activity by facilitating activation of PKD at an AKAP scaffold (cyan circle indicates activated PKD). For a 2nd pool of PKD, β -AR/PKA activation prevents G_q -dependent PKD activation and translocation primarily by PKA-dependent phosphorylation of PKD at S427E. We do not exclude that additional mechanisms (such as PLC inhibition or PP2A activation) could contribute to this inhibitory effect of PKA.