DECODING THE CARDIAC ACTIONS OF PROTEIN KINASE D ISOFORMS

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RUNNING TITLE PAGE
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List of nonstandard abbreviations:
\(\alpha_1\)-AR: \(\alpha_1\)-adrenergic receptor
AKAP: A kinase anchoring protein
AL: Activation loop
Bcl-2: B-cell lymphoma-2
CHD: congenital heart disease
CREB: cyclic-nucleotide regulatory element binding protein
cTnI: cardiac troponin I
DAG: diacylglycerol
DLP1: dynamic-like/related protein 1
EDG receptor: endothelial differentiation gene receptor sphingosine 1-phosphate (S1P)
ERK: Extracellular signal regulated kinase
GEF: Guanine nucleotide exchange factor
GPCR: G protein-coupled receptor
HDAC: histone deacetylase
LIMK: LIM kinase
LPL: lipoprotein lipase
MEF2: myocyte enhancer factor 2
MnSOD: Manganese superoxide dismutase
mPTP: mitochondrial permeability transition pore
NFkB: nuclear factor kB
NHERF-1: Na+/H+ exchanger regulatory factor 1
NTDD: N-terminal dimerization domain
PAR: protease-activated receptor
PdBu: Phorbol 12,13-dibutyrate
PDZ domain: PSD-95/disks large/zona occludens domain
PH domain: Pleckstrin homology domain
PKC: Protein kinase C
PKD: Protein kinase D
PMA: Phorbol 12-myristate 13-acetate
RhoA: rat sarcoma virus homolog family member A
ROS: Reactive oxygen species
S1P: sphingosine 1-phosphate
SSH1L: slingshot 1L
TGN: trans-Golgi network
ABSTRACT
Protein kinase D (PKD) consists of a family of three structurally related enzymes that play key roles in a wide range of biological functions that contribute to the evolution of cardiac hypertrophy and heart failure. PKD1 (the founding member of this enzyme family) has been implicated in the phosphorylation of substrates that regulate cardiac hypertrophy, contraction, and susceptibility to ischemia/reperfusion injury and de novo PRKD1 mutations have been identified in patients with syndromic congenital heart disease. However, cardiomyocytes co-express all three PKDs. While stimulus-specific activation patterns for PKD1, PKD2, and PKD3 have been identified in cardiomyocytes, progress toward identifying PKD isoform-specific functions in the heart have been hampered by significant gaps in our understanding of the molecular mechanisms that regulate PKD activity. This review incorporates recent conceptual breakthroughs in our understanding of various alternative mechanisms for PKD activation, with an emphasis on recent evidence that PKDs activate certain effector responses as dimers, to consider the role of PKD isoforms in signaling pathways that drive cardiac hypertrophy and ischemia/reperfusion injury. The focus is on whether the recently identified activation mechanisms that enhance the signaling repertoire of PKD family enzymes provide novel therapeutic strategies to target PKD enzymes and prevent or slow the evolution of cardiac injury and pathological cardiac remodeling.

SIGNIFICANCE STATEMENT. PKD isoforms regulate a large number of fundamental biological processes, but our understanding of the biological actions of individual PKDs (based upon studies using adenoviral overexpression or gene silencing methods) remains incomplete. This review focuses on dimerization, a recently identified mechanism for PKD activation, and the notion that this mechanism provides a strategy to develop novel PKD-targeted pharmaceuticals that restrict proliferation, invasion, or angiogenesis in cancer and prevent or slow the evolution of cardiac injury and pathological cardiac remodeling.
INTRODUCTION

Protein kinase D (PKD) consists of a family of 3 structurally-related stress-activated enzymes (PKD1 or PKDμ, PKD2, and PKD3 or PKDν) that regulate a large number of fundamental biological processes involved in cell proliferation, differentiation, apoptosis, immune regulation, cardiac contraction, cardiac hypertrophy, angiogenesis, and cancer (Harrison et al., 2006; Papur et al., 2018; Ren, 2016; Renton et al., 2020; Roy et al., 2017; Rozengurt, 2011; Youssef and Ricort, 2019; Zhang et al., 2021). The traditional model for PKD activation, based upon a series of early studies from the Rozengurt laboratory, holds that PKD isoforms are activated by receptor-driven pathways that promote DAG accumulation and co-localize PKD with its upstream activator protein kinase C (PKC) at the plasma membrane (Rozengurt et al., 2005). This facilitates PKC-dependent transphosphorylation of PKD at conserved serine residues in the activation loop (AL). However, it is now evident that PKDs also can be activated through a PKC-independent AL autophosphorylation mechanism (Jacamo et al., 2008; Rybin et al., 2009; Steinberg, 2012), PKDs are activated via a distinct tyrosine phosphorylation-dependent mechanism during oxidative stress (Cobbaut et al., 2017; Cobbaut and Van Lint, 2018; Doppler and Storz, 2017; Storz et al., 2003; Storz et al., 2005; Zhang et al., 2005a), and PKD1 activity can be modulated as a result of proteolytic cleavage by caspase-3 in the setting of apoptosis (Endo et al., 2000; Haussermann et al., 1999). While early studies suggested that PKD family members (enzymes that are co-expressed in many cell types and exhibit similar *in vitro* substrate specificities) recruit many common downstream signaling pathways and act in a functionally redundant manner in many model cell lines, there is growing evidence for isoform specific PKD responses in more differentiated cell types, with some studies even showing antithetical roles for PKD1 vs PKD2/PKD3 in the control of cell proliferation and invasion and the pathogenesis of certain cancers (Roy et al., 2017). Of note, PKD isoform-specific functions have generally been defined based on cell or tissue-specific differences in PKD isoform expression, subcellular localization, or induction in the context of certain diseases. The identification of
substrates, downstream signaling responses, and biological functions of individual PKD isoforms using standard adenoviral overexpression or gene silencing approaches has been more challenging. Studies using these traditional approaches have frequently yielded ambiguous or inconclusive results, an impasse generally attributed to imperfect specificity/efficiency of the PKD knockdown, cellular responses that are activated in a functionally redundant fashion by multiple PKDs or the presence of compensatory changes in other PKD isoforms (Guo et al., 2011; Harrison et al., 2006; Steiner et al., 2010). Recent evidence that PKDs activate certain effector responses as dimers (with two laboratories recently identifying the structural basis for PKD dimerization and implicating dimerization and AL trans-autophosphorylation as a key feature in the mechanism for PKD activation (Aicart-Ramos et al., 2016; Elsner et al., 2019)) suggests that the experimental approaches used in previous studies - based on the concept PKD enzymes signal as monomers and that the cellular actions of individual PKD isoforms are independent of other family members - may be conceptually flawed. Gene silencing approaches (a method that leads to the loss of both protein and kinase activity) are ill-suited to detect responses activated by PKD dimers. This review summarizes our current understanding of the various alternative mechanisms for PKD activation with a focus on whether newer concepts regarding PKD activation through dimerization could contribute to our understanding of PKD-dependent actions in the heart.

**Protein kinase D (PKD) structure; the canonical mechanism for PKD activation.** PKD isoforms share a common modular domain structure consisting of a C-terminal kinase domain and an N-terminal regulatory domain (Fig 1). Studies to date have focused on the tandem C1A/C1B motifs in the regulatory domain that anchor full-length PKD to lipid membranes and the pleckstrin homology (PH) motif that participates in an intramolecular autoinhibitory interaction that regulates enzyme activity (Rozengurt et al., 2005; Steinberg, 2012). The classical model for PKD activation involves receptor-dependent pathways that promote DAG
accumulation and co-localize PKD with allosterically-activated novel protein kinase C isoforms (nPKCs) at the plasma membrane, leading to nPKC-dependent trans-phosphorylation of PKD at conserved serine residues in the AL. Activated forms of PKD1, PKD2, and PKD3 then phosphorylate target substrates. PKD1 and PKD2 also autophosphorylate at a PKD consensus phosphorylation motif (LxxRxS/T, in a PDZ domain-binding motif) at the extreme C-terminus - this modification regulates PKD1/2 interactions with PDZ domain containing scaffolding proteins, PKD1/2 trafficking, and the amplitude/tempo of PKD1/2 signaling responses (Kunkel et al., 2009; Sanchez-Ruiloba et al., 2006). PKD3 lacks a C-tail autophosphorylation site and is not regulated in this manner.

**PKC-independent AL autophosphorylation.** PKD activation is typically associated with an increase in AL phosphorylation, a post-translational modification originally ascribed to a nPKC-dependent mechanism based upon a large number of studies showing that AL phosphorylation is effectively blocked by PKC inhibitors that do not directly inhibit PKD activity. However, it is worth noting that there is only very limited direct in vitro experimental evidence that nPKC isoforms actually phosphorylate PKD1 at its AL sites (at Ser^744 and/or Ser^748; nomenclature based upon rodent sequence corresponding to Ser^738 and Ser^742 in human PKD1).

Our current understanding of the mechanisms that regulate AL phosphorylation also is confounded by the fact that most studies have relied on a phosphorylation state specific antibody (PSSA) labeled anti-PKD1-pS^744/pS^748 that actually tracks phosphorylation primarily at Ser^744 (not Ser^748). With the development of a different PSSA that specifically recognizes Ser^748 phosphorylation, it became apparent that the mechanisms and consequences of PKD1 phosphorylation at Ser^744 versus Ser^748 differ. Specifically, cell-based studies show that the catalytically-inactive PKD1 mutant enzyme is phosphorylated at Ser^744, but not at Ser^748 in cells treated with PMA; this result argues that kinase dead PKD1 is phosphorylated at Ser^744 in trans by an endogenous cellular Ser/Thr kinase (namely PKC) – and that PKD1-
Ser\textsuperscript{748} phosphorylation in cells requires an autophosphorylation reaction that is defective in the catalytically inactive enzyme (Rybin et al., 2009). Conversely, the constitutively active PKD1-ΔPH deletion mutant (lacking the PH domain) is recovered from COS-7 cells with a high level of AL phosphorylation at Ser\textsuperscript{748} (but not at Ser\textsuperscript{744}) even when cells are treated with a pan-PKC inhibitor (Rybin et al., 2009). These results indicate that PKD1-ΔPH-Ser\textsuperscript{748} phosphorylation is attributable to a PKC-independent autocatalytic reaction. It is worth noting that this particular feature of PKD regulation is evolutionally conserved; the AL sequence of DFK-1 (the *Caenorhabditis elegans* PKD homolog) contains a single phospho-acceptor site at QFRKT\textsuperscript{588} (corresponding to Ser\textsuperscript{748} in PKD1) that is a targeted for a PKC-independent autocatalytic reaction (Feng et al., 2006).

There has been some progress in defining the molecular controls for AL autophosphorylation. We showed that a Ser-Ala substitution at the C-tail autophosphorylation site (at Ser\textsuperscript{916}) prevents PKD1 autophosphorylation at Ser\textsuperscript{748} (i.e., the major AL autocatalytic site); the C-tail autophosphorylation site Ser-Ala substitution has little effect on autophosphorylation at Ser\textsuperscript{744} (Rybin et al., 2009). This result indicates that a C-tail autophosphorylation reaction is required (i.e., in some way primes or structures the kinase core of the enzyme) for autophosphorylation at Ser\textsuperscript{748}. Other recent studies suggest that AL phosphorylation requires dimerization and the physical association of two PKD molecules, an interaction that facilitates kinase domain trans-autophosphorylation (Elsner et al., 2019).

**PKD isoform activation during oxidative stress.** PKD isoforms are activated in response to oxidative stress via a mechanism that differs from the canonical GPCR-dependent pathway and is distinct for individual PKD isoforms (see Fig 2) (Cobbaut and Van Lint, 2018).

**PKD1:** The redox-dependent mechanism for PKD1 activation (the isoform most intensively studied in this context) involves a hierarchical series of phosphorylations involving c-Abl and Src (two non-receptor tyrosine kinases that are activated by oxidative stress): c-Abl-dependent...
phosphorylation at Y{463} in the PH domain leads to a conformational change that is permissive for Src-mediated phosphorylation at Y{95} in the N-terminus. pY{95} serves as a docking site for the PKCδ-C2 domain, which then phosphorylates PKD1’s AL at Ser{738}/Ser{742} (Doppler and Storz, 2007; Storz et al., 2003; Storz and Toker, 2003b). While ROS-activated PKD1 has been implicated in the activation of the apoptosis signal-regulating kinase 1 (ASK1)-JNK pathway (a cell death pathway (Zhang et al., 2005a)), and a previous study from our laboratory implicated PKD1 in a H_{2}O_{2}-activated pathway that decreases the abundance of the transcription factor cyclic-nucleotide regulatory element binding protein (CREB) thereby disrupting Cre-dependent gene expression in cardiomyocytes (Ozgen et al., 2009), most studies (performed in non-cardiomyocytes) have focused on ROS-activated PKD1 localization to mitochondria where it triggers a mitochondrion-to-nucleus signaling pathway involving nuclear factor κB (NFκB). This PKD1-NFκB pathway induces expression of antioxidant/anti-apoptotic genes such as MnSOD that detoxify cellular ROS and promote cell survival (Storz et al., 2005; Storz and Toker, 2003a).

Of note, the canonical growth factor-dependent pathway for PKD1 activation does not decrease CREB protein abundance, stimulate NFκB or induce MnSOD. These results emphasized that [1] ROS-activated PKD1 can serve as a nodal point in signaling networks that trigger either pro-survival or pro-apoptotic responses and [2] the signaling repertoire and cellular actions of PKD1 are highly contextual.

**PKD2:** Tyrosine phosphorylation and PKCδ also are required for ROS-dependent activation of PKD2, but the mechanism differs. Here, an initial PKCδ-dependent phosphorylation of AL serine residues primes the enzyme for subsequent c-Abl-dependent phosphorylation at Y{717} in the activation segment P+1 loop (a sequence that is highly conserved in all PKD isoforms, but phosphorylated only in PKD2); the PKCδ-PKD2 interaction does not require phosphorylation of the conserved putative PKCδ-C2 domain docking site (Y{87}) in PKD2’s N-terminus (Cobbaut et al., 2017). Redox-activated PKD2 contributes to NFκB activation, but via a mechanism that does not require PKD2 kinase activity (Cobbaut and Van Lint, 2018; Mihailovic et al., 2004).
Rather, PKD2’s kinase-independent role has been attributed to the formation of PKD1/PKD2 heterodimers, with PKD2 acting as a scaffold to facilitate PKD1 activation (either by trapping activators such as PKCδ, Abl, and Src or by protecting PKD1 from phosphatase inactivation). While the Y87 phosphorylation site in the PKD2 N-terminus is not required for ROS-dependent PKD2 activation, Y87 phosphorylation (via a RhoA-Src pathway) is required for PKD2 localization to focal adhesions, PKD2 interaction with focal adhesion kinase, and PKD2 regulation of cell adhesion/migration (Durand et al., 2017). Of note, PKD1 and PKD2 N-terminal tyrosine phosphorylation sites sit in close proximity to the recently described dimerization interface (vide infra); the notion that N-terminal tyrosine phosphorylation interferes with PKD dimerization has not been considered.

PKD3: There is a limited amount of evidence for redox-regulation of PKD3. Specifically, our studies using Mn2+-Phos-tag sodium dodecyl sulfate polyacrylamide gel electrophoresis (a method that exaggerates phosphorylation-dependent mobility shifts) show that H2O2 activates all three PKD isoforms (PKD1, PKD2, and PKD3) in cardiomyocytes (Qiu and Steinberg, 2016). The structural basis for ROS-dependent activation of PKD3 remains uncertain since PKD3 contains a Phe in place of Tyr at the putative N-terminal PKCδ-C2 domain-docking site.

PKD1 cleavage by caspase-3 in the setting of apoptosis. Caspase-3 cleaves PKD1 at a site in the C1-PH interdomain (that is conserved in PKD1, but not PKD2 or PKD3), generating a catalytic fragment that retains the autoinhibitory PH, but not the C1, domain (Endo et al., 2000). The functional consequences of caspase-3-dependent PKD1 cleavage have been disputed. While early studies concluded that PKD1 is a proteolytically-activated enzyme, this was based on in vitro kinase assays showing that the PKD1 C-terminal cleavage product generated during apoptosis displays enhanced lipid-independent phosphorylation of a peptide substrate (Endo et al., 2000; Hausermann et al., 1999). Studies from our laboratory challenged this conclusion by showing that peptide kinase assays do not necessarily provide valid surrogate readouts of PKD1 activity toward physiologically relevant protein substrates (Rybin et al., 2009). We showed
that the PKD1-Δ1–321 truncation mutant (which models the C-terminal fragment generated as a result of caspase-3 cleavage) displays constitutive lipid-independent S916-autocatalytic activity and it phosphorylates small peptide substrates (such as CREBtide), but it does not autophosphorylate at its AL or phosphorylate protein substrates such as cyclic-nucleotide regulatory element binding protein (CREB) or cardiac troponin I (Rybin et al., 2012). Cell-based studies provide further evidence that PKD1 cleavage provides a mechanism to limit its activity, showing that PKD1-Δ1–321 does not substitute for wild-type PKD1 as an in vivo activator of the CREB or ERK phosphorylation pathways (Rybin et al., 2012). Other studies showing that PKD1’s actions to regulate lipoprotein lipase (LPL)-mediated triglyceride accumulation in cardiomyocytes is lost in the diabetic heart as a result of activation of caspase-3 and caspase-3-dependent cleavage/inactivation of PKD1 lend further support to the notion that caspase-3-dependent cleavage provides a mechanism to inhibit PKD1 (Kim et al., 2009).

What is the significance of the contextual differences in PKD isoform activation? The conventional allosteric model for PKD activation by lipid cofactors - which focuses on translocation events that deliver the enzyme in an active conformation to target substrates in membranes - assumes that PKD substrates are restricted to DAG-enriched membranes. This common stereotypical model for PKD activation does not explain observed differences in the subcellular compartmentalization patterns and spatiotemporal regulation of individual PKD isoforms that have been described in various cell types and it does not adequately explain PKD phosphorylation of substrates in other cellular compartments (such as mitochondria, nucleus, or sarcomere (Bossuyt et al., 2011; Rey et al., 2001; Rey et al., 2003)). The conventional allosteric model for PKD activation by lipid cofactors also assumes that PKD activity is an inherent/immutable property of the enzyme that is not altered by the activation process. However, there is growing evidence that stimulus-induced changes in phosphorylation can alter the enzymology (either activity, co-factor requirements, or evidence substrate-specificity) of
certain protein kinases. We previously described such a mechanism for a different lipid-regulated protein kinase, namely PKCδ. We identified differences in the enzymology of the PMA- versus the redox-activated PKCδ enzyme; PKCδ becomes a lipid-independent kinase with altered substrate specificity when tyrosine phosphorylated by Src (Steinberg, 2015). In this context, it is interesting to note that there is only very limited information on the enzymology of redox-activated (Tyr-phosphorylated) PKDs. Available studies have not considered whether the redox-mode for PKD activation (that is accompanied by a distinct set of phosphorylations on the enzyme) ‘fine-tunes’ PKD’s activity. Context-dependent changes in PKD’s enzymology could underlie observed differences in the signaling properties of the redox-activated PKD1 versus PKD2 enzymes and the observation that PKD2 phosphorylates the interferon-α/β receptor chain 1 at QTSQDP535 - a site that does not conform to the PKD LxRxxpS/T consensus phosphorylation motif and is not phosphorylated by PKD1 or PKD3 (Zheng et al., 2011). Finally, it’s worth noting that the Newton laboratory has taken advantage of genetically encoded fluorescence resonance energy transfer-based kinase activity reporters for PKD (DKAR) to identify differences in the spatiotemporal control of PKD activation at Na⁺/H⁺ exchanger regulatory factor 1 (NHERF-1, the PDZ domain-containing scaffolding protein that interacts with PKD1 and PKD2) when compared to PKD activity in the bulk cytosol or at the plasma membrane; the phorbol ester (PdBu) or receptor (histamine) evoked increases in PKD activity at NHERF-1 are more rapid and sustained (although decrease in overall amplitude) when compared to the PKD activation profiles in the other cellular compartments (Kunkel et al., 2009). These studies raise the intriguing notion that other mechanisms for PKD activation (during oxidative stress or as a result of proteolytic cleavage) might also lead to the accumulation of pools of enzyme with distinct catalytic properties and that PKD activation signatures are stimulus-specific. Given that signaling output reflects the ensemble effects of both the individual PKD isoforms co-expressed by a particular cell type and their distinct activation profiles in
different subcellular compartments, such a mechanism would vastly increase the signaling repertoire of PKD family enzymes.

**PKD dimerization.** There is evidence that PKDs (in most cases, PKD2 and PKD3) activate effector responses such as protein transport from the trans-Golgi network [TGN] to the cell surface and F-actin-directed cell motility as dimers (Bossard et al., 2007; Doppler et al., 2014); PKD2 is also reported to play a kinase-independent role (in heterodimeric complexes with PKD1) in reactive oxygen species- (ROS-) dependent activation of NF-κB (Cobbaut and Van Lint, 2018; Mihailovic et al., 2004). However, the notion that dimerization plays a more general role to regulate PKD activity (and the identification of the structural basis for PKD dimerization) is quite recent and is summarized in this section.

The Rubin laboratory identified a highly conserved 92 amino acid sequence N-terminal to the C1a DAG binding site of mammalian PKD1-2 and Dkf-2A (a *C. elegans* PKD homolog) that functions independently of other regulatory or catalytic domain regions to direct PKD monomer incorporation into homo- or heterodimeric complexes (Fig 1 (Aicart-Ramos et al., 2016)). Importantly, this study also demonstrated that overexpression of the isolated N-terminal dimerization domain (NTDD) is sufficient to disrupt PKD dimerization, PKD phosphorylation of a cellular substrate (phosphatidylinositol 4-kinase IIIβ), and a PKD-mediated response (protein transport from the TGN to the cell surface) in HEK293 cells. It also showed that DKF-2A homodimerization is essential for activation of the innate immune response (a PKD-regulated function) in *C. elegans* (Aicart-Ramos et al., 2016).

A subsequent study from the Leonard laboratory resolved the crystal structure of the *C. elegans* NTDD, showing that it adopts a ubiquitin-like fold with a hydrophobic patch that serves as a dimerization interface for all three PKDs (Elsner et al., 2019). They showed that introduction of a negative charge (a Phe-Glu substitution) at a critical site in the NTDD interface or selective deletion of the entire NTDD is sufficient to abrogate PKD1 homodimerization, PKD1
heterodimerization with PKD2 or PKD3 and agonist-dependent PKD1 AL phosphorylation in cells (Elsner et al., 2019). The NTDD-mediated dimerization mechanism identified in these studies would explain the enzymatic profile of PKD1-Δ1–321, the truncation mutant that models the PKD1 C-terminal fragment generated in cells as a result of caspase-3 cleavage. This truncation mutant (that lacks the NTDD) does not autophosphorylate at its AL and it has reduced catalytic activity (Rybin et al., 2012). This model also provides a plausible explanation for the strong dominant negative effect resulting from overexpression of KD-PKD or PKD truncation mutants consisting of only the regulatory domain (mutants that model the N-terminal species formed as a result of caspase-3 cleavage during apoptosis (Endo et al., 2000; Haussermann et al., 1999) or the PRKD1 truncation mutant identified in certain patients with syndromic CHD (Shaheen et al., 2015); vide infra). These species would act as dominant negative inhibitors by preventing the formation of homodimers of full-length PKDs.

These studies provide elegant, detailed, and convincing structural insights into the role of the NTDD as a mediator of PKD dimerization/activation, but it is worth noting that studies to date have focused on dimerization as a mechanism to control AL autophosphorylation on PKD1, with the implicit assumption that a similar AL autophosphorylation mechanism holds for PKD2 and PKD3. In fact, there is still only limited information on the mechanisms that regulate PKD2 and almost no information on the enzymology of PKD3. While PKD1 and PKD2 share many structural features, PKD3 lacks a C-tail autophosphorylation site and (like the PKD1-S916A mutant) it does not autophosphorylate at its major AL autophosphorylation site. This result emphasizes that some aspects of PKD3’s enzymology differ – and it raises the intriguing possibility that PKD3 may achieve full catalytic competence only when heterodimerized with PKD1 or PKD2 (i.e., that the early evidence that dimers of PKD2/3, and not other isoforms, are required for certain cellular responses was not serendipitous, but rather was indicative of this property). It is also important to emphasize that our current understanding of the functional significance of PKD dimerization is based upon a very limited set of studies in mammalian cells.
The notion that dimerization provides a mechanism to control or diversify physiologically relevant PKD signaling or functional responses (i.e., to enhance the signaling repertoire of PKD family enzymes) in differentiated mammalian cells deserves further study.

**PKD ISOFORM-SPECIFIC FUNCTIONS IN CARDIOMYOCYTES.**

PKD isoforms are activated in an isoform-specific manner in cardiomyocytes. Cardiomyocytes co-express PKD1, PKD2, and PKD3. While there is evidence that some cellular responses are activated in a functionally redundant fashion by multiple PKDs, studies from our laboratory expose a mechanism for isoform specificity showing that PKD isoforms are activated in an agonist- and isoform-specific manner in neonatal cardiomyocyte cultures. Specifically, we showed that \( \alpha_1 \)-adrenergic receptor (\( \alpha_1 \)-AR) agonists specifically activate PKD1 (not PKD2 or PKD3), whereas the protease-activated receptor (PAR) agonist thrombin or the endothelial differentiation gene (EDG) receptor agonist sphingosine 1-phosphate (S1P) preferentially activating PKD2 and PKD3 (Fig 3 (Guo et al., 2011; Qiu and Steinberg, 2016)). Inhibitor studies link the \( \alpha_1 \)-AR-PKD1 pathway to increased phosphorylation of CREB, an increase in Cre-dependent gene expression, and induction of Bcl-2 (a CREB target gene; i.e. PKD1 links \( \alpha_1 \)-AR activation to CREB-S\(^{133} \) phosphorylation in CMs (Guo et al., 2011)). In contrast, thrombin and S1P activate a different Rho-dependent pathway that preferentially activates PKD2/PKD3 and promotes CREB-S\(^{133} \) phosphorylation; Rho is not required for \( \alpha_1 \)-AR-dependent-PKD1-CREB-S\(^{133} \) phosphorylation (Qiu and Steinberg, 2016). The observation that Rho recruits a signaling pathway that is specific for PKD2/PKD3 resonates with a previous study from the Storz laboratory implicating PKD2-PKD3 complexes in the Rho-dependent pathway that regulates cofilin phosphorylation and cell migration (Doppler et al., 2014).

**Hypertrophy/Heart failure.** Early studies focused on PKD1’s role in pathologic cardiac remodeling, showing that PKD1 knockdown attenuates agonist-induced hypertrophy of cultured
cardiomyocytes, cardiac-specific PKD1-S744E/S748E overexpression induces hypertrophy which transitions to HF, and that cardiac-specific PKD1 deletion prevents transverse aortic constriction- (TAC-) induced hypertrophy and cardiac fibrosis (Fielitz et al., 2008; Harrison et al., 2006). These cardiac actions of PKD have been attributed to phosphorylation of HDAC5, a class IIa histone deacetylase that acts as an inhibitory regulator of myocyte enhancer factor 2 (MEF2). PKD-dependent HDAC5 phosphorylation at S^{259}/S^{498} creates docking sites for 14-3-3 proteins that escort HDAC5 from the nucleus, freeing nuclear MEF2 to activate pathologic gene programs (Fig 4). While all 3 PKD isoforms possess in vitro HDAC5 kinase activity, studies to date have focused on the cellular actions of PKD1, showing that siRNA knockdown of PKD1 blunts (but does not fully abrogate) receptor-dependent HDAC5 phosphorylation (Huynh and McKinsey, 2006; Matthews et al., 2006; Vega et al., 2004). These results could suggest that PKD isoforms regulate HDAC5 in a functionally redundant manner, although the alternative interpretation - that this is regulated by PKD dimers - also is possible and has never been considered.

There is evidence that the PKD1/HDAC5/MEF2 pathway is amplified through interactions with scaffolding proteins such as A kinase anchoring protein-Lbc (AKAP-Lbc) or the muscle A-kinase anchoring protein β (mAKAPβ), two scaffolds that serve as platforms to coordinate PKD activation by PKC (Carnegie et al., 2004; Carnegie et al., 2008; Diviani et al., 2001; Dodge-Kafka et al., 2018; Kritzer et al., 2014). AKAP-Lbc is expressed at high levels in cardiomyocytes, is upregulated in response to hypertrophic stimuli, and is unique among AKAPs in that it has Rho-GEF activity; it enhances signaling efficiency through the PKD/HDAC5/MEF2 pathway that activates the fetal gene program (Carnegie et al., 2004; Carnegie et al., 2008; Diviani et al., 2001). Similarly, mAKAPβ is a muscle-specific scaffolding protein that serves as a platform to organize a signaling module involving PKD1, its upstream activator phospholipase Cε (which generates a local pool of DAG from phosphatidylinositol 4-phosphate in the perinuclear Golgi apparatus (Zhang et al., 2013)) and HDAC4/5; targeted deletion of mAKAPβ
disrupts in vivo pressure overload-induced PKD activation and PKD-dependent HDAC4/5 phosphorylation (Dodge-Kafka et al., 2018; Kritzer et al., 2014). Studies to date have focused on AKAP-Lbc or mAKAPβ as scaffolds for PKD1. The notion that these proteins also bind/scaffold PKD2/3 has never been considered.

Finally, PKD also phosphorylates CREB, a bZip transcription factor that binds CREs in the regulatory region of CREB target genes and contributes to the maintenance of normal ventricular structure and function (Fentzke et al., 1998; Ozgen et al., 2008). PKD-dependent CREB-S\textsuperscript{133} phosphorylation leads to the recruitment of co-activators (such as CREB-binding protein/p300, also a PKD substrate) and increased expression of CREB target genes, including Bcl-2, atrial natriuretic factor, and brain natriuretic peptide (Zhang et al., 2005b). We previously showed that \(\alpha_1\)-ARs activate a PKC\(\delta\)-PKD pathway leading to CREB-S\textsuperscript{133} phosphorylation, increased Cre-dependent gene expression and induction of Bcl-2 in cardiomyocytes (Ozgen et al., 2008). However, the relative importance of PKD-dependent pathways involving CREB vs. HDAC5 in adverse cardiac remodeling has never been directly examined and remains uncertain.

**Phosphorylation of sarcomeric proteins.** PKD regulates cardiac myofilament function (and thereby contraction) through the coordinated phosphorylation of multiple sarcomeric proteins at sites that also are targets for phosphorylation by PKA. PKD decreases myofilament Ca\textsuperscript{2+} sensitivity by phosphorylating cardiac troponin I (cTnI) at Ser\textsuperscript{22}/Ser\textsuperscript{23} (or Ser\textsuperscript{23}/Ser\textsuperscript{24}, if the initiating methionine is counted) and it accelerates cross-bridge cycle kinetics at least in part by phosphorylating the thick filament protein cardiac myosin binding protein-C at Ser\textsuperscript{302} (Bardswell et al., 2010; Martin-Garrido et al., 2018). Of note, these PKD phosphorylation sites also are targets for phosphorylation by PKA, fueling speculation that PKD-dependent regulation of cardiac contractility may assume greater functional significance in settings such as heart failure, where the PKA pathway is down-regulated. Moreover, while PKA phosphorylates cTnI at both Ser\textsuperscript{22} and Ser\textsuperscript{23}, the prevailing notion that phosphorylation at both sites is required to decrease
myofibrillar Ca$^{2+}$ sensitivity was challenged by studies from the Avkiran and Kentish laboratories showing that PKD phosphorylates cTnI primarily at Ser$^{23}$ and this PKD-dependent monophosphorylation of cTnI is sufficient to decrease myofibrillar Ca$^{2+}$ sensitivity (Martin-Garrido et al., 2018). Again, all 3 PKD isoforms share this in vitro kinase activity, but their individual roles in the regulation of contraction and the notion that a dimerization mechanism may influence sarcomeric protein phosphorylation have never been considered.

**Cardiac adaptation to metabolic derangements in the diabetic heart.** PKD’s regulate vesicles fission from Golgi membranes and thereby control trafficking of LPL to surface membranes (Kim et al., 2008). In the setting of moderate hypoinsulinemia, (with impaired glucose uptake and glycolysis), a PKD-dependent pathway that requires the formation of a functional complex between PKD, LPL, and β3 integrin increases cardiac LPL, allowing for enhanced hydrolysis of circulating triglycerides and the liberation of free fatty acids that can be used as an alternative energy source by cardiomyocytes (Kim et al., 2008; Xiao et al., 2020). However, in the setting of severe hypoinsulinemia, a different PKD-dependent pathway - that involves caspase-3-dependent cleavage and inactivation of PKD1 (the only isoform that contains a caspase-3 cleavage site) - functions to limit PKD-mediated delivery of LPL to the surface membrane and prevent cardiac triglyceride overload (Kim et al., 2009).

**PKD isoforms and cardiac injury vs. cardioprotection.** PKDs have been implicated in both ischemia/reperfusion injury and cardioprotection. Studies from our laboratory show that oxidative stress (a stimulus that plays an important role in the evolution of HF syndromes) leads to the activation of PKD1, PKD2, and PKD3 in cardiomyocytes and cardiac fibroblasts (Qiu and Steinberg, 2016) and that H$_2$O$_2$-dependent activation of PKD1 leads to a decrease in the abundance of CREB (a transcription factor that is important for the maintenance of normal physiologic function) in cardiomyocytes (Ozgen et al., 2009). However, other studies implicate PKD in an S1P receptor pathway that regulates cofillin phosphorylation and confers cardioprotection. The PKD-dependent pathways that regulate cofillin (a mechanism investigated
most comprehensively in studies that interrogate cofilin’s role as an actin-binding protein that regulates of cell motility and cell migration) are described below.

Cofilin’s role in the regulation of cell migration is controlled through a complex/elaborate set of phosphorylations. Cofilin phosphorylation by LIM kinase (LIMK) leads to a decrease in cell migration, whereas cell migration is restored when cofilin is dephosphorylated by the protein phosphatase slingshot 1 (SSH1L). Importantly, SSH1L is a substrate for (and is negatively regulated by) PKD. PKD-phosphorylated SSH1L interacts with 14-3-3 adaptor proteins that sequester SSH1L in the cytosol away from cofilin and F-actin (Eiseler et al., 2009; Niwa et al., 2002; Peterburs et al., 2009) (see schematics; Figs 2 and 4). However, PKDs can also increase cofilin phosphorylation by phosphorylating and activating p21-activated kinase (PAK4) an upstream activator of LIMK (Spratley et al., 2011).

Studies in cardiomyocytes have focused on the S1P-RhoA-PKD signaling pathway; this pathway inactivates SSH1L, increases cofilin phosphorylation and thereby prevents cofilin translocation to mitochondria, prevents the cofilin interaction with the proapoptotic molecule Bax, and blocks redox-induced cell death (see schematic in Fig 2 (Chua et al., 2003; Xiang et al., 2013)). While this pathway would confer cardioprotection, there also is evidence that this mechanism might be counterbalanced by an α₁-AR-PKD pathway that leads to phosphorylation of the mitochondrial fission protein dynamic-like/related protein 1 (DLP1, also known as DRP-1) at its PKD consensus site (S⁶₃⁷); PKD-phosphorylated DLP1 is localized to the outer mitochondrial membrane where it triggers ROS generation, activates the mitochondrial permeability transition pore (mPTP), promotes mitochondrial fragmentation and activates apoptotic signaling (cytochrome c release, caspase cleavage) (Jhun et al., 2018). In theory, these results could suggest that PKD might play opposing roles to fine-tune the cardiomyocyte apoptotic response through the S1PR-pathway that confers cardioprotection and the α₁-AR-pathway that promotes apoptosis.
**PRKD1 mutations and syndromic congenital heart disease (CHD).** Genome-wide association studies link the *PRKD1* gene to elevated body mass index (a cardiovascular risk) and *PRKD1* has also been implicated in certain forms of syndromic congenital heart disease (CHD) (Alter et al., 2020; Graff et al., 2013; Shaheen et al., 2015; Sifrim et al., 2016; Speliotes et al., 2010). Shaheen *et al.* implicated a homozygous truncation mutation in *PRKD1* that results in the generation of a catalytically inactive protein (containing the entire N-terminal regulatory domain but only the first 35 residues of the kinase domain N-terminus) in truncus arteriosus (Shaheen et al., 2015). Sifrim *et al.* and our group identified heterozygous de novo missense mutations in *PRKD1* in 5 patients with syndromic CHD associated with skin and limb abnormalities and developmental delay (Alter et al., 2020; Sifrim et al., 2016). 4 of the 5 missense mutations map to the kinase domain. The implicit assumption of previous studies (that did not perform biochemical assays) was that disease-causing de novo *PRKD1* mutations would be inactivating; this assumption was based largely on literature showing that homozygous *PRKD1* knockout in mice is embryonic lethal (Fielitz et al., 2008). However, we showed that the kinase domain R603H substitution identified in one patient is a gain-of-function mutation; PKD1-R603H displays high levels of constitutive/lipid-independent autocatalytic and CREB kinase activity (Alter et al., 2020). The G592R mutation identified in the 3 other patients is at the 2nd glycine in the highly conserved Gly-rich loop (or G-loop), a residue conserved in more than 99% of eukaryotic protein kinases and plays a critical role to bind and orient ATP in an optimal position for catalysis (Hemmer et al., 1997; Steinberg, 2018). While modelling studies based upon structural data suggest that other residues at this position would result in steric clash and disrupt catalytic activity (Hemmer et al., 1997), we showed that PKD1-G592R is recovered from HEK293 cells with a considerable amount of lipid-dependent autocatalytic activity and reduced (but not absent) CREB-Ser\(^{133}\) kinase activity (Alter et al., 2020). These results emphasize that phenotypes described in gene knockout models (where there is a loss of protein and activity) may not be relevant to heterozygous CHD-causing PKD1 truncation or missense mutations.
where there is a change in enzyme activity, but preserved full-length or truncated protein (protein that can still exert a kinase-independent functions in PKD homo- or hetero-dimers or other multiprotein complexes involving PKD).

**CONCLUSION AND CLINICAL IMPLICATIONS**

In the years since PKDs first emerged as signaling enzymes that play pivotal roles in biological mechanisms that influence cell growth, cell differentiation, pro-apoptotic and survival pathways, and angiogenesis, PKDs have been implicated in the pathogenesis of many inflammatory and metabolic disorders, the evolution of cardiac hypertrophy, ischemia/reperfusion injury and heart failure syndromes, and the pathogenesis and progression of many cancers. The evidence that PKD isoform expression is deregulated in many cancers (with *PRKD1* gene mutation identified in over 2.2% of cancer samples in The Cancer Genome Atlas and high frequency somatic mutations in *PRKD* genes identified in polymorphus low-grade adenocarcinoma and in angiolipomas (Weinreb et al., 2014)) has provided a rationale to use high throughput screens to develop compounds that interdict signaling by PKDs. Several laboratories have identified potent, selective small-molecule pan-PKD inhibitors that show considerable promise in preclinical cancer models (Gamber et al., 2011; Guha et al., 2010; Lavalle et al., 2010; Meredith et al., 2010; Monovich et al., 2010; Roy et al., 2017; Sharlow et al., 2008). However, newer insights into the mechanisms and actions of PKD family enzyme that are summarized in this review raise certain caveats that must be considered in assessing the efficacy and safety associated with the use of pan-PKD inhibitors. First, cells typically co-express multiple PKD family members. While PKD isoforms exhibit some functionally redundant actions, this review describes a number of functionally important differences in the activation mechanisms and signaling repertoires for individual PKD isoforms. A pan-PKD inhibitor may be of limited clinical utility in conditions where PKD1 and PKD2/3 exert antithetical actions, such as in certain cancers (including breast, prostate, and gastric cancer) where PKD1
acts as a tumor suppressor and PKD2/PKD3 increase proliferation, invasion and chemoresistance. A similar limitation may apply to the use of inhibitors that lack isoform-specificity to prevent ischemia/reperfusion injury or adverse cardiac remodeling, where differences in the activation patterns and cellular actions of PKD1 versus PKD2/3 in cardiomyocytes have been identified. These concerns provide a strong rationale for the development of isoform-selective PKD inhibitors.

It is also worth emphasizing that PKD-targeted pharmaceuticals that restrict proliferation, invasion, or angiogenesis in cancer might be predicted to have clinically important 'off-target' effects by inhibiting PKD-dependent pro-survival pathways in other tissues. The field of cardio-oncology has emerged in recent years in response to growing concerns that cancer therapies that promote tumor cell apoptosis also limit prosurvival pathways in cardiomyocytes and lead to a cardiotoxicity that limits their use in a clinical setting (Sheng et al., 2016). This issue also much be considered.

Finally, this review focuses on the newer concept that a dimerization mechanism is required for at least certain PKD-driven responses. The mapping of a functionally important PKD dimerization interface provides the basis for future studies that use high-throughput screening and structure-based drug design to develop peptide inhibitors of this protein-protein interaction, compounds that can be exploited as research tools and could serve as prototypes for future therapeutics.

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**FIGURE LEGENDS**

**Fig 1. Domain structure of PKD1, PKD2, and PKD3.** The cartoon depicts the domain structure of PKD isoforms with conserved NTDD, C1A-C1B, and PH domains in the regulatory region and the kinase domain in the catalytic region. Sites for tyrosine phosphorylation in NTDD (conserved in PKD1 and PKD2) and the PH domain (conserved in all three PKDs), the kinase domain activation loop phosphorylation motif (conserved in all three PKDs), and the C-terminal autophosphorylation site (conserved in PKD1 and PKD2, but not PKD3) are depicted.

**Fig 2. PKD activation loop phosphorylation mechanisms.** The conventional model ascribes PKD phosphorylation at S$^{744}$ and S$^{748}$ in the activation loop to novel isoforms of PKC, but the PKD-S$^{748}$ activation loop site can also be phosphorylated via an autocatalytic mechanism.
Fig 3. Schematic of the oxidative stress pathways that activate PKD isoforms and the PKD-dependent mechanisms that regulate apoptosis. Oxidative stress activates PKD through a mechanism involving coordinate phosphorylations by the redox-activated non-receptor tyrosine kinases c-Abl, Src and by PKC. While ROS-activated PKD1 has been implicated in a pathway that decreases the abundance of the transcription factor cyclic-nucleotide regulatory element binding protein (CREB) thereby disrupting Cre-dependent gene expression in cardiomyocytes, other studies have focused on the ROS-activated PKD1-NF-κB pathway that induces expression of antioxidant/anti-apoptotic genes such as MnSOD that detoxify cellular ROS and promote cell survival. PKD also has been implicated in a mechanism that regulates cofilin phosphorylation by the protein phosphatase slingshot 1 (SSH1L); this mechanism prevents cofilin translocation to mitochondria, prevents the cofilin interaction with the proapoptotic molecule Bax, and confers cardioprotection. PKD also has been implicated in an α₁-AR-dependent pathway that leads to phosphorylation of the mitochondrial fission protein dynamic-like/related protein 1 (DLP1) that localizes to the mitochondrial membrane, triggers ROS generation, and induces cellular apoptosis. PKD and proteins known to be direct substrates of PKD are shaded pink.

Fig 4. PKD isoforms are activated in an agonist specific manner in cardiomyocytes. The schematic shows that α₁-ARs promote CREB-S\(^{133}\) phosphorylation via a G\(_{q}\)-dependent pathway that activates phospholipase C-β (PLCβ) and selectively recruits PKD1 (but not PKD2 or PKD3), whereas S1P and thrombin promote CREB-S\(^{133}\) phosphorylation via a RhoA-dependent mechanism involving phosphatidylycholine-selective phospholipase D (PLD) or phospholipase C-ε (PLCε) that activates PKD2 and PKD3. These results argue that PKD isoforms are not entirely functionally redundant in the heart (i.e., that PKD1 cannot substitute for PKD2 and PKD3 in certain agonist-dependent pathways that influence cardiac remodeling).

*Based on JMCC 99:14, 2016.*
Fig 5. Schematic of the receptor-driven pathways involving PKD that influence cardiomyocyte remodeling. Proteins known to be direct substrates of PKD that regulate cofilin phosphorylation (PAK4 and SSH1L) and apoptosis, that influence cardiomyocyte contractile function (cTnI and cMyBP-C), and that influence cardiomyocyte hypertrophy (HDAC and CREB) are shaded pink.

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Figure 1
CONVENTIONAL MODEL FOR PKD1 ACTIVATION

NTDD  C1A  C1B  PH Domain  Kinase Domain

nPKC → ACTIVATION LOOP
S^{744}/S^{748}

AUTOPHOSPHORYLATION

PKD1 ACTIVATION LOOP AUTOPHOSPHORYLATION

anti-PKD1-pS^{744}/pS^{748} – preferentially recognizes pS^{748} (not pS^{748})

nPKC → S^{744}

anti-PKD1-pS^{748}

AUTOPHOSPHORYLATION AT S^{748} AND S^{916}

Figure 2
Figure 4

Diagram showing the interaction of NE (α₁-AR) and S1P Thr with their respective pathways involving Gαq, PLCβ, RhoA, PLCε, PLD, DAG/PKC, PKD1, PKD2, PKD3, and CREB-pSer133.