Title

Molecular mechanisms of Protein Kinase D1 regulation of calcium handling in ventricular myocytes

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Molecular mechanisms of Protein Kinase D1 regulation of calcium handling in ventricular myocytes

By

JULIANA MIRA HERNANDEZ
DISSERTATION

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of the

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Approved:

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Donald M. Bers, Chair

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Crystal Ripplinger

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Manuel F. Navedo

Committee in Charge

2022

Dedication

To my parents, **Augusto** and **Maria Helena**, for firmly believing in the fundamental value of a high-quality education, for their effort to give me such education and for the unconditional love and the support in every project I decide to follow.

To my sisters, **Natalia**, **Carolina** and **Valeria**, for the love and the permanent support. To **Carolina** for the essential help during the application for the Fulbright scholarship, the GRE preparation and the 101 guidelines for living in the US. To **Natalia** for teaching me about discipline and that how far I will get only depends on me. “Uno es lo que es y esta donde esta por lo que lleva en la mente” (you are who you are, and you are where you are because of what you have in your mind). To **Valeria** for always being my friend, my partner, and my biggest lesson in life.

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Abstract

Protein kinase D (PKD) is serine/threonine kinase (member of the CaM kinase superfamily) well recognized for its roles in cell proliferation, cell survival, inflammation, immunity, and cancer. In the heart, PKD is strongly linked to cardiac remodeling and myofilament calcium (Ca) sensitivity, inducing the expression of fetal genes during pressure overload induced hypertrophy and reducing myofilament affinity for Ca in response to G_q-protein coupled receptor activation. PKD has been also demonstrated to be required for the pathologic hypertrophic remodeling induced by chronic G_s-protein coupled receptor stimulation with isoproterenol (β-AR receptor agonist) in mice. Unlike some other kinases PKD's effects on physiological cardiomyocyte function during excitation-contraction coupling (ECC) remain unclear. This work presents an up-to-date literature review of PKD functions in the body and specifically in the heart (**Chapter 1**), the effects of PKD in the cardiomyocyte response to β-adrenergic stimulation (**Chapter 2**), the effects of angiotensin II-induced activation of PKD in the cardiomyocyte ECC (**Chapter 3**) and future directions (**Chapter 4**).

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Chapter 1: Protein Kinase D in systemic and heart physiology

Protein Kinase D structure and isoforms

The protein kinase D (PKD) family consist of three serine/threonine isoforms: PKD1 (gene *PRKD1*), PKD2 (gene *PRKD2*) and PKD3 (gene *PRKD3*) (Johannes et al., 1994; Valverde et al., 1994). Despite their high homology (~90%), the isoforms differ in molecular weight, abundance (according to the tissue) and functions; being PKD1 the most abundant in cardiomyocytes (Haworth et al., 2000). All PKDs contain a highly preserved catalytic domain and an N-terminal regulatory domain (Johannes et al., 1994; Valverde et al., 1994). Because of the structural and enzymatic properties of the catalytic domain, the PKD family is classified as member of the Ca^{2+}/calmodulin- dependent protein kinase (CaMK) superfamily (Avkiran et al., 2008); however, PKD is not dependent on Ca^{2+} for its activation but may be regulated by Ca^{2+} indirectly through alterations in diacylglycerol (DAG) production (Kunkel et al., 2007). The N-terminal regulatory domain comprises two cysteine-rich, zinc finger-like motifs (C1a and C1b; **Figure 1**) where DAG and phorbol esters bind (and activate PKD), a pleckstrin homology (PH) domain that at baseline inhibits catalytic activity (Valverde et al., 1994; Van Lint et al., 1995), an alanine–proline rich (AP) region (for PKD1), a proline-rich (P) region (for PKD2), and an acidic amino-acid-rich region (AD) between Clb and PH domains, whose functions remain unclear (Sundram et al., 2011). In addition, a ubiquitin-like domain (ULD) at the N-terminal (following the AP or P region), that is present in all the isoforms, was recently identified (Elsner et al., 2019). On the catalytic domain, PKD can be activated by Protein Kinase C (PKC) trans-phosphorylation of Ser744 and Ser748 in the activation loop (Rozengurt et

al., 1997; Zugaza et al., 1996). PKD can also autophosphorylate itself on C-terminal domain at Ser910 (Ser916 in mouse) and on N-terminal C1a-C1b interdomain (Ser205/Ser208/Ser219/Ser223) (Rozengurt et al., 2005; Steinberg, 2012).

PKD expression in the myocardium is highest in the perinatal period and declines substantially in the first several weeks of life (Haworth et al., 2000). Work from the Avkiran lab and others confirmed that PKD is expressed in neonatal and adult rat ventricular myocytes, adult mouse, human and rabbit myocardial tissue (Bossuyt et al., 2008; Harrison et al., 2006; Haworth et al., 2007; Iwata et al., 2005; Roberts et al., 2005; Tsybouleva et al., 2004). Similar to other genes, PKD expression in the adult heart reverts toward a fetal phenotype and is upregulated during disease (i.e. heart failure in rabbit, rat and human) (Avkiran et al., 2008; Bossuyt et al., 2008; Harrison et al., 2006).

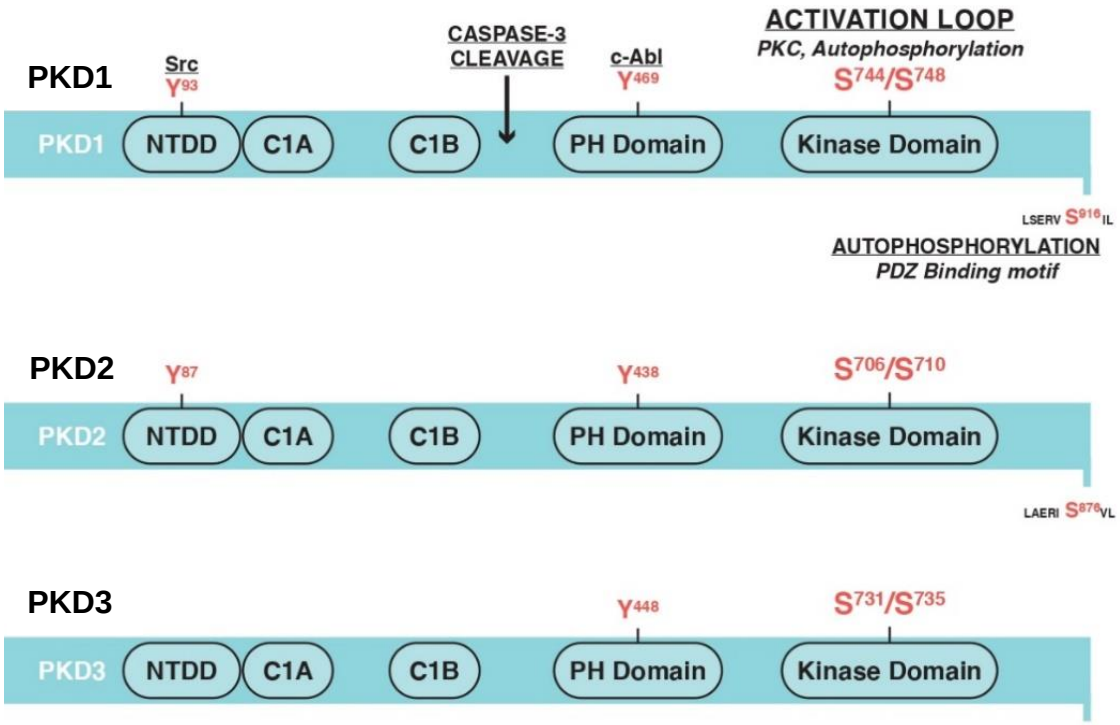


Figure 1. Protein kinase D isoforms structure and domains: C1A-C1B, and PH domains in the regulatory region and the kinase domain in the catalytic region. Sites for tyrosine phosphorylation in NTDD (conserved in PKD1 and PKD2) and the PH domain (conserved in all three PKDs), the kinase-domain activation loop phosphorylation motif (conserved in all three PKDs), and the C-terminal autophosphorylation site (conserved in PKD1 and PKD2 but not PKD3). Adapted from Steinberg, 2021.

PKD signaling and mechanisms of activation

In cardiomyocytes, the canonical pathway of PKD activation is as follows: after Gq-coupled receptor activation (GPCR) by angiotensin II, endothelin-1, norepinephrine or

growth factors, phospholipase Cβ is activated and cleaves membrane phosphotidylinositol 1,4 bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG can then activate PKD directly or by activation of Protein Kinase C (PKC). PKC phosphorylates PKD at S744/S748 (in mouse PKD1) and S738/S742 (in human) in the catalytic domain, relieving the autoinhibition by the PH domain. PKD can autophosphorylate at S916 (in mouse PKD1) and analogous S910 (in human), and in the C1a-C1b interdomain region (**Figure 2**; (Avkiran et al., 2008; Rozengurt et al., 2005; Steinberg, 2012; Waldron & Rozengurt, 2003)). Some activation differences are observed: PKD1 activation after α1-adrenergic receptor (α1-AR) stimulation is completely PKC-dependent, while endothelin (ET)-triggered PKD activation only requires PKC in the early phase (Guo et al., 2011; Haworth et al., 2000). Also, it is well known that PKDs can be activated in a PKC-independent manner through activation loop (AL) autophosphorylation (Jacamo et al., 2008; Steinberg, 2012), tyrosine-dependent phosphorylation during oxidative stress (Cobbaut et al., 2017; Cobbaut et al., 2018; Cobbaut & Van Lint, 2018; Storz et al., 2005; Storz & Toker, 2003; W. Zhang et al., 2005) and caspase-3 proteolytic cleavage during apoptosis (Endo et al., 2000; Haussermann et al., 1999; Steinberg, 2012). Recently, dimerization of two PKD molecules has been suggested as mechanism of activation by AL trans-autophosphorylation (Cobbaut & Van Lint, 2018; Elsner et al., 2019).

Recently, several authors suggested that PKD activation is not limited to DAG-enriched membranes (translocation after activation) because this does not explain the differences in regulation of PKD isoforms in subcellular compartments (mitochondria, nucleus, sarcomere) (Bossuyt et al., 2011; Rey et al., 2001). Bossuyt *et al.* observed that

upon α-adrenergic activation by phenylephrine (PE) PKD is rapidly recruited to the sarcolemma, activated and then translocates rapidly into the nucleus where it phosphorylates HDAC5, but endothelin-1 (ET-1) activated PKD remains largely at the sarcolemma (Bossuyt et al., 2011). This supports the idea that stimulus-specificity can modify the subcellular location and activity of some protein kinases including PKD (Steinberg, 2012).

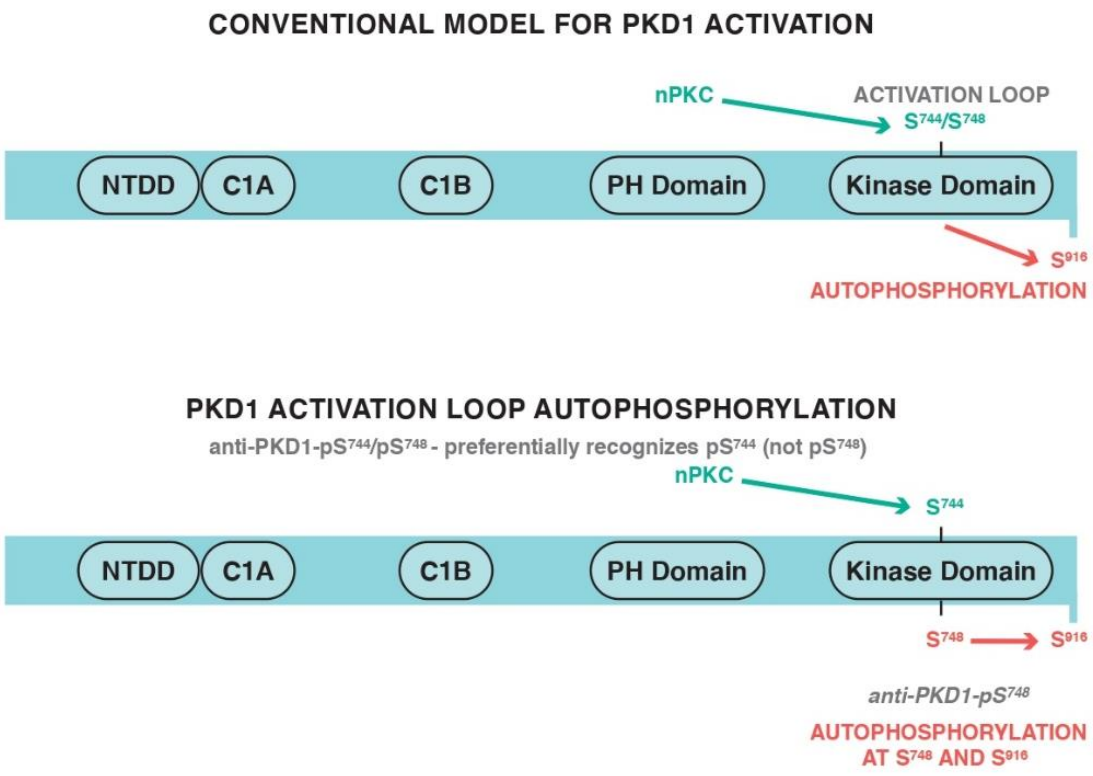


Figure 2. Representation of Protein Kinase D activation. Novel PKCs (nPKC) can activate PKD at S744 and S748 (also phosphorylated via an autocatalytic mechanism) in the activation loop. Adapted from Steinberg, 2021.

Neurohumoral activation

As mentioned before, PKDs can be activated in an agonist-specific manner in neonatal and adult cardiomyocytes and fibroblasts by stimulation of **Gq-coupled receptors (GqPCRs)** like **α-adrenergic receptors (α1-AR)** and **endothelin-1 (ET-1)** receptors. On one side, α1-AR (i.e. agonist phenylephrine) can induce a fast increase in PKD activation that can last for an hour and this is PKC-dependent. PKD can then translocate to the nucleus and phosphorylate class IIa histone deacetylase 5 (HDAC5). This phosphorylation drives HDAC5 nuclear export and consequent de-repression of MEF2-regulated transcription. On the other hand, ET-1 receptors induce a transient activation of membrane localized PKD that is PKC-dependent, but then is followed by a sustained activation of PKD that is PKC-independent with less prominent PKD-driven HDAC5 phosphorylation and nuclear export (Bossuyt et al., 2011; Bossuyt et al., 2008; Guo et al., 2011; Haworth et al., 2000; Steinberg, 2012). There are also differences in activation of the isoforms, for example, norepinephrine (α/β-AR agonist) selectively activates PKD1 in neonatal cardiomyocytes and fibroblasts, while ET-1, thrombin (thrombin receptor, ThR) and platelet derived growth factor (PDGF, PDGF receptor) facilitate PKD2 and PKD3 activation (Guo et al., 2011; Qiu & Steinberg, 2016).

In H9c2 and neonatal cardiac myocytes, G_qPCR stimulation induced by α1-adrenergic stimulation mediates mitochondrial fragmentation in a PKD-dependent manner. After activation, PKD translocates to the outer mitochondrial membrane (OMM) and phosphorylates dynamin-like protein 1 (DLP1) at S637 (mitochondrial fission protein). This allows DLP-1 association with OMM and mitochondrial fragmentation, superoxide generation, mitochondrial permeability transition pore opening and apoptosis signaling activation (Jhun et al., 2018).

Up to now, the results related to **β-adrenergic receptor (β-AR)** regulation of PKD signaling have been conflicting or unconclusive. In vitro, no effect of β-AR or protein kinase A (PKA) stimulation on PKD activity was detected (Harrison et al., 2006). However, the same group reported that PKD1 cardiac-specific knock-out mice (PKD1 cKO) were protected against isoproterenol-induced (β-AR agonist) cardiac hypertrophy, indicating that PKD is necessary for chronic β-AR mediated hypertrophy in vivo (Fielitz et al., 2008). The Avkiran group reported that heterologous PKD expression in adult rat ventricular myocytes (ARVM) had no effect on isoproterenol-induced phosphorylation of phospholamban (PLB), phospholeman (PLM) and myosin-binding protein C (MyBP-C) or on isoproterenol-induced increases in sarcomere shortening, relaxation rate and Ca transient amplitude (Cuello et al., 2007). In contrast, A kinase anchoring protein (AKAP)-Lbc was reported to function as a scaffold for PKA, PKC and PKD, mediating PKD1 activity and hypertrophy induction (Carnegie et al., 2004). Additionally, others found that β-AR stimulation causes PKD translocation and nuclear signaling and inhibits G_qPCR-mediated PKD1 activation by altering its intracellular translocation (Nichols et al., 2014). In agreement with this, in other studies PKD activity was found to be reduced by β-AR agonists or PKA activation (Haworth et al., 2011; Sucharov et al., 2011). Here, phosphodiesterase 3 and 4 (PDE3 and PDE4) were detected as the key regulators of the PKA activity that inhibits PKD activation (Haworth et al., 2011). These results again show complex mechanisms of PKD signaling at different subcellular compartments but also highlight potential interaction between PKD regulation and β-AR signaling.

Moreover, profibrotic agonists like aldosterone and **angiotensin II (AngII)** also can activate PKD1 in cardiac myocytes, supporting the role of PKD in the control of cardiac

fibrosis and remodeling (Iwata et al., 2005; Tsybouleva et al., 2004). This was further proven using PKD1 cKO mice that were markedly resistant to fibrosis during chronic exposure to AngII (Fielitz et al., 2008). Despite this influence in fibrosis outcome during chronic AngII, the specific mechanism by which hypertrophy is induced remains unclear. Several groups, demonstrated that PKD can promote aldosterone synthesis in the adrenal gland which implies that PKD enhances a positive feedback in fibrosis induction in the heart (Olala et al., 2014; Romero et al., 2006).

In the heart, **AngII** induces hypertrophy directly by AT1 receptor activation or intracellular RAS activation (Kumar et al., 2008) and subsequent activation of kinases like Protein Kinase D1 (PKD1) in cardiomyocytes, that has been demonstrated as a downstream effector of AngII (Fielitz et al., 2008; Iwata et al., 2005) and in vessels (Tan et al., 2004). In addition, AngII induces cardiac remodeling indirectly by the induction of chronic hypertension, increasing the afterload (Kumar et al., 2008) and impairment of the arterial baroreceptor reflex in the central nervous system (Miller & Arnold, 2019).

Oxidative stress (ischemia) activation

During ischemic situations, cell levels of reactive oxygen species (ROS) increase leading to what is known as oxidative stress. This condition destabilizes the intracellular redox balance causing damage to proteins and other molecules and can lead to cell death. This disturbance is associated with cardiovascular disease, fibrosis, neurological disease, and cancer. PKD has been found as one of the downstream effectors of high levels of ROS in cardiomyocytes (Cobbaut et al., 2018; Cobbaut & Van Lint, 2018).

During oxidative stress, PKD1 is activated through nonreceptor tyrosine kinases (c-Abl and Src) and PKCδ (Steinberg, 2012). Phosphorylation of Y463 by c-Abl allows the phosphorylation of Y95 by Src in the N-terminal. This works as a docking site for PKCδ that then can phosphorylate PKD1 at Ser738/742 (Doppler & Storz, 2007; Storz et al., 2005; Storz & Toker, 2003). In the nucleus and mitochondria, PKD activity impact cell survival through nuclear factor kappa B (NFκB) (antioxidative genes activation), cyclic-nucleotide regulatory element binding protein (CREB), RhoA, and c-Jun N-terminal kinase (JNK) (**Figure 3**; (Ozgen et al., 2009; Steinberg, 2012; Xiang et al., 2013; W. Zhang et al., 2005)). PKD1 oxidative protection can be also RhoA-mediated via sphingosine 1-phosphate (S1P)/Gα12/13-coupled receptor (cardioprotectant released during ischemia-reperfusion). Activated RhoA can then activate PLCε that will lead to increased DAG levels and PKD1 activation. In PKD1 KO mice, S1P-mediated cardioprotection during ischemia-reperfusion was reduced (Xiang et al., 2013). PKD can also regulate the response to ischemia by phosphorylation of transient receptor potential vanilloid type 1 (TRPV1) channel and heat shock protein (HSP27) which have been implicated as modulators of myocardial ischemic injury (Avkiran et al., 2008; Doppler et al., 2005; Hollander et al., 2004; Wang & Wang, 2005; Wang et al., 2004).

For PKD2 a PKCδ-dependent phosphorylation of AL serine residues facilitates the subsequent c-Abl-dependent phosphorylation at Y717 (Cobbaut et al., 2017). This PKD2 activation contributes to NFκB activation by the formation of heterodimers with PKD1, facilitating its activation and protecting it from phosphatases (Cobbaut & Van Lint, 2018; Steinberg, 2021).

PKD3 was thought to not be activated by oxidative stress because it lacks the implicated tyrosine residues; however, it has been demonstrated that all three isoforms are activated by H_2O_2 and that during acute oxidative stress in fibroblasts PKC activates PKD3 independently of tyrosine kinases (Cobbaut & Van Lint, 2018; Qiu & Steinberg, 2016; Steinberg, 2021).

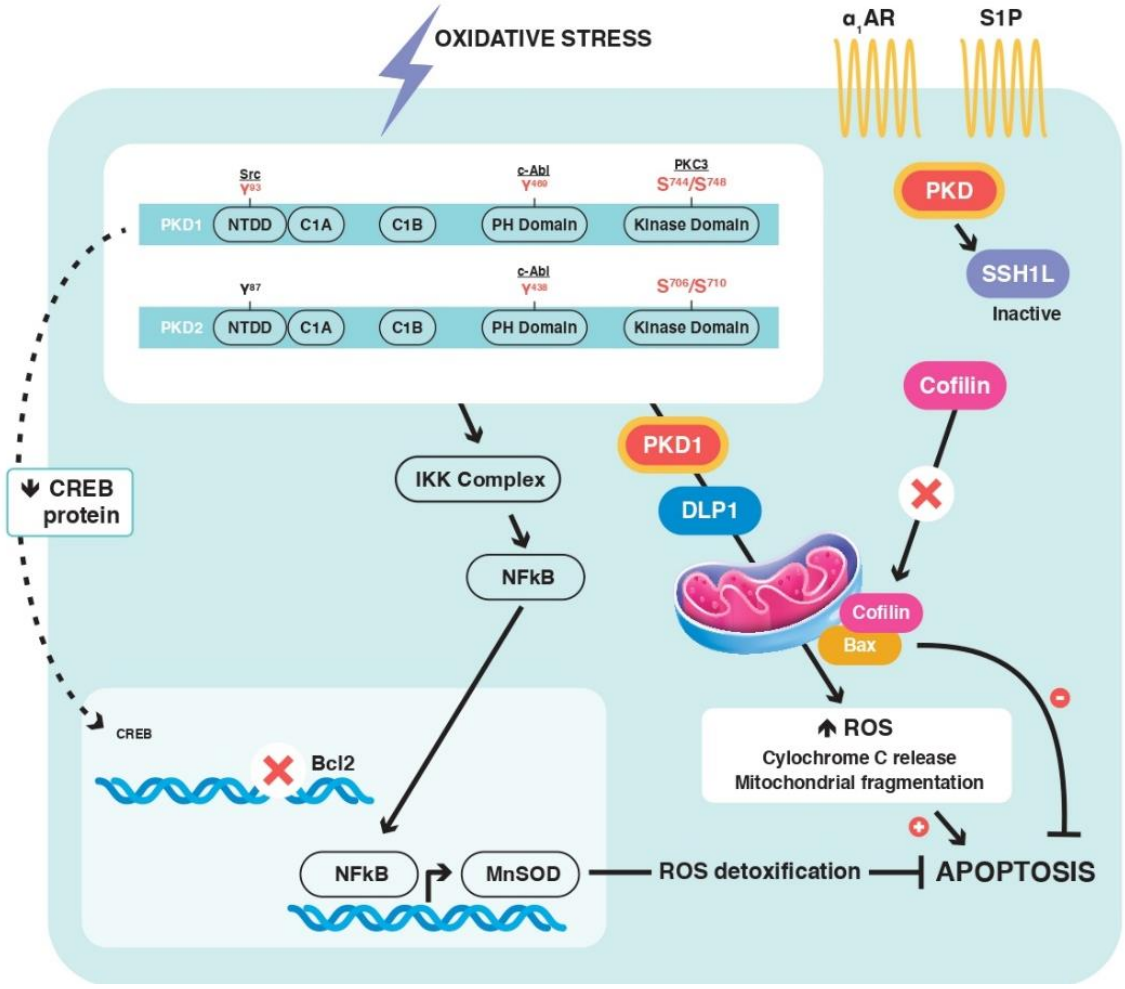


Figure 3. PKD activation by oxidative stress. Redox-activated nonreceptor tyrosine kinases c-Abl, Src, and PKC phosphorylate PKD at the sites indicated. ROS-activated PKD1 has been implicated in a pathway that decreases the abundance of the

transcription factor CREB, thereby disrupting Cre-dependent gene expression in cardiomyocytes and also has been implicated in the NFκB pathway that induces expression of antioxidant/antiapoptotic genes, such as MnSOD (Mn-superoxide dismutase), that detoxify cellular ROS and promote cell survival. PKD also has been implicated in a mechanism that regulates cofilin phosphorylation by the cofilin-phosphatase slingshot1L (SSH1L); this prevents cofilin translocation to mitochondria and its interaction with Bax (proapoptotic). PKD is also involved in an α1-AR–dependent pathway that leads to phosphorylation of mitochondrial DLP1. IKK, IκB kinase. Adapted from Steinberg, 2021.

Metabolic activation

Recently, PKD has been highlighted as a novel nutrient sensor. It is well known that PKD is activated by the lipid intermediate DAG and that feeding leads to PKD activation in the liver, heart and adipose tissue. In these same tissues during obesity, PKD signaling is linked to reduce insulin signaling and function (Renton et al., 2021).

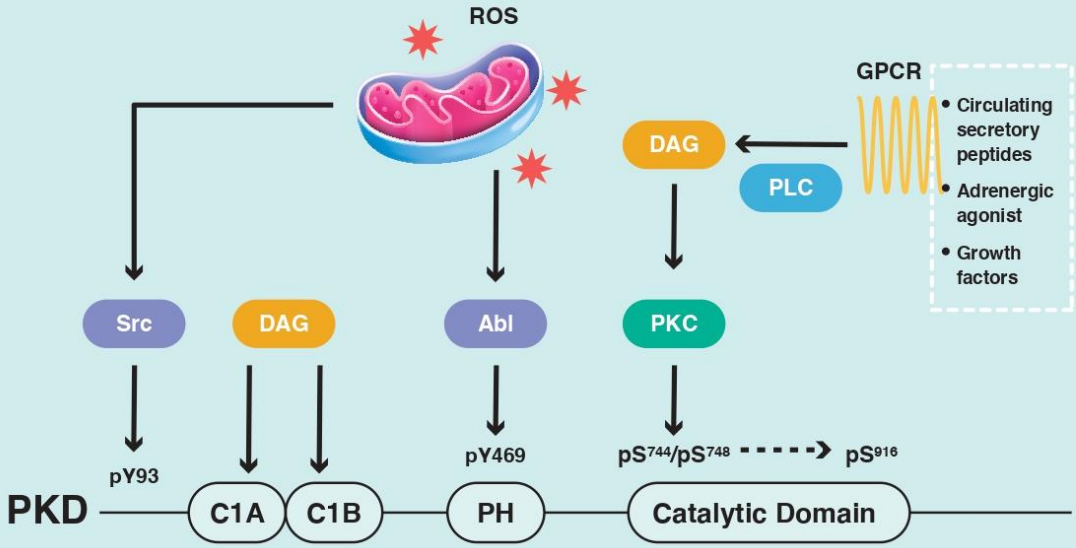
In pancreatic β-cells, PKD1 has a key role for insulin secretion in response to glucose stimulation of the GPR40 receptor (long-chain fatty acid receptor) and can be inhibited by MAPK p38δ (Ferdaoussi et al., 2012; Sumara et al., 2009). Knock-out of MAPK p38δ allows PKD1 to be highly active and prevent insulin resistance and oxidative stress in β-cells during high fat diet exposure (Sumara et al., 2009).

In the heart, PKD enhances myofilament Ca sensitivity, contraction and glucose uptake (inducing translocation of the glucose transporter type 4, GLUT4 to the

membrane) coordinated by contraction-induced ROS production and subsequent death-activated kinase (DAPK) and PKD activation (Dirkx, Schwenk, et al., 2012; Luiken et al., 2008). In PKD1 cKO cardiomyocytes, glucose uptake during pacing was not increased (Dirkx, Schwenk, et al., 2012) while overexpression of constitutively active PKD increased glucose uptake in vivo (Dirkx et al., 2014). Recently, De Jong et al. (2020) demonstrated that in cardiomyocytes from obese cardiac dominant-negative PKD (DN-PKD) mice, PKD activity is key for normal basal glucose clearance but has no effect on major metabolic pathways after glucose oral ingestion. This suggests that other pathways compensate for the induced downregulation in PKD activity during obesity in DN-PKD mice. In these obese DN-PKD mice, cardiac function was preserved with respect to WT controls. This reinforces that PKD is deleterious for heart remodeling and function during obesity.

In mice expressing constitutively active PKD and fed with high fat diet, lipid overload and insulin resistance were prevented (Dirkx et al., 2014). However, other studies showed that during diabetes there is an increase in lipoprotein lipase (LPL) secretion that leads to the altered accumulation of cardiac lipids and dysfunction. This happens through activation of PKD (PKCδ after dissociation from HSP25) by and enhanced vesicle trafficking and release of LPL (Kim et al., 2008; Kim et al., 2009; Wang & Rodrigues, 2015). In db/db mice (type 2 diabetes model), the administration of CID755673 (PKD inhibitor) ameliorated the diastolic and systolic dysfunction independently of changes on glucose and insulin regulation and body weight (Venardos et al., 2015). This was also the case in a type 1 diabetes model induced by STZ injections and high fat diet in rats (Liu et al., 2015). **Figure 4** illustrates several PKD activation pathways, targets and organ-specific effects.

ACTIVATION



KEY TARGETS

MnSOD	AMPK	PI4KIIIβ	RIN1	NFkB
↑ ROS clearance	↓ Insulin signalling	↑ Golgi vesicle secretion	↑ Cell proliferation	↑ Inflamation

ACTIONS

↑ Lipogenesis ↓ Energy expediture ↓ White adipose tissue beiging ↑ Inflamation	↓ Insulin mediated glucose uptake?	↓ Lipogenesis ↓ Triglyceride accumulation ↓ Fibrosis	↑ Glucose-stimulated insulin secretion ↓ Stress-induced nescent insulin granule degradation ↓ β-cell failure?	↓ Diastolic function ↓ Systolic function ↑ Fibrosis ↑ Hypertrophy

Figure 4. PKD activation mechanisms, targets and functions in different tissues. Key targets of PKD include manganese superoxide dismutase (MnSOD), 50 adenosine monophosphate-activated protein kinase (AMPK), phosphatidylinositol 4-kinase III β (PI4KIIIβ), Ras and Rab interactor 1 (RIN1), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Adapted from Renton, 2020.

PKD functions in the body tissues

PKD is expressed almost in all body tissues and is critical for multiple biological processes during normal tissue function (Fu & Rubin, 2011; Rozengurt, 2011; Rozengurt et al., 2005). One of the processes that has been deeply studied is the regulation of vesicle transport in the trans-Golgi network mediated by phosphatidylinositol 4-kinase III β (PI4KIIIβ) (Fu & Rubin, 2011; Hausser et al., 2005). As mentioned in the previous section, in the pancreas PKD mediates Golgi fission that leads to the glucose-stimulated insulin secretion by GPR40 stimulation (Bergeron et al., 2018; Khan et al., 2019; Sumara et al., 2009). PKD is also involved in cell proliferation of various cell types (especially cancer cells) (Renton et al., 2021; Rozengurt, 2011; Rozengurt et al., 2005) and cell survival during oxidative stress as pointed before in the activation section. Here, PKD increases the expression of manganese superoxide dismutase (MnSOD, antioxidant) (Doppler & Storz, 2007; Renton et al., 2021; Storz et al., 2005; Storz & Toker, 2003).

<u>Vesicle trafficking in the trans-Golgi apparatus, secretion, and cell motility</u>

Some PKD1 and PKD2 localize at the Golgi complex where they mediate vesicle budding. Here they phosphorylate Golgi-localized substrates like PI4KIIIβ needed for fission of the

trans-Golgi network to plasma membrane carriers (Hausser et al., 2005; Liljedahl et al., 2001). For more extensive details about PKD involvement in vesicle trafficking, see (Fu & Rubin, 2011).

PKD has also been implicated in secretion. In H295R cells (human adrenocortical cell line), PKD regulates angiotensin II-induced cortisol and aldosterone secretion (Romero et al., 2006) and in neuronal cells stimulates the secretion of neurotensin (Cabrera-Poch et al., 2004; Iglesias et al., 2000).

Depending on the context, PKD can enhance or inhibit cell motility (Rozengurt, 2011). In fibroblasts, PKD has been implicated in Rac-1 activity and integrin recruitment to newly formed focal adhesions (Prigozhina & Waterman-Storer, 2004; Woods et al., 2004). Other groups demonstrated that PKD mediates cofilin and cortactin phosphorylation reducing cell motility (Eiseler et al., 2009; Eiseler et al., 2010).

Immune response

In the immune system, the PKC/PKD pathway has been demonstrated in B and T lymphocytes during response to antigen stimulus (Rozengurt et al., 2005). In inactivated T cells, PKD localizes in the cytosol and upon antigen stimulation is recruited to the immunological synapse by DAG accumulation (Spitaler et al., 2006). Like in other cells, in lymphocytes PKD phosphorylates HDACs to regulate gene expression (Matthews et al., 2006). PKD can also regulate integrin activity in T cells through Rap1 (Medeiros et al., 2005) and IL-2 promoter in response to TCR stimulation (Irie et al., 2006).

PKD has been also shown to mediate toll-like receptors (TLRs) 2, 5 and 9 function in various cells (Rozengurt, 2011). In epithelial cells mediates the phosphorylation of TLR5 and subsequent activation of p38MAPK and cytokine production (Ivison et al., 2007). In macrophages and mast cells, PKD is part of the downstream signaling pathway after activation of TLR 9 and 2, respectively (Murphy et al., 2007; Park et al., 2008). However, further investigation is needed to elucidate the specific mechanisms by which PKD may regulate TLR signaling and innate immune response (Rozengurt, 2011).

Cell survival during oxidative stress and inflammation

The nuclear factor kappa B (NFκB) is an important transcription factor that is activated downstream of several receptors for the regulation of gene expression in innate and adaptive immune responses. PKD has been demonstrated as a mediator of NFκB activation after GPCR stimulation or oxidative stress (**Figure 5**; (Chiu et al., 2007; Doppler & Storz, 2007; Mihailovic et al., 2004; Storz & Toker, 2003)). In pancreatic acinar cells stimulated by CCK-8, PKD (after PKCδ and PKCε activation) was shown to be necessary for the downstream activation of NFκB during acute pancreatitis (Yuan et al., 2008). In human colonic epithelial NCM460 cells, PKD2 (major isoform in these cells) was shown to be essential for lipid lysophosphatidic acid (LPA)-stimulated NFκB activation and IL-8 production (Chiu et al., 2007). This was also the case in myeloid leukemia cells (Mihailovic et al., 2004). As mentioned before, PKD1 opposes the apoptotic effect of oxidative stress in several cell types.

Other studies showed that PKD1 knockdown in colonic myofibroblasts reduced the TNF-α and bradykinin-induced increase in COX-2 (prostaglandin downstream mediator)

expression, limiting colon cancer progression (Plummer et al., 2020; Rodriguez Perez et al., 2011; Yoo et al., 2011). Additionally, PKD is also involved in pain transmission during inflammation through the vanilloid receptor type 1 (VR1 or TRPV1) (Amadesi et al., 2009; Zhu et al., 2008).

Cell proliferation and cancer

One of the most important features of cancer is the accelerated cell proliferation. PKD has been widely recognized as a mediator of cell proliferation and cancer development since early days of PKD research (Rennecke et al., 1999; Zhang et al., 2021; Zhukova et al., 2001). Several GPCRs (bombesin, vasopressin, endothelin, bradykinin) can activate PKD and enhance proliferation through the phosphorylation of HDACIIa and β-catenin transcription factor (**Figure 5**; (Sinnett-Smith et al., 2014; Wang et al., 2016; Zhukova et al., 2001)). PKD is a regulator of the cell cycle at G2-M and mitotic phases in normal cells (Martinez-Leon et al., 2019; Papazyan et al., 2008). Roy, (2018) reported that PKD2 depletion delays the mitotic entry by Aurora A kinase degradation induction. PKD1 has been also implicated in multiple proliferative processes like neurotensin (NT)-induced cell proliferation by the suppression of JNK/c-Jun activation and prolongation of NT-stimulated ERK activation in pancreatic cancer cells, this results in accumulation of c-FOS, increase of DNA synthesis, and proliferation of pancreatic carcinoma cells (Kisfalvi et al., 2010). For further reviews of the role of PKD in cell proliferation and cancer see (Zhang et al., 2021) and (Rozengurt, 2011).

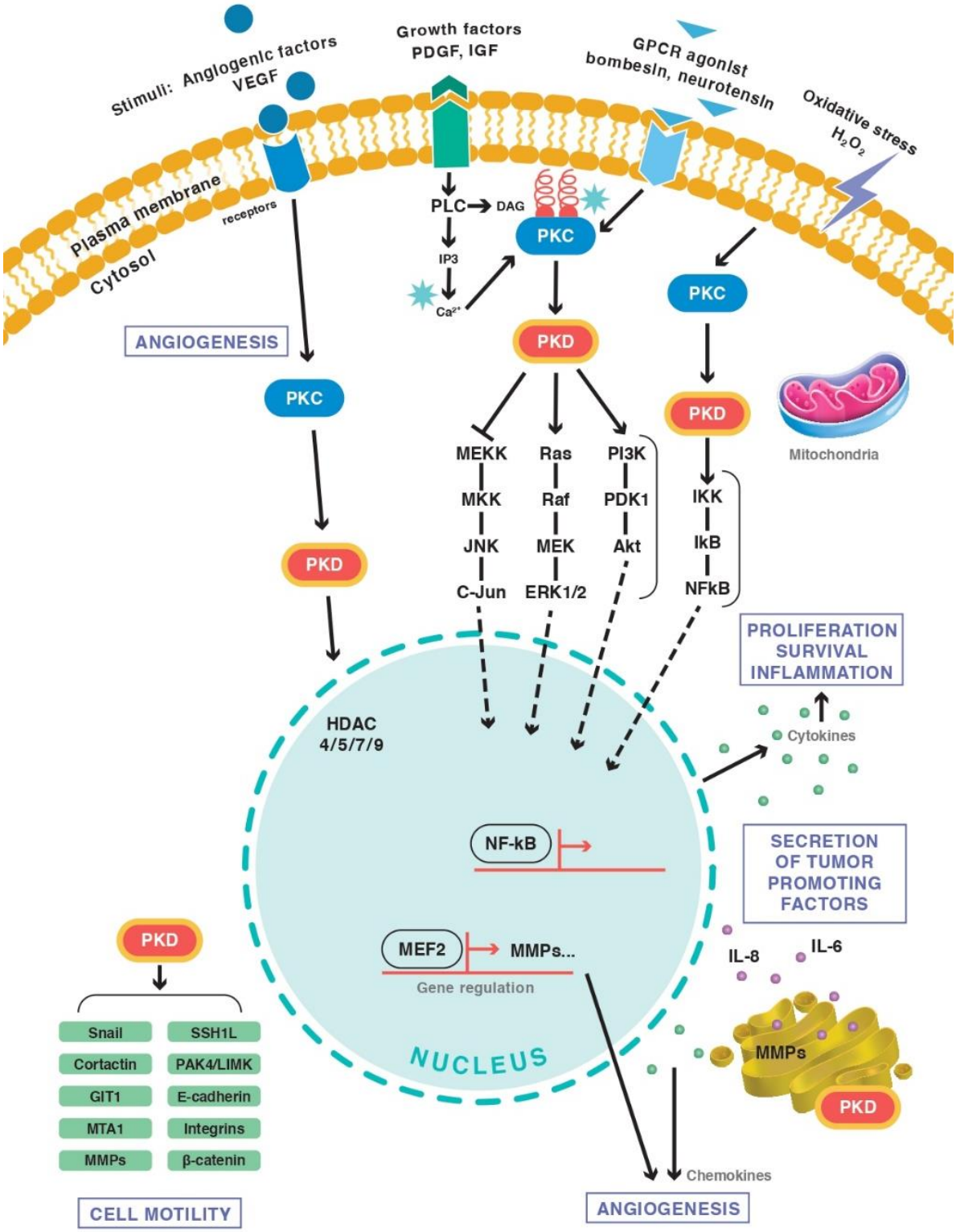

Stimuli: Angiogenic factors
VEGF
Growth factors
PDGF, IGF
GPCR agonist
bombesin, neurotensin
Oxidative stress
H_2O_2
Plasma membrane
Cytosol
receptors
PLC
DAG
IP3
Ca^{2+}
PKC
PKD
ANGIOGENESIS
MEKK
MKK
JNK
C-Jun
Ras
Raf
MEK
ERK1/2
PI3K
PDK1
Akt
IKK
IkB
NFkB
Mitochondria
PROLIFERATION
SURVIVAL
INFLAMMATION
Cytokines
HDAC
4/5/7/9
NF-kB
MEF2
MMPs...
Gene regulation
NUCLEUS
SECRETION
OF TUMOR
PROMOTING
FACTORS
IL-8
IL-6
MMPs
Snail
Cortactin
GIT1
MTA1
MMPs
SSH1L
PAK4/LIMK
E-cadherin
Integrins
β-catenin
CELL MOTILITY
Chemokines
ANGIOGENESIS

Figure 5. Signaling pathways and pathological processes regulated by PKD. Activated PKD regulates several pathological processes including cell proliferation, survival, migration, invasion, gene transcription, inflammation, angiogenesis, and secretion of tumor-associated factors through several major signaling pathways. Abbreviations: matrix metalloproteinase (MMP), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), metastasis-associated 1 (MTA1), slingshot-1L (SSH1L), GPCR kinase-interacting protein 1 (GIT1). Adapted from Zhang, 2021.

PKD and cardiac remodeling/hypertrophy

PKD's role in cardiac pathological remodeling has been well studied before. Harrison et al. (2006) demonstrated that expression of activated PKD1 induced dilated cardiomyopathy development in transgenic mice and Fielitz et al. (2008) showed that cardiac-specific PKD1 deletion prevents hypertrophy and cardiac fibrosis in transverse aortic constriction (TAC) mice. More specifically, in the heart PKD regulates the activity of cyclic-nucleotide regulatory element binding protein (CREB) and MEF2 transcription factors (Steinberg, 2021; Wood & Bossuyt, 2017). MEF2 activity is regulated by PKD1 phosphorylation of HDAC5 and its nuclear export, releasing the inhibition on the transcription factor and inducing activation of fetal genes (**Figure 6**; (McKinsey & Olson, 2005; McKinsey et al., 2001; Wood & Bossuyt, 2017)). This PKD1/HDAC5/MEF2 pathway can be amplified by interacting with A-kinase anchoring proteins (AKAP) (like AKAP-Lbc or mAKAPb) because these proteins work as scaffolds to regulate PKC-induced PKD activation (Carnegie et al., 2004; Carnegie et al., 2008; Kritzer et al., 2014). PKD-

dependent CREB phosphorylation increases the expression of CREB target genes, such as Bcl-2, atrial natriuretic factor, and brain natriuretic peptide, that will then regulate ventricular structure and function (Ozgen et al., 2008; Steinberg, 2021; X. Zhang et al., 2005).

PKD regulation of contractile proteins and channels in the heart

Currently, PKD is well known for its role in myofilament function regulation through phosphorylation of troponin I (TnI) at serine 22/23 (shared phosphosites with PKA) and myosin binding protein C (MyBPC) at serine 302/315 (shared phosphosites with PKA) which reduce myofilament calcium sensitivity and accelerate cross-bridge cycling kinetics, respectively (**Figure 6**; (Bardswell et al., 2010; Cuello et al., 2007; Dirkx, Cazorla, et al., 2012)). Recently, Martin-Garrido et al. (2018) showed that monophosphorylation of TnI (Ser 22/23) is sufficient to reduce myofilament Ca sensitivity, leaving PKD as a key regulator of cardiac contractility.

PKD has also been described as a regulator of L-type calcium channel (LTCC) activity. PKD can phosphorylate LTCC at Ser 1884 and increase its open probability (**Figure 6**; (Aita et al., 2011; Maturana et al., 2008)). In addition, PKD can modulate LTCC expression and trafficking to the T-tubules by phosphorylation and subsequent inhibition of GTP-binding protein Rem1 (that inhibits LTCC) (Jhun et al., 2012).

During obesity, PKD autonomous activation is increased, reducing CREB activation and subsequently K^+ channels expression, specifically $K_v1.5$ and $K_v2.1$ (Huang et al., 2013). When co-expressed with I_{Kr} pore-forming subunit ($K_v11.1$, hERG), PKD1

can reduce the hERG current amplitude without changes in the expression or kinetic of the channel (Steffensen et al., 2018). When activated acutely with sphingomyelin synthase, PKD can increase I_K without changes in the biophysics of the channel but with possible changes in expression or trafficking (Wu et al., 2016). Recent work from our lab, demonstrated in PKD1 cKO TAC mice that there were larger K^+ currents via the transient outward current (I_{to}), sustained current (I_{sus}), inward rectifier K^+ current (I_{K1}), and rapid delayed rectifier K^+ current (I_{Kr}), with increased expression of these channels. Additionally, the sham PKD1 cKO mice exhibited a larger I_{to} and expression of this channel.

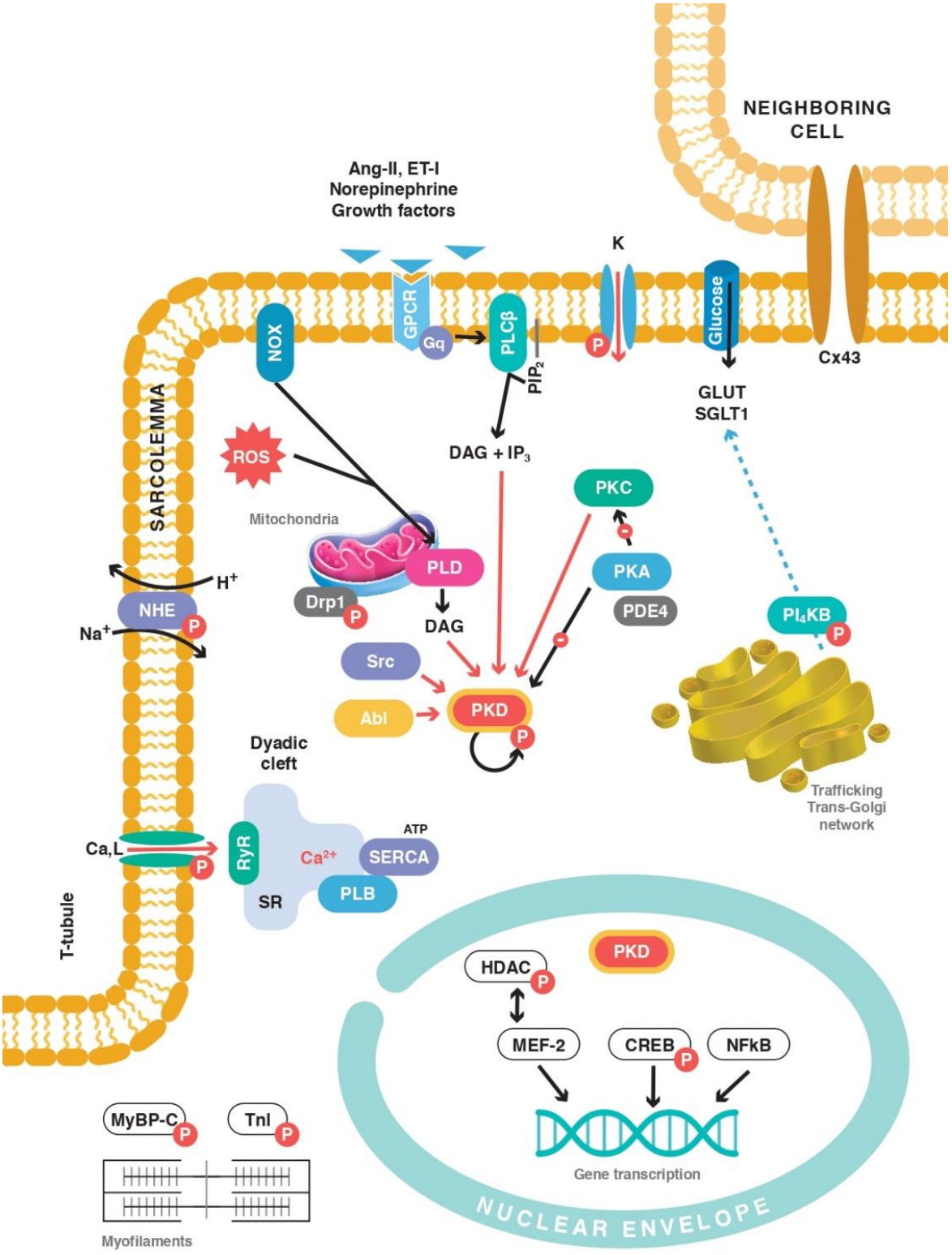

NEIGHBORING CELL
Ang-II, ET-I
Norepinephrine
Growth factors
K
GPCR
Gq
PLCβ
PIP2
NOX
Glucose
Cx43
GLUT
SGLT1
SARCOLEMMA
ROS
DAG + IP3
PKC
Mitochondria
PLD
PKA
Drp1
PDE4
DAG
PI4KB
H+
NHE
Na+
Src
Abl
PKD
Dyadic
cleft
Trafficking
Trans-Golgi
network
ATP
Ca,L
RyR
Ca2+
SERCA
PLB
SR
T-tubule
PKD
HDAC
MEF-2
CREB
NFkB
Gene transcription
NUCLEAR ENVELOPE
MyBP-C
TnI
Myofilaments

Figure 6. PKD described functions in cardiomyocytes. After PKD activation through GPCRs or oxidative stress, multiple targets can be phosphorylated as indicated on the figure with red circles marked with a P. Some of these targets are L-type Ca channels, troponin I (TnI), myosin binding protein C (MyBP-C), Drp1 and HDAC. Adapted from unpublished image from Bence Hegyi.

In summary, PKD is a multifunctional kinase involved in several functions in different tissues: for example, cell proliferation and survival, motility, vesicle trafficking, immune response, metabolism, and remodeling and contractility in the heart. Due to this broad spectrum of functions, PKD represents an important therapeutic target for multiple diseases in different body systems (i.e., the heart) and cancer. However, this kinase has been less studied under physiological (and not remodeling) conditions and with specific common stressors in the heart like β-AR agonists and AngII. In the next two chapters I will present the results of the effects of PKD in the cardiomyocyte response to β-adrenergic stimulation (**Chapter 2**) and the effects of angiotensin II-induced activation of PKD in the cardiomyocyte ECC (**Chapter 3**).

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Chapter 2: Cardiac Protein Kinase D1 ablation alters the β-adrenergic response in myocytes

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ABSTRACT

The β-adrenergic (β-AR) signaling pathway is essential for the adaptation of the heart to exercise and stress. Chronic stress leads to the activation of CaM kinase superfamily members such as Ca/CaM-dependent kinase II (CaMKII) and protein kinase D (PKD) that regulate excitation-contraction coupling (ECC). Unlike CaMKII the effects of PKD on ECC remain unclear. To elucidate the mechanisms of PKD-dependent regulation of ECC, we used hearts from cardiac-specific PKD1 knockout (cKO) mice and their WT littermates. We measured calcium transients (CaT), Ca sparks, contraction and L-type Ca current in cardiomyocytes at 0.5 and 1 Hz pacing under acute β-AR stimulation with isoproterenol (100 nM). Sarcoplasmic reticulum (SR) Ca load was assessed by rapid caffeine (10 mM) induced Ca release. Expression and phosphorylation of ECC proteins (PLB, TnI, RyR2, SERCA) were evaluated by western blotting. At baseline, CaT amplitude and decay tau, Ca spark frequency, SR Ca load, Ca current, contractility and expression and phosphorylation level of ECC proteins were similar in WT vs. PKD1 cKO. However, PKD1 cKO cardiomyocytes presented a diminished response to isoproterenol vs. WT with less increase in CaT amplitude ($\Delta F/F_0$, 5.2 ± 0.44 vs. 8.4 ± 0.55), slower $[Ca]_i$ decline (tau decay, 305.3 ± 19.56 vs. 205.7 ± 14.05, $CaTD_{90}$, 745 ± 44.54 vs. 572 ± 53.32 ms) and

lower Ca spark rate (0.38 ± 0.29 vs. 0.62 ± 0.34 sparks/100μm/sec), but with comparable SR Ca loads ($\Delta F/F_0$, 7.98 ± 3.17 vs. 8.13 ± 3.7), L-type Ca currents, contraction (shortening) and ECC proteins phosphorylation. Our findings suggest that basal PKD1 may allow enhanced cardiomyocyte responsiveness to β-adrenergic stimulation without changing basal SR Ca load, L-type Ca current, PLB and TnI phosphorylation and contractility. This PKD1-dependent enhancement of β-AR responsiveness might occur through some regulation of phophodiesterases (PDEs) or phosphatases (PPs) at the nanodomain level but further studies are necessary to elucidate these mechanisms.

INTRODUCTION

The β-adrenergic (β-AR) signaling pathway is critical for the survival mechanism referred to as the sympathetic fight-or-flight response, but under physiological basal conditions, the sympathetic nervous system also regulates the function of several organs, including the heart. The sympathetic reflex modulates (through β-AR pathway) the heart's response to changes in afterload by increasing heart rate and contractility (Boron & Boulpaep, 2012). This is accomplished through alterations in the activities of proteins involved in excitation-contraction coupling (ECC) including L-type Ca channels, ryanodine receptors (RyRs), phospholambam (PLB) and troponin I (TnI) and myosin binding protein C (MyBP-C) mostly by protein kinase A (PKA) (Bers et al., 2019). In cardiac hypertrophy and failure there is chronic activation of β-AR pathway and activation of CaM kinase superfamily members such as Ca/CaM-dependent kinase II (CaMKII) and protein kinase D (PKD) that can modulate ECC (Anderson et al., 2011; Cuello et al., 2007; Haworth et al., 2004;

Martin-Garrido et al., 2018). While CaMKII effects on ECC have been studied in detail, PKD has been somewhat less well-studied.

PKD is a serine/threonine kinase that has been strongly implicated in cardiac remodeling during, for example, pressure overload induced hypertrophy (Fielitz et al., 2008) and also decreasing myofilament Ca sensitivity in response to G_q-protein coupled receptor activation; e.g endothelin-1 (Bardswell et al., 2010; Haworth et al., 2004). Moreover, PKD is required for the pathologic hypertrophic remodeling induced by chronic neurohumoral stimulation with isoproterenol (β-AR receptor agonist) in mice (Fielitz et al., 2008), indicating the interaction between PKD and the β-AR signaling pathway. However, the acute mechanisms by which PKD modulates β-AR responses at the ECC level are not well understood. Indeed, prior studies on the molecular interaction between β-AR/PKA signaling and PKD have produced conflicting results: Harrison et al. (2006) detected no effect on PKD phosphorylation after β-AR or PKA stimulation; Carnegie et al. (2004) showed that AKAP-Lbc operates as a scaffold for PKA and PKC enabling PKD activation and HDAC5 phosphorylation; and Haworth et al. (2011) found that PKA inhibits PKD activation by endothelin-1 (ET-1) or phenylephrine (PE) and this is mediated by phosphodiesterase 3 and 4 (PDE 3 and 4). Nichols *et al.* (2014) identified that β-AR/PKA signaling triggers a novel nuclear PKD activation, while also suppressing the canonical PKC-dependent sarcolemmal PKD activation, demonstrating crosstalk between these two signaling pathways (Nichols et al., 2014).

Here, we used a cardiac specific knockout of the major cardiac isoform, PKD1 (PKD1 cKO; (Fielitz et al., 2008)) to test how PKD1 alters adult murine ventricular myocyte

Ca handling in the absence and presence of β-AR activation. These mice exhibit no prominent baseline phenotype with respect to cardiac size or function, but cellular Ca handling and contraction had not been assessed. We found little differences in ECC in PKD1 cKO myocyte, but blunted β-AR response with respect to Ca transient amplitude and rate of $[Ca]_i$ decline. The β-AR-induced increase in arrhythmogenic SR Ca release events (Ca sparks) was also reduced in the PKD1 cKO mouse myocytes. No significant changes were observed in L-type Ca current, contractility or ECC protein phosphorylation.

Our findings suggest that basal PKD1 activity may enhance cardiomyocyte responsiveness to β-adrenergic stimulation without appreciably changing SR Ca load, L-type Ca current, PLB and TnI phosphorylation and contractility. This leaves the door opened for new studies that can assess the potential modulation of phosphodiesterases and phosphatases or other players in the β-adrenergic signaling pathway and the regulation of ECC.

METHODS

Animal models and cell isolation

Healthy adult (8-12 weeks) C57BL/6J (WT, Jackson Laboratory, Stock No. 000664), Protein Kinase D1 cardiac specific knock-out (PKD1 cKO, obtained by crossing $PKD1^{loxP/loxP}$ mice (Jackson Laboratory, stock No.: 014181) with $PKD1^{loxP/loxP;\ \alpha\text{-MHC-Cre}}$ (Fielitz et al., 2008)) and wild type (WT) littermates were used.

Isolated cardiomyocytes were obtained by enzymatic digestion of mouse left ventricles with 89 mg of type II collagenase (Worthington Biochemical Company, Cat#LS004177)

and 4 mg of type XIV protease (from Streptomyces griseus, Sigma-Aldrich, Cat#P5147). Briefly, heparin injected mice (400 U/kg body weight) were anesthetized with isoflurane (5% for induction and 3-3.5% for maintenance) and heart excision was performed. After aortic cannulation, hearts were perfused on constant flow Langendorff apparatus (37ºC) with 50 mL 1X MEM (in mM: NaCl 135, KCl 4.7, KH_2PO_4 0.6, Na_2HPO_4 0.6, $MgSO_4$ 1.2, Hepes-free acid 20, taurine 30, and 10 µM Ca^{2+} with enzymes and 100% O_2, pH 7.4) for 15-20 minutes. Subsequently, myocytes were disaggregated by gentle pipetting, filtered (nylon mesh) and sedimented repeatedly increasing Ca^{2+} (0.125, 0.25 and 0.5 mmol/L). Before experiments, myocytes are kept in normal Tyrode (NT) solution (in mM: NaCl 140, KCl 4, $MgCl_2$ 1, Hepes-Na 5, Hepes-H-free 5, glucose 5.5, 0.5 mmol/L [Ca^{2+}], pH 7.4) at room temperature (22-23°C).

All animal procedures were approved by the Institutional Animal Care and Use Committee at University of California, Davis (protocol #22824) in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8^{th} edition, 2011).

Calcium imaging and contractility analysis

Isolated myocytes were indicator-loaded by incubation with Fluo-4 AM (10 µmol/L, Invitrogen with Pluronic F-127 0.02%, Invitrogen) in NT for 30 min and then washed for 10 min in NT. Then CaT and diastolic events (sparks) were measured at 0.5 Hz and 1 Hz pacing under acute β-adrenergic stimulation (isoproterenol, 100 nM). Experiments were performed in paired cells (control and ISO on the same cell) at room temperature in NT solution with 1.8 mM Ca^{2+} and 5.5 mM glucose. Line scan recordings were obtained

using confocal microscopy (Bio-Rad Radiance 2100, 40x objective, 6 ms/line), exciting Fluo-4 AM with an Argon laser at 488 nm and collecting emission with a 500-530 nm bandpass filter. For SR Ca^{2+} load determination, 10 mM caffeine rapid delivery was used. Diastolic [Ca] variations (at rest and during pacing) were assessed by measurements of initial Ca baseline (rest), diastolic Ca baseline (during pacing) and the ratio of diastolic/resting fluorescence (F/F_0). Recordings were analyzed with Image J and the plugin SparkMaster (Image J-Fiji™), with later data processing with our custom-made Python based software for CaT and SR Ca load (CaTransient_Analyzer Beta 4.1 Lite by Christopher Y. Ko) and for contractility/fractional shortening (LineMaster by Kim Hellgren).

Cellular electrophysiology

Freshly isolated ventricular cardiomyocytes were transferred to a measuring chamber (QR-40LP, Warner Instruments) mounted on a Leica DMI3000 B inverted microscope, and continuously perfused (2 mL/min) with a modified NT solution (K^+ was replaced with Cs^+) containing (in mmol/L): NaCl 140, CsCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-HEPES 5, glucose 5.5, 4-aminopyridine 5, tetrodotoxin citrate 0.01, with pH=7.40 (using HCl). Patch electrodes were fabricated from borosilicate glass (World Precision Instruments) having tip resistances of 2–3 MΩ when filled with a Cs-based pipette solution containing (in mmol/L): CsCl 110, tetraethylammonium chloride 20, MgATP 5, HEPES 10, phosphocreatine disodium salt 5, calmodulin 0.0001, EGTA 10, $CaCl_2$ 3.6 (free $[Ca^{2+}]$=100 nmol/L), with pH=7.20 (using CsOH). The electrodes were connected to the input of an Axopatch 200B amplifier (Axon Instruments). Outputs from the amplifier were digitized at 50 kHz using Digidata 1332A A/D card (Axon Instruments) under software

control (pClamp 10). Series resistance (on cell) was typically 3–5 MΩ and it was compensated by 85%. Experiments were discarded when the series resistance was high or increased by >20%.

Whole-cell L-type Ca^{2+} channel currents (I_{CaL}) were measured using 500 ms long voltage steps from holding potential of –80 mV to test potentials (between –40 and +20 mV) every 5 s with a 50-ms pre-step to –40 mV to inactivate Na^{+} channels. At the end of each experiment, I_{CaL} was inhibited using 10 µmol/L nifedipine. Ionic currents were normalized to cell capacitance, determined in each cell using short (10 ms) hyperpolarizing pulses from −10 mV to −20 mV. Cell capacitance was 148±3 pF in wild-type (13 cells/6 animals) and 153±4 pF in PKD1 cKO (15 cells/6 animals). All experiments were conducted at 21±1°C.

Inotropic stimulation and western blotting

Acute inotropic stimulation was performed in anesthetized mice (intraperitoneal (IP) injection of 90 mg/kg of ketamine and 4.5 mg/kg of xylazine) by IP injection of 1.5 mg/kg of isoproterenol for 15 min before hearts from these mice were rapidly excised, rinsed with 0 Ca^{2+} NT solution, cut into 6-8 pieces and flash-frozen in liquid nitrogen. Samples were stored at -80°C until use. Hearts were lysed in ice-cold buffer containing (in mmol/L): NaCl 300, TrisHCl (pH 7.4) 40, NaF 20, sodium pyrophosphate 2, MgCl2 1, EGTA 2, EDTA 2, 4% NP40, and protease and phosphatase inhibitors (EMD Millipore, set III and V, respectively). Protein concentration was assessed in heart lysates with a Pierce™ BCA protein assay (Thermo Fisher Scientific, Cat#: 23225). Proteins were divided using Tris-Bis and Tris-HCl SDS-PAGE electrophoresis gels (4-12% Criterion XT Bio-Rad for PLB

and 4-15% Criterion TGX Bio-Rad for TnI, SERCA, RyR and GAPDH) and then transferred to a 0.2 µm (PLB) or 0.45 µm (TnI and others) nitrocellulose membrane that was then blocked with 5% blotting-grade blocker (Bio-Rad). Protein transfer success was checked with Ponceau staining. Blots were incubated overnight at 4°C with primary antibodies: **PLB** (Badrilla, PLN, mAB A1, Cat#: A010-14, 1:2000, mouse), **PLB pS16** (Badrilla, Cat#: A010-12AP, 1:2000, rabbit), **TnI** (Cell signaling, Cat# 4002S, 1:2000, rabbit), **TnI pS22/23** (Abcam, EPR1059(2), Cat#1906971:2000, rabbit), **RyR** (Invitrogen, C3-33, Cat# MA3-916, 1:1000, mouse), **SERCA2** (Invitrogen, Cat#MA3-919, 1:2000, rabbit) and **GAPDH** (Bio-Rad, Cat# MCA4739, 1:2000, mouse). After primary antibodies incubation, membranes were rinsed with TBS-tween (TBST) and then incubated at room temperature with secondary antibodies (1:10000) for 2 hours: Alexa Fluor 647 (Invitrogen, goat anti-rabbit, Cat# A32733) and Alexa Fluor 790 (Invitrogen, goat anti-mouse, Cat# A11357). Later, the blots were rinsed in TBST and then left washing in more TBST for 30 min until reading. Blots were imaged in the ChemiDoc™ MP Imaging System (Bio-Rad) and analyzed with Image Lab (Bio-Rad).

Statistical analysis

Data is presented as mean ± SEM. Data distribution was determined by D'Agostino-Pearson test. Two-tailed Student's t-test (paired or unpaired) and two-way ANOVA followed by post-hoc multiple comparison test (Tukey's) was used and are noted in each figure legend. When data was not normally distributed or N<6, non-parametric test were used. Differences were considered statistically significant if $P < 0.05$. Exact P-values are noted in figures or results section. GraphPad Prism 9 software was used for data analysis.

RESULTS

In the absence of PKD1, cardiomyocytes response to β-adrenergic stimulation is diminished

To test whether baseline Ca handling is modified by the absence of Protein Kinase D1, CaT amplitude, tau decay, time to peak and durations were assessed. Fluo-4 AM loaded mouse ventricular myocytes were used to record CaTs by confocal microscopy. No statistically significant differences at baseline were detected between the WT and PKD1 cKO mice (CaT amplitude P=0.52; time constants (τ) of $[Ca]_i$ decline P=0.12). Paired to the baseline (control) measurements, β-AR effects (ISO) in CaT parameters were evaluated. As shown in **Figure 1**, the responsiveness to β-AR stimulation (induced by isoproterenol 100 nM) is diminished in the PKD1 cKO mice compared to the WT mice, with smaller CaT amplitudes and slower kinetics represented in longer time constants (τ) of $[Ca]_i$ decline and CaT duration 90 (**Figure 1 D**). Diastolic $[Ca]_i$ (during pacing, F/F_0) had comparable changes with respect to baseline $[Ca]_i$ (at rest, $F_0=F_{cell}-F_{background}$) before and after ISO in both genotypes (P=0.87; **Supplementary figure S1A**).

β-AR stimulation-induced increase in Ca spark rate is reduced in PKD1 cKO cardiomyocytes

To assess whether PKD1 modulates spontaneous SR Ca release through ryanodine receptors (RyR) or SR Ca load, spark rate and caffeine-induced CaT were measured. No statistically significant difference in Ca spark rate at baseline was detected between the WT and PKD1 cKO mice (P=0.81). In both WT and PKD1 cKO myocytes, ISO induced

an increase in Ca spark frequency (CaSp frequency; **Figure 2C**). However, the CaSp frequency achieved in the PKD1 cKO myocytes was significantly less than that observed in WT. The SR Ca load at the end of the Ca spark recording period was also not different between WT and PKD1 cKO myocytes, both before and after ISO. The apparent ISO-induced decrease in SR Ca load could have been due to the increase in Ca spark frequency and net Ca extrusion via Na/Ca exchanger during rest. To test this possibility, we repeated the pacing protocol (in separate cells) and tested SR Ca load immediately after stimulation was stopped, and in most cells an increase in SR Ca load with ISO was observed (**Figure 2 F**). Normalizing Ca spark frequency to these SR Ca loads (**Figure 2G**) did not change the conclusions from **Figure 2E**.

Unaltered β-adrenergic regulation of L-type Ca current (I_{CaL}) in PKD1 cKO ventricular myocytes

The L-type Ca^{2+} channel is an important downstream target of β-adrenergic signaling pathway and critically contributes to increasing CaT. As expected, isoproterenol markedly increased the L-type Ca^{2+} current (I_{CaL}) in WT murine ventricular myocytes (**Fig. 3A-B**) and induced a characteristic leftward shift in activation voltage (**Fig. 3C**). Isoproterenol also slightly slowed I_{CaL} decay and enhanced I_{CaL} recovery from inactivation in WT, whereas voltage-dependence of inactivation was unchanged (**Fig. 3B-C**). Importantly, isoproterenol induced identical increase in I_{CaL} density, activation voltage shift, slowing of inactivation, and enhanced recovery in PKD1 cKO as in WT (**Fig. 3A-C**). Baseline I_{CaL} magnitude and biophysical parameters were also indistinguishable in PKD1 cKO *vs.* WT. These data suggest that the impaired β-adrenergic CaT response in PKD1 cKO is not

due to I_{CaL} hyporesponsiveness. In line with this, PKD1 has already been shown to regulate L-type Ca^{2+} channel in neonatal rat cardiomyocytes; however, this regulation occurred only upon stimulation with α-adrenergic rather than β-adrenergic agonists (Maturana et al., 2008)

β–AR induced changes in myocyte contractility were similar in WT and PKD1 cKO ventricular myocytes

It has been described that PKD can phosphorylate cardiac myofilaments, more specifically troponin I at serine 22/23 in mice (Cuello et al., 2007). Notably, these are same sites known to be targets for the PKA-dependent phosphorylation that reduces myofilament Ca sensitivity and contributes to faster relaxation. To determine whether the absence of PKD1 would alter baseline or ISO-induced myocyte contraction we measured contraction and relaxation in confocal images for the same cells used for CaT imaging in **Figure 1.** **Figure 4** shows that there were no statistically significant differences in any of the contraction or relaxation parameters between the WT and PKD1 cKO ventricular myocytes either at baseline upon β–AR stimulation. However, both WT and PKD1 cKO myocytes showed the expected ISO-induced increase in contraction amplitude and rate of relaxation (**Figure 4B**).

Phosphorylation levels of phospholamban (PLB) and cardiac troponin I (cTnI) increase similarly in WT and PKD1 cKO cells after ISO stimulation

As mentioned above, PKD monophosphorylation of serine 22/23 was reported to be sufficient to cause marked reduction in myofilament Ca sensitivity (similar to PKA)

(Bardswell et al., 2010; Cuello et al., 2007) and that could alter ISO-induced responses. **Figure 5A-B** shows that the in vivo ISO intraperitoneal injection of isoproterenol (1.5 mg/kg) produced robust increases in the phosphorylation of PLB at Ser16 and cTnI at Ser22/23 and was similar in both WT and PKD1 cKO. Notably there was also no difference in either expression levels or baseline phosphorylation levels of PLB or cTnI in WT vs. PKD1 cKO (P=0.89 and P=0.58, respectively). This suggests that under baseline conditions in WT mice, there may be insufficient PKD1 activity to significantly phosphorylated cTnI S22/23 sites. The levels of SERCA2 and RyR2 expression were also not different in WT or PKD1 cKO hearts (P=0.7 and P=0.78, respectively; **Figure 5C-D**). There was a slight trend toward a weaker PLB Ser16 phosphorylation in the PKD1 cKO vs. the WT (P=0.21), which would be in the direction consistent with the slower ISO-induced $[Ca]_i$ decline kinetics and CaT amplitude in the PKD1 cKO myocytes (**Figure 1D**). However, no such trend was apparent in the cTnI phosphorylation data in **Figure 5B**.

DISCUSSION

Protein kinases regulate a wide variety of functions in cells and are one of the largest supergene families described. In the heart, protein kinases are essential regulators of cardiomyocyte hypertrophy, protein expression and excitation-contraction coupling (ECC) modulation in physiological and pathophysiological conditions like pressure overload and heart failure (Fuller et al., 2015). Kinases also have a wide variety of intracellular distributions (compartments) and functions in the myocyte that have been described, but still there are many unknowns and it is difficult to accurately state that a specific kinase does not has a specific function, location, or target until it is tested experimentally.

Additionally, the levels of expression and function of a kinase can vary with aging and stress stimuli or disease conditions, this increases the complexity of the study of these proteins (Fuller et al., 2015). One of the most remarkable stimuli altering the heart function is the sympathetic nervous system through the adrenergic signaling (Boron & Boulpaep, 2012). This signaling pathway modulates ECC, protein expression and hypertrophy, and it is mediated by several protein kinases like Protein Kinase A (PKA) and CaM kinase superfamily members (CaMKII and PKD) (Anderson et al., 2011; Bers et al., 2019; Cuello et al., 2007). Despite its key role in cardiac hypertrophy (similar to other kinases), PKD is less studied in relation to ECC. In this study we tested several mechanisms to elucidate the PKD-dependent regulation of ECC in physiological conditions in mouse adult ventricular myocytes stimulated with isoproterenol (ISO; β-AR agonist).

Under physiological conditions, stimulation with β-AR agonists causes an increase in I_{CaL} and SERCA2 pump rate, and modifies RyR gating, these changes are reflected in larger and faster CaTs, and higher Ca spark frequency. Due to the increase in I_{CaL}, it is expected that more Ca enters the cell and more cytosolic Ca is pumped into the SR due increased SERCA2 pump rate. Therefore, a larger SR Ca load is expected (Bers, 2001). Here we showed for the first time that PKD1 ablation in cardiomyocytes blunts the response to β-AR stimulation (ISO) by limiting the rise in CaT amplitude and the acceleration of CaT decay and reducing the ISO-induced increase in Ca spark frequency, but with no changes in SR Ca load. These findings suggest that PKD1 may somehow enhance the cardiomyocyte response to β-AR agonists in adult mouse ventricular myocytes.

Mechanisms that may contribute to reduced β-AR responsiveness in PKD1 cKO

The first step in cardiac ECC is the action potential (AP) induced activation of L-type Ca current, which is normally robustly increased in response to β-AR activation. To test whether the limited CaT amplitude increase observed might be due to reduced Ca entering the cell via I_{CaL} we measured this current in both WT and PKD1 cKO myocytes. We detected no significant differences in either baseline I_{CaL} or its response to ISO stimulation. WT and PKD1 cKO cells both showed a nearly identical increase in I_{CaL} density and classical shift in the I-V relationship. In both genotypes, ISO induced a very similar negative shift in I_{CaL} activation voltage-dependence, no change in I_{CaL} inactivation voltage-dependence, and slightly enhanced I_{CaL} recovery. Some prior data suggested that PKD may modulate $I_{Ca,L}$ upon α-adrenergic receptor activation which is primarily via Gq-coupled receptor that activates PKC and PKD (Aita et al., 2011; Jhun et al., 2012; Maturana et al., 2008). That α-AR-induced effect was suppressed by a dominant negative PKD1 and may involve partner proteins, such as Rad-Gem/Kir-related (RGKs) and enigma homolog 1 (ENH1), but PKD has not been implicated in β-AR regulation of $I_{Ca,L}$ in heart. Together we conclude that PKD1 ablation does not limit CaT amplitude and kinetics through alterations on L-type Ca channel properties in myocytes. However, the total influx of Ca via $I_{Ca,L}$ can also be influenced by the AP duration (APD) or altered Ca-dependent inactivation, points to be addressed later.

The next step in ECC is via $I_{Ca,L}$ triggering SR Ca release that is mediated by the RyR channel and the intra-SR Ca content that is controlled by RyR, SERCA and PLB. Of note, we found no differences in the expression levels of these three key SR proteins in

WT vs. PKD1 cKO mice, nor did we detect significant differences in SR Ca load between the genotypes (in the absence or presence of ISO). In an effort to partially isolate RyR sensitivity as a cause of reduced ISO-induced SR Ca release, we measured Ca spark frequency normalized to SR Ca load (Figure 2E and G). We observed a similar limitation in the ISO-induced increase in Ca spark frequency as was seen for CaT amplitude, raising the possibility that PKD1 activity might slightly increase RyR sensitivity, such that smaller SR Ca release in the PKD1 cKO would limit the ISO-induced increase in CaTs. RyR sensitivity is susceptible to many factors, including pH, [Mg], CaM binding, redox state and phosphorylation at multiple sites (Bers, 2001). So, altered RyR function remains a candidate to explain the reduced ISO-induced increase in CaTs. However, against this simple idea is the situation that moderate changes in RyR sensitivity do not appreciably alter steady state CaTs (Eisner et al., 2000; Trafford et al., 2000). That is, a small decrease in RyR sensitivity would reduce SR Ca release, but that would also reduce Ca extrusion from the cell and increase SR Ca load for the next beat, such that in the steady state the less sensitive RyR case would have slightly higher SR Ca load and a slightly lower fractional SR Ca release, but result in an unaltered CaT amplitude.

Considering whether SERCA or PLB might explain the reduced ISO-induced increase in CaT and rate of $[Ca]_i$ decline in PKD1 cKO mice, there were no differences in SERCA2 or PLB expression and similar changes in PLB phosphorylation during ISO exposure. However, there was a trend ($P=0.21$) for a lower PLB phosphorylation ratio in PKD1 cKO hearts, that while not statistically significant in the 24 hearts analyzed (6 per treatment and genotype), would agree with the lower ISO-induced acceleration of $[Ca]_i$ decline that is a relatively direct readout of SR Ca uptake rate, especially in mouse

myocytes (Bers 2001). We conclude that a limitation in SR Ca uptake rate in response to ISO contributes directly to the slower $[Ca]_i$ decline observed in PKD1 cKO myocytes, and that limited SR Ca uptake could also limit SR Ca load and so, contribute to the limited CaT response to ISO in PKD1 cKO myocytes.

To assess if diastolic $[Ca]_i$ (at rest and during pacing) was significantly different in the PKD1 cKO myocytes, we measured baseline $[Ca]_i$ (at rest) and diastolic $[Ca]_i$ (during pacing), and the ratio of diastolic/baseline Ca. Both genotypes had a comparable slight change in diastolic $[Ca]_i$ and diastolic/rest $[Ca]_i$ ratio after ISO stimulation. Thus, we conclude that altered resting or pacing diastolic $[Ca]_i$ are unlikely to explain the blunted ISO response in PKD1 cKO vs. WT mice.

β-AR stimulation also decreases myofilament Ca sensitivity by PKA-dependent phosphorylation of troponin I (at Ser 22/23). This causes a faster dissociation of Ca from troponin C, which can speed relaxation, although this effect is more prominent when myocytes are contracting against a significant afterload (Li et al., 2000). However, in myocytes that are not mechanically tethered (as here) ISO-induced acceleration of relaxation was shown to depend entirely on PLB phosphorylation. PKD shares with PKA the phosphorylation site serine 22/23 on TnI (Cuello et al., 2007). Phosphorylation of this site by PKD has been demonstrated to be sufficient to reduce myofilament Ca sensitivity in mouse skinned trabeculae and adult ventricular myocytes (AVRM) overexpressing PKD (Martin-Garrido et al., 2018). Less phosphorylation of TnI during β-AR stimulation could also potentially alter Ca buffering by troponin C. However, we saw no genotype differences in TnI phosphorylation (or myocyte relaxation rates) at baseline, suggesting

that PKD1 does not significantly elevate baseline cTnI phosphorylation at the regulatory Ser 22/23 sites. The ISO-induced increases in TnI phosphorylation were also not altered in PKD1 cKO vs. WT mice, suggesting that the presence of PKD1 did not influence cTnI phosphorylation in response to ISO.

These results may not be surprising, because we did not do anything to directly activate PKD (e.g. by activating Gq-coupled receptors) in either the myocytes or the intact animal. Since the phosphorylation measurements were from intact animals, it also suggests that ambient PKD1 activation levels are likely quite low, even upon ISO challenge. Notably, the baseline and ISO responses for contraction and relaxation were quite similar between genotypes, which largely rules out a differential change in myofilament Ca buffering as a complicating factor for the Ca analysis. We conclude that altered myofilament properties in the PKD1 cKO vs. WT mice are unlikely to contribute to the reduced ISO-induced effects on myocyte Ca transients.

The consequences of chronic β-AR activation have been extensively studied in the search of therapeutic targets that can ameliorate the deleterious changes during heart failure. Our results suggest that endogenous PKD1 has a small beneficial effect on the ability of β-AR activation to increase CaT amplitude and decay kinetics. Since PKD expression and activity is upregulated in heart failure, this potentially beneficial effect might be stronger and contribute to a degree of compensatory benefit. A potential counterpoint to that is that the more responsive β-AR effects on Ca handling in the chronic setting could be more proarrhythmic and also drive progressive remodeling more strongly.

Likely integrated mechanisms for blunted β-AR response in PKD1 cKO

A recent study from our group has shown that the mouse action potential duration (APD) is shorter in PKD cKO, and that may be attributable to a significant increase in transient outward K^+ current (I_{to}) (Bossuyt et al., 2022). That shorter APD would limit the amount of Ca entry via $I_{Ca,L}$ even without altering Ca channel expression or properties. It would also increase the time at diastolic voltages between beats when most Ca extrusion occurs via Na/Ca exchange. This situation may suffice to create a slight reduction in basal myocyte Ca loading and CaT amplitude. That may be difficult to detect statistically, but there was a tendency for baseline CaT amplitude to be smaller in PKD1 cKO vs. WT myocytes (Figure 1D). A small Ca influx/efflux difference in PKD1 cKO vs. WT could be worsened by a lower baseline SERCA function (based on a slightly slower mean τ of $[Ca]_i$ decline (Fig 1D) and slightly lower baseline PLB phosphorylation (Fig 5A) and a marginally lower steady state SR Ca content (Fig 2F). So, we cannot rule out the possibility that the limited ISO-induced increase in CaT amplitude $[Ca]_i$ decline rate is due mainly to the culmination of small changes in baseline APD and SERCA function that limit the integrated ISO effects on CaT properties in PKD1 cKO myocytes.

Conclusion

Here we showed that PKD1-ablated cardiomyocytes behave as “quieter” cells upon acute β-AR induced stress. This could agree with the previous demonstrated protective effect of PKD1 cKO for TAC or chronic neurohormonal stimulation resistance in PKD1 cKO mice. These myocytes have less proarrhythmic spontaneous Ca release from the SR by RyR and a slower SERCA pump rate, without substantial compromise in overall contractility and relaxation. The specific mechanism by which RyRs appear less

sensitized and SERCA responses blunted remain to be elucidated. β-AR signaling involves a wide array of proteins with functions that can be altered by interactions with several other proteins, making this pathway complex and multifactorial. Additionally, the β-AR and PKD signaling pathways have already been described to be compartmentalized within cardiac myocytes (Bers et al., 2019; Nichols et al., 2014), with PKD being less well understood.

AFFILIATIONS

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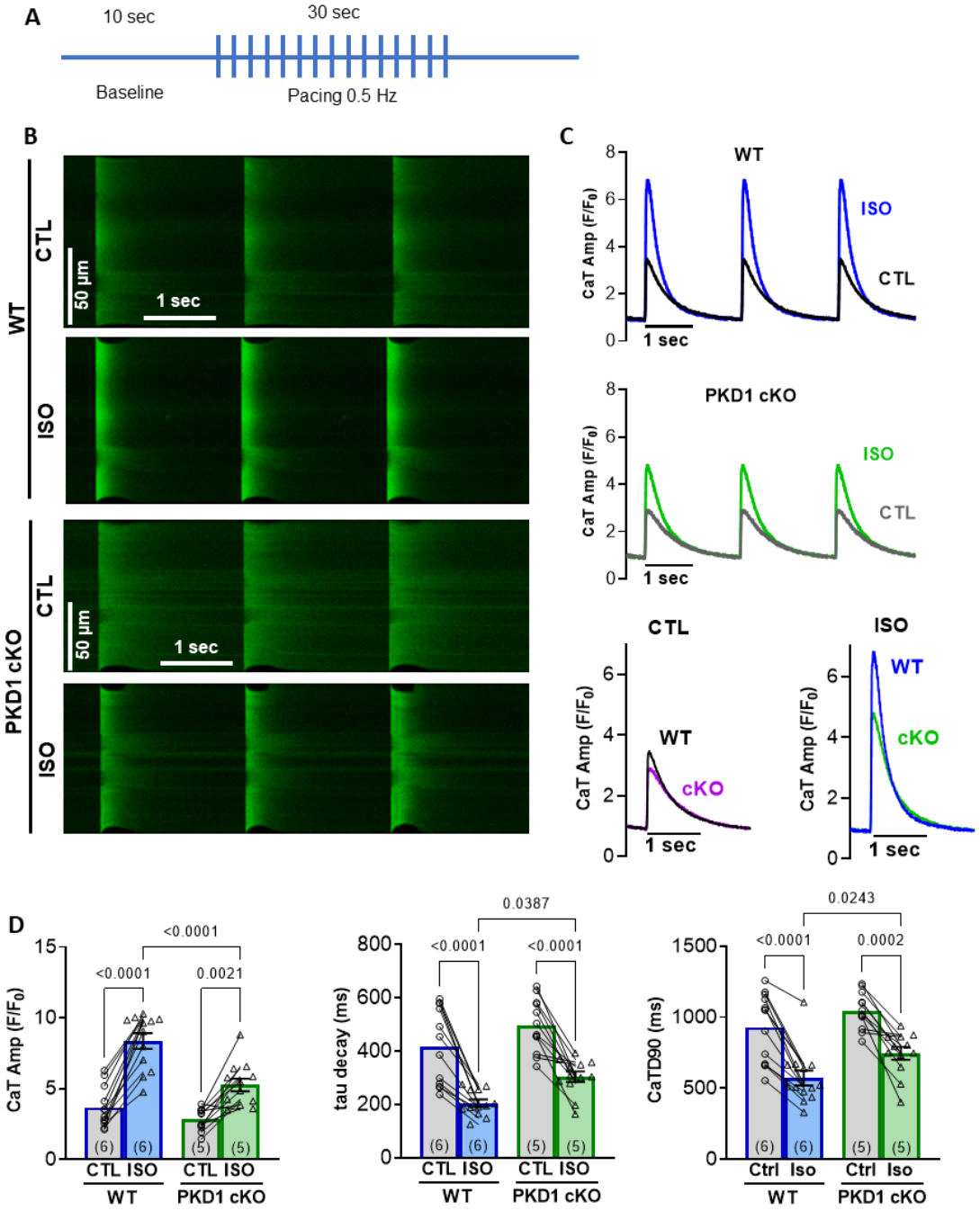

FIGURE 1. Upon β–AR stimulation (isoproterenol), PKD1 cKO cardiomyocytes exhibited smaller Ca^{2+} transient (CaT) amplitudes and slower kinetics compared to WT litter mates. A, Experimental protocol for Ca^{2+} imaging. **B,** Representative confocal images and **C,** representative traces of intracellular Ca^{2+} transients (CaT) in both WT and PKD1 cKO Fluo-4 AM loaded myocytes at baseline and after 5 minute incubation with 100 nM isoproterenol (ISO). **D,** CaT amplitude increase after ISO treatment was lower in the PKD1 cKO, along with slower CaT tau decay. No significant changes in CaT time to peak in both genotypes. Data points represent cells (WT n=12, KO n=12) and numbers on the bars the mice (WT N=6, KO N=5). Ordinary two-way ANOVA, followed by Tukey's multiple comparisons test.

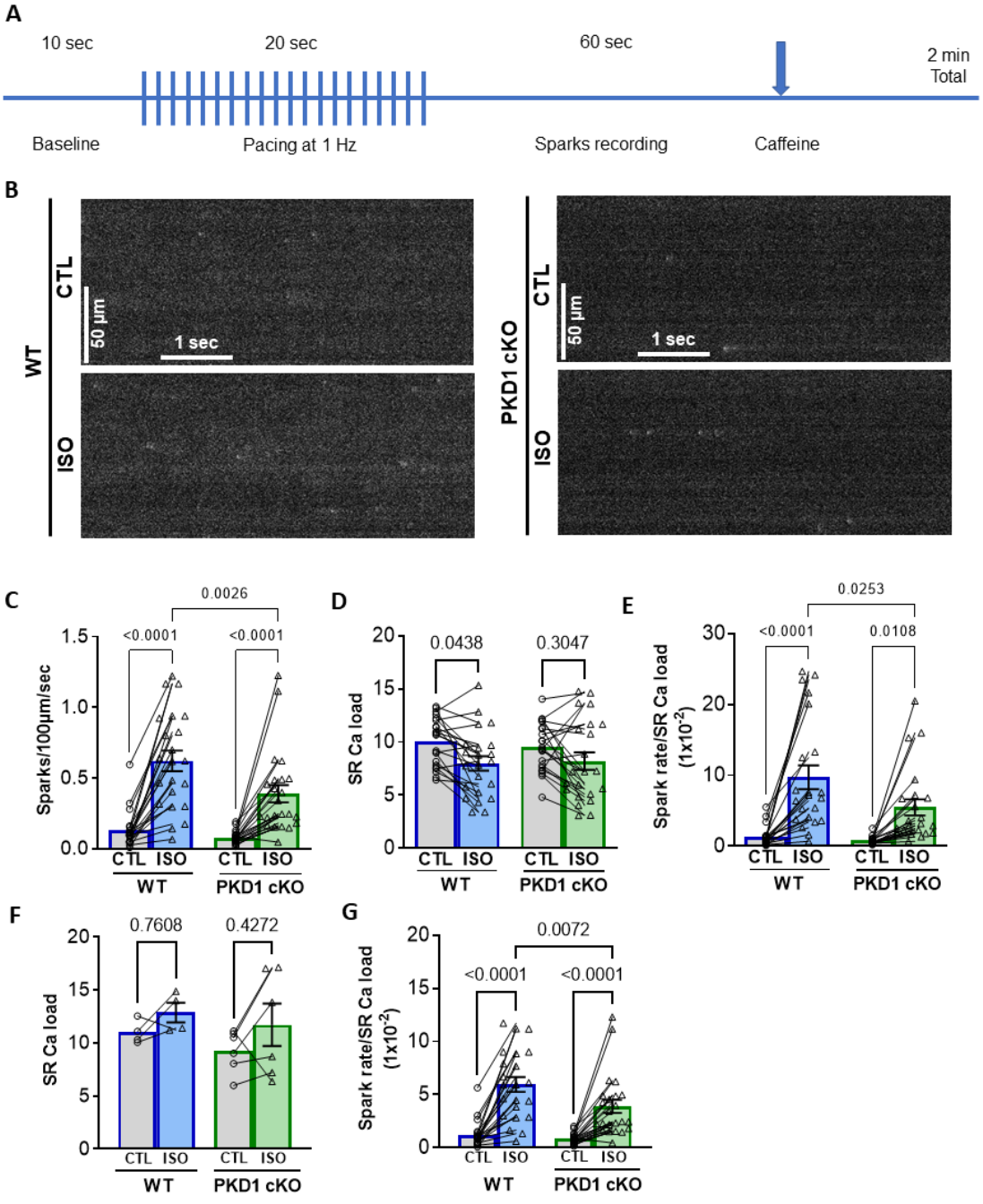

FIGURE 2. ISO-induced increase in spark rate is blunted in PKD1 cKO ventricular myocytes. A, Experimental protocol for diastolic Ca^{2+} imaging (sparks). **B,** Representative confocal images of diastolic Ca^{2+} sparks in both WT and PKD1 cKO myocytes at baseline and after 5-minute incubation with 100 nM isoproterenol (ISO). **C,** Diastolic Ca^{2+} sparks rate significantly increases more in WT myocytes. **D,** ISO-induced SR Ca^{2+} load decrease is similar in WT and PKD1 cKO ventricular myocytes. **E,** Normalization of spark rate by SR Ca^{2+} load in WT and PKD1 cKO. **F,** SR Ca^{2+} load right after pacing. **G,** Normalization of spark rate by SR Ca^{2+} load in figure F. Data points represent cells (WT n=22, KO n=23). Mice: WT N=11, KO N=12. Ordinary two-way ANOVA , followed by Tukey's multiple comparisons test.

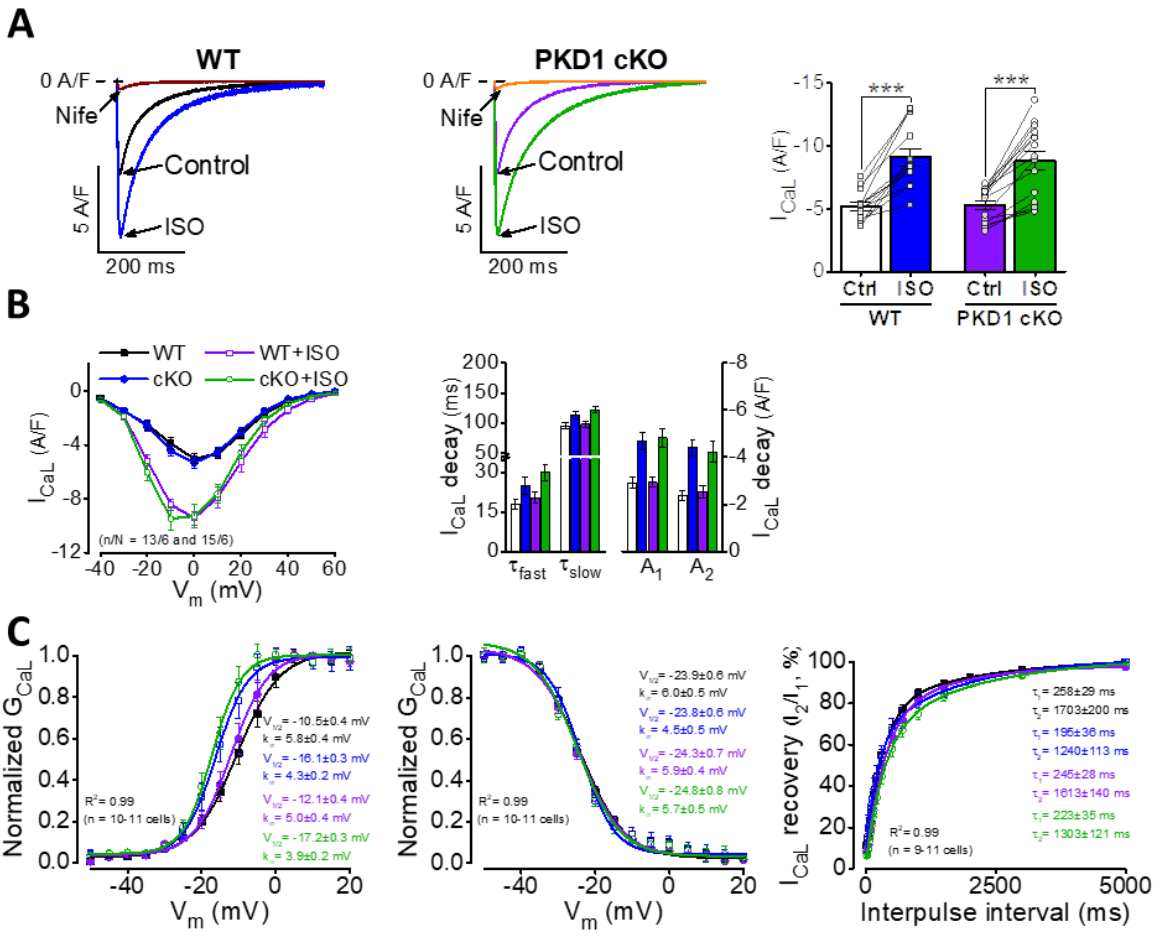

FIGURE 3. ISO-induced L-type Ca^{2+} current (I_{CaL}) increase is unaltered in PKD1 cKO ventricular myocytes. A, Representative I_{CaL} traces and average I_{CaL} density in WT and PKD1 cKO myocytes at baseline and following 5 min treatment with 100 nM isoproterenol (ISO). Ordinary two-way ANOVA, followed by Tukey's multiple comparisons test. **B,** No difference in the ISO effect on I_{CaL} I-V relationship (*left*) and I_{CaL} decay (*middle*) in WT and PKD1 cKO. **C,** ISO-induced a negative shift in I_{CaL} activation voltage-dependence (*left*), no change in I_{CaL} inactivation voltage-dependence (*middle*), and slightly enhanced I_{CaL} recovery (*right*) in a similar way in WT and PKD1 cKO.

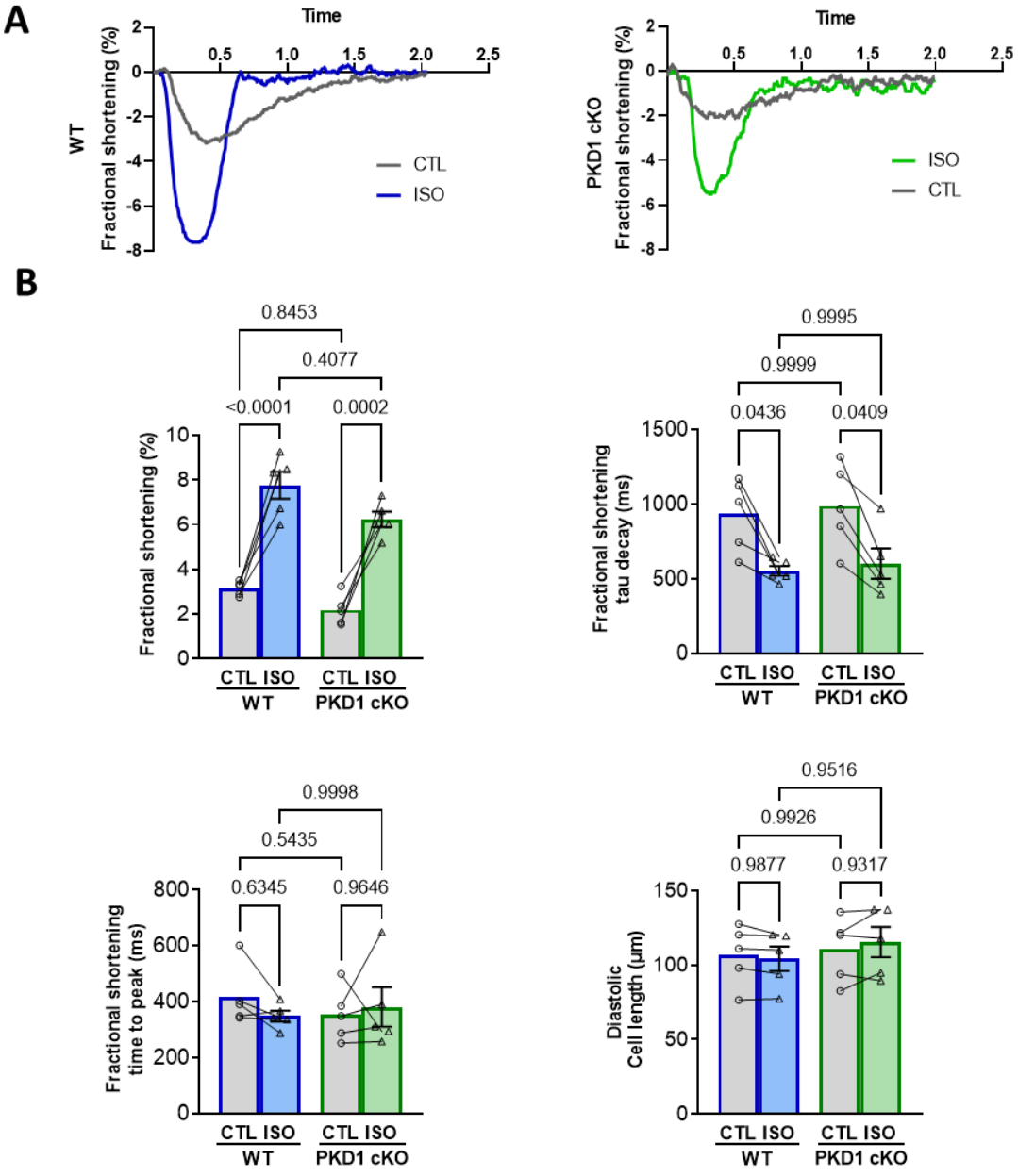

FIGURE 4. Both WT and PKD1 cKO ventricular myocytes increase contractility similarly after β–AR stimulation (isoproterenol). A, Representative traces of cell shortening in WT and PKD1 cKO myocytes. **B,** Fractional shortening raw amplitude, tau decay, time to peak of contraction and diastolic cell length were similar in both WT and PKD1 cKO Fluo-4 AM loaded myocytes at baseline and after 5-minute incubation with 100 nM isoproterenol (ISO). Data points represent cells (WT n=6, KO n=6). Mice: WT N=4, KO N=4. Ordinary two-way ANOVA, followed by Tukey's multiple comparisons test.

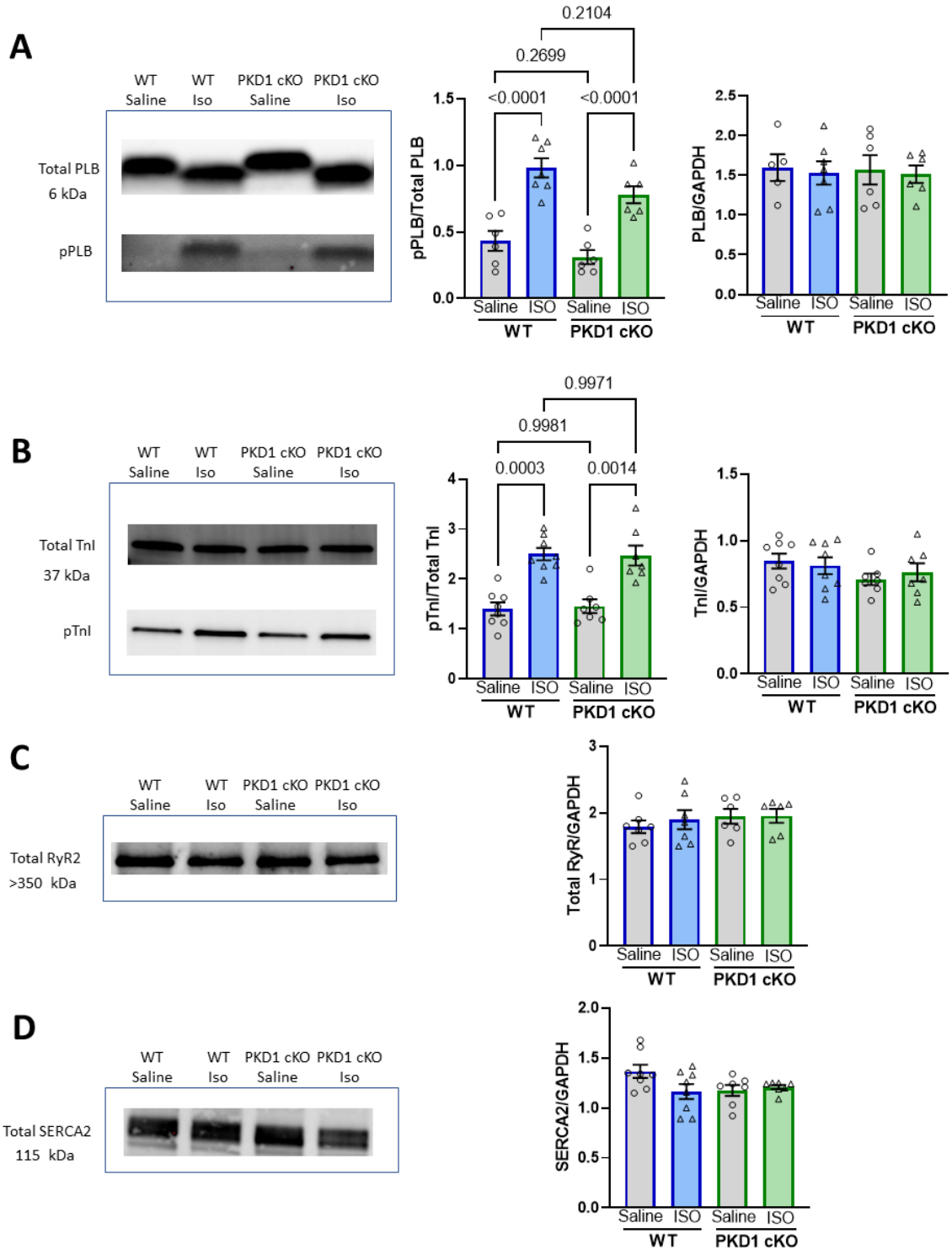

FIGURE 5. Phospholamban (PLB) and cardiac troponin I (cTnI) ISO-induced phosphorylation increase was similar in both genotypes. A, Representative blots for total PLB and pPLB (Ser 16), and ratios of pPLB/Total PLB and total PLB/GAPDH. **B,** Representative blots for total TnI and pTnI (Ser 22/23), and ratios of pTnI/Total TnI and total TnI/GAPDH. **C,** Representative blots for total RyR2 and ratios for total RyR/GAPDH. **D,** Representative blots for total SERCA2 and ratios for SERCA2/GAPDH. Data points represent hearts (WT n=6-8, KO n=6-8). Ordinary two-way ANOVA, followed by Tukey's multiple comparisons test.

Supplementary data

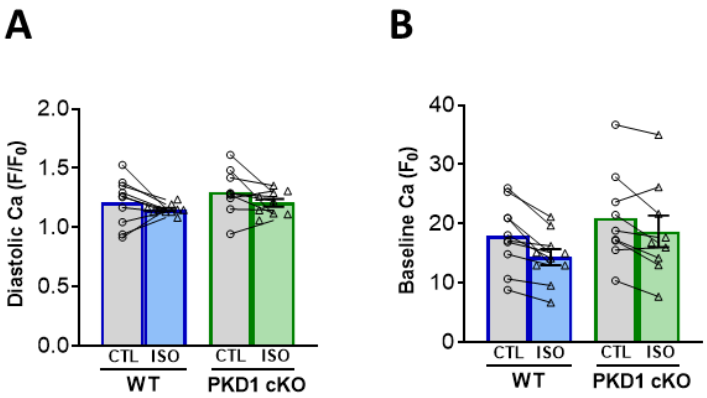

FIGURE S1. Diastolic Ca changes were comparable in both WT and PKD1 cKO myocytes. A, Diastolic Ca during pacing. **B,** baseline Ca (at rest). Same recordings from Figure 1. Data points represent cells (WT n=9, KO n=8). Mice: WT N=5, KO N=4. Ordinary two-way ANOVA, followed by Tukey's multiple comparisons test.

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Chapter 3: PKD1 is necessary for AngII-induced cardiac hypertrophy independent of calcium handling changes

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ABSTRACT

The renin-angiotensin system (RAS) or renin-angiotensin-aldosterone system (RAAS) regulates blood pressure, extracellular fluid and electrolyte balance, cardiac inotropy (cardiac output), and vascular resistance, allowing necessary adjustments to systemic and central circulation in low plasma volume conditions (i.e., exercise, dehydration). However, chronic activation of this system can lead to abnormal vascular and cardiac remodeling that alters the adequate function of the heart. Protein Kinase D (PKD) has been demonstrated as a downstream effector of the angiotensin II (AngII) signaling pathway in blood vessel cells, fibroblasts and cardiomyocytes. In the heart, AngII induces hypertrophy by direct signaling activation through kinases and indirectly by vascular hypertension. Despite PKD's role in cardiac hypertrophy induced by AngII stimulation, little is known about the acute and chronic effects of AngII-induced PKD activation on calcium (Ca) handling. To gain more insights into the acute and chronic effects of AngII/PKD signaling effects on Ca handling, we used hearts from cardiac-specific PKD1 knockout (cKO) mice and their WT littermates. We measured Ca transients (CaT) and Ca spark (CaSp) frequency in cardiomyocytes at 0.5 and 1 Hz pacing under acute and chronic AngII stimulation (100 nM and 3 mg/kg/day, respectively). Sarcoplasmic reticulum (SR) Ca load was assessed by rapid caffeine-induced Ca release. At baseline and after

acute AngII treatment, CaT amplitude and tau decay, spark frequency and SR Ca load were similar in WT vs. PKD1 cKO. This was also the case for chronic exposure to AngII except that the tau decay was significantly slower in PKD1 cKO myocytes. In agreement with previous reports, echocardiographic measurements revealed that PKD1 cKO mice were also more resistant to chronic AngII-induced hypertrophy (LVPW increase 24.69 ± 2.74% vs. 54.73 ± 7.73 %) and ejection fraction reduction (4.08 ± 1.5% vs. 12.67 ± 0.52%). We conclude that despite PKD being necessary for the remodeling changes induced by AngII, it does not alter the amplitude and kinetics of CaT and spontaneous Ca release (sparks), except for the slowed decay kinetics in the PKD1cKO after chronic AngII treatment and the acute SR Ca load.

INTRODUCTION

The renin-angiotensin-aldosterone system (RAAS) is characterized by its essential role in maintaining vascular tone through the balance of the fluid volume and blood pressure, along with the cardiovascular control of neural and endocrine functions (Miller & Arnold, 2019; Patel et al., 2017). Uncontrolled and chronic activation of this system can progress to cardiovascular diseases like hypertension, heart failure, obesity, chronic kidney disease, coronary artery disease, and stroke (Miller & Arnold, 2019). This system contains several biologically active components such as renin, prorenin, prorenin receptor, angiotensinogen, angiotensin (AngI), angiotensin II (AngII), angiotensin III (AngIII), angiotensin IV (AngIV), aldosterone and others. From these components, AngII is the most remarkable one for cardiovascular function regulation (Miller & Arnold, 2019). In the heart, AngII induces hypertrophy directly by AT1 receptor activation and intracellular RAS

activation (Kumar et al., 2008) and subsequent activation of kinases like Protein Kinase D1 (PKD1) in cardiomyocytes (Fielitz et al., 2008; Iwata et al., 2005) and in vessels (Tan et al., 2004). In addition, AngII induces cardiac remodeling indirectly by the induction of chronic hypertension, by its vasoconstrictive effect that increase cardiac afterload (Kumar et al., 2008), promotion of aldosterone release (volume retention) and impairment of the arterial baroreceptor reflex in the central nervous system (Miller & Arnold, 2019).

PKD is a serine/threonine kinase that regulates cardiac remodeling during diseases like pressure overload induced hypertrophy (Fielitz et al., 2008) and also decreases myofilament Ca sensitivity in response to endothelin-1 activation (Bardswell et al., 2010; Haworth et al., 2004). PKD has been demonstrated to be required for the pathologic hypertrophic remodeling induced by chronic AngII and isoproterenol stimulation (β-AR receptor agonist) in mice (Fielitz et al., 2008). However, little is known about the acute and chronic effects of AngII-induced PKD activation on Ca handling.

In this study, we tested how PKD1 alters adult murine ventricular myocyte Ca handling under acute and chronic AngII stimulation. For this we used a cardiac specific knockout of the major cardiac isoform, PKD1 (PKD1 cKO; Fielitz et al., 2008). We found little differences in Ca transient (CaT) amplitude and Ca spark frequency (CaSp) in cardiomyocytes at 0.5 and 1 Hz pacing under acute and chronic AngII stimulation (100 nM and 3 mg/kg/day, respectively). In contrast to chronic AngII exposure, after acute AngII stimulation sarcoplasmic reticulum (SR) Ca load was reduced in both WT and PKD1 cKO mice, with a larger reduction in WT myocytes. In addition, the PKD1 cKO mice exhibited slower kinetics (tau decay) after chronic exposure to AngII. Our findings suggest

that PKD1 is necessary for cardiac remodeling, but not for Ca handling under AngII stimulation.

METHODS

Animal models and cell isolation

Healthy adult (8-12 weeks) C57BL/6J (WT, Jackson Laboratory, Stock No. 000664) mice, Protein Kinase D1 cardiac specific knock-out (PKD1 cKO, obtained by crossing $PKD1^{loxP/loxP}$ mice (Jackson Laboratory, stock No.: 014181) with $PKD1^{\alpha\text{-MHC-Cre}}$ (Fielitz et al., 2008)) and wild type (WT) littermates were used. For chronic exposure to angiotensin II (Ang II). The mice were subjected to a surgical implantation of subcutaneous osmotic minipumps (Alzet®, model 2006) that delivered a dose of 3 mg/kg/day of AngII over 2 weeks.

All animal procedures were approved by the Institutional Animal Care and Use Committee at University of California, Davis (protocol #22824) in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8^{th} edition, 2011).

Isolated cardiomyocytes were obtained by enzymatic digestion of mouse left ventricles with 89 mg of type II collagenase (Worthington Biochemical Company, Cat#LS004177) and 4 mg of type XIV protease (from Streptomyces griseus, Sigma-Aldrich, Cat#P5147). Briefly, heparin injected mice (400 U/kg body weight) were anesthetized with isoflurane (5% for induction and 3-3.5% for maintenance) and heart excision was performed. After aortic cannulation, hearts were perfused on constant flow

Langendorff apparatus (37ºC) with 50 mL 1X MEM (in mM NaCl 135, KCl 4.7, KH_2PO_4 0.6, Na_2HPO_4 0.6, $MgSO_4$ 1.2, Hepes-free acid 20, taurine 30, glucose 5.5 and 10 µM Ca^{2+} with enzymes and equilibrated with 100% O_2, pH 7.4) for 15-20 min. Subsequently, myocytes were disaggregated by gentle pipetting, filtered (nylon mesh) and sedimented repeatedly increasing Ca^{2+} concentration (0.125, 0.25 and 0.5 mmol/L). Before experiments, myocytes are kept in normal Tyrode's (NT) solution (in mM: NaCl 140, KCl 4, $MgCl_2$ 1, Hepes-Na 5, Hepes-H-free 5, glucose 5.5, 0.5 mmol/L [Ca^{2+}], pH 7.4) at room temperature (22-23°C).

Calcium imaging and contractility analysis

Isolated myocytes were indicator-loaded by incubation with Fluo-4 AM (10 µmol/L, Invitrogen with Pluronic F-127 0.02%, Invitrogen) in NT for 30 min and then washed for 10 min in NT. Then CaT and diastolic Ca release events (sparks) were measured at 0.5 Hz and 1 Hz pacing under either control conditions or acute AngII stimulation (100 nM) and additional experiments were done after in-vivo chronic AngII exposure for 2 weeks (3 mg/kg/day, respectively). Experiments were performed in paired cells (control and acute AngII on the same cell) at room temperature in NT solution (with 1.8 mM Ca^{2+} and 5.5 mM glucose). Line scan recordings were obtained using confocal microscopy (Bio-Rad Radiance 2100, 40x objective, 6 ms/line), exciting Fluo-4 AM with an Argon laser at 488 nm and collecting emission with a 500-530 nm bandpass filter. For SR Ca load determination, 10 mM caffeine was applied by rapid local delivery via pipette tip very close to the myocyte, and which caused intracellular [Ca] ($[Ca]_i$) rise rate comparable to an electrically evoked CaT. Recordings were analyzed with Image J and the plugin

SparkMaster (Image J-Fiji™(Picht et al., 2007)), with later data processing with our custom-made Python based script for CaT and Ca SR load (CaTransient_Analyzer Beta 4.1 Lite by Christopher Y. Ko).

Statistical analysis

Data is presented as mean ± SEM. Data distribution was determined by D'Agostino-Pearson test. Two-tailed Student's t-test (paired or unpaired) and one-way or two-way ANOVA followed by post-hoc multiple comparison test (Tukey's) was used and are noted in each figure legend. When data was not normally distributed or N<6, non-parametric test were used. Differences were considered statistically significant if $P < 0.05$. Exact P-values are noted in figures or legends. GraphPad Prism 9 software was used for data analysis.

RESULTS

Ca transient amplitudes and kinetics are not altered by AngII in WT or PKD1 cKO

PKD is classically activated by several Gq-coupled receptor agonists, including AngII, endothelin-1 and α-adrenergic agonists (e.g. phenylephrine; (Avkiran et al., 2008; Rozengurt et al., 2005)). **Figure 1** shows steady state CaT at 0.5 Hz pacing in WT and PKD1 cKO myocytes before and 5 min after exposure to 100 nM AngII. Baseline Ca handling was not modified by PKD1, as observed before by our group (Chapter 2 unpublished data). Both WT and PKD1 cKO cardiomyocytes showed similar CaT amplitudes and kinetics at baseline (without AngII; **Figure 1D**). There was also no

significant change in CaT properties induced by AngII exposure in either WT or PKD1 cKO mouse myocytes (**Figure 1D**).

Ca Spark rate was not altered by acute AngII treatment in WT or PKD1 cKO

To evaluate whether AngII-induced PKD activation alters diastolic SR Ca leak we measured spontaneous SR Ca release events (Ca sparks) and also SR Ca content (caffeine-induced CaT) immediately after the spark recording period (**Figure 2A**). No significant differences were observed between WT and PKD1 cKO at baseline. AngII treatment showed no significant changes in Ca spark rate in either genotype (**Figure 2B-C**), demonstrating that the acute AngII/PKD1 pathway is not important in regulating basal spontaneous Ca release from the SR. In contrast, SR Ca content was significantly reduced after acute AngII treatment in both WT and PKD1 cKO, with a larger reduction in WT myocytes. This may reflect a small AngII-induced increase in SR Ca leak that is more apparent when the Ca spark frequency is normalized to the SR Ca load (**Figure 2C**, right) accounting for the influence of SR Ca content on Ca leak (Bers, 2014; Shannon et al., 2000).

PKD1 cKO mice were more resistant to chronic AngII-induced hypertrophy and their cardiomyocytes showed slower Ca kinetics

To test whether chronic AngII stimulation and subsequent PKD1 activation modifies cardiac hypertrophy and function, we implanted AngII-releasing minipumps and measured echocardiograms before and 2 weeks after the implants (**Figure 3A-B**). In agreement with previous studies (Fielitz et al., 2008), echocardiographic measures

revealed that PKD1 cKO mice were more resistant to chronic AngII-induced hypertrophy (assessed here by changes in LV posterior wall thickness in diastole; LVPWd) and decrease in ejection fraction (EF; **Figure 3C-G**).

We also assessed Ca handling in ventricular myocytes from mice which had either AngII or saline minipump implantation for two weeks. **Figure 4A-C** shows cytosolic CaT amplitude and kinetics paced at 0.5 Hz in chronically AngII-exposed WT and PKD1 cKO cardiomyocytes. No statistically significant differences were seen in CaT amplitude or time to peak in either saline or chronic AngII-exposed myocytes, independently of the genotype (**Figure 4**). However, myocytes from PKD1 cKO mice exhibited a significant slowing of CaT tau of decay after chronic AngII exposure that was not seen in WT (**Figure 4C-D**).

Spontaneous Ca release after chronic AngII exposure was reduced in WT but not PKD1 cKO mice

To assess whether SR Ca leak was altered by chronic exposure to AngII, we measured diastolic Ca release (spark rate) and SR Ca load measured by caffeine-induced CaT in chronically AngII-exposed WT and PKD1 cKO cardiomyocytes (**Figure 5**). No significant genotype differences were observed in myocytes from saline-implant mice, but in chronically AngII-exposed WT myocytes exhibited a significant reduction in Ca spark rate without altered SR Ca load. In PKD1 cKO myocytes, chronic AngII did not significantly alter either Ca spark rate or SR Ca load. These results would suggest that chronic AngII does something in control myocytes, involving PKD1 signaling, that limits SR Ca leak.

Acute AngII in chronic AngII-exposed myocytes did not alter Ca handling in either genotype

To assess whether a pre-activation of PKD1 during chronic AngII exposure can modify the effects of an acute stimulation with AngII, we measured cytosolic CaT amplitude and kinetics (with Fluo4-AM) at 0.5 Hz pacing rate in chronically AngII-exposed WT and PKD1 cKO cardiomyocytes upon acute incubation with 100 nM of AngII (5 min; **Figure 6**). In both genotypes the cells showed similar CaT amplitudes, tau decay and time to peak to the ones observed without acute AngII treatment (with saline minipumps). Thus, as for naïve mice (without AngII minipumps) acute AngII had no significant effect on CaT properties.

DISCUSSION

AngII's best described effect in the heart is the induction of remodeling (hypertrophy) and heart failure, indirectly by hypertension and directly by AT_1R activation (Kumar et al., 2008). AngII has been also implicated in the modulation of cardiac contractility (Vila Petroff & Mattiazzi, 2001). However, studies in this area are controversial. Several authors have reported that AngII can increase (positive inotropy)(Ishihata & Endoh, 1995; Koch-Weser, 1965), decrease (Li et al., 1994), or not change contractility (Lefroy et al., 1996). This variability between studies may come in part from the intrinsic variations between models (species), cellular and whole heart experiments, incubation times and overall experimental conditions (Vila Petroff & Mattiazzi, 2001). Despite the differences, most studies agree in some cardiac positive inotropy induced by AngII. This effect is produced by increasing $[Ca]_i$ and/or increasing myofilament responsiveness to Ca (Vila

Petroff & Mattiazzi, 2001). AngII signaling pathway for cardiac remodeling includes Protein Kinase D activation but the effects of this kinase on Ca handling upon AngII stimulation remain unclear.

Here we showed that acute incubation (5 min) of AngII did not alter CaT amplitude or decay in either WT or PKD1 cKO myocytes. The AngII signaling pathway activation and subsequent EC-coupling proteins phosphorylation by downstream effectors takes several minutes. We wanted to assess the very acute short effects of AngII in the presence and absence of PKD1, and we found no remarkable effects. As we mentioned before, AngII effects may vary according to experimental conditions, but we can conclude that the WT and PKD1 cKO myocytes responded similarly to the acute stimulation with AngII. CaTs are expected to increase after AngII stimulation through the mechanism of I_{CaL} increase (Talukder & Endoh, 1997); however, I_{CaL} measurement results have been also controversial with some finding it also to be decreased or unchanged (Vila Petroff & Mattiazzi, 2001).

Like CaTs, CaSp frequency was unchanged after AngII treatment in WT and PKD1 cKO cells. However, SR Ca load was significantly reduced in both genotypes with a larger reduction in the WT. This differs from the previously reported AngII-induced increase in NCX reverse activity that increases $[Ca]_i$ by increased NHE activity and subsequent increase in $[Na]_i$ (Gusev et al., 2009; Vila Petroff & Mattiazzi, 2001). It is possible that the early effects of acute AngII stimulation reduce NHE and NCX activity, and this is later replaced by the opposite effect ending in the increase of $[Ca]_i$ as previously described. A lower SR Ca load will lead to a lower Ca spontaneous release (CaSp frequency) like the

observed here (Bers, 2001, 2014). Our findings of an AngII-induced decrease in SR Ca load with an elevated Ca spark frequency when normalized to SR Ca load would be consistent with an AngII-induced slight RyR2 sensitization that lowers SR Ca load but preserves CaT amplitude and kinetics (larger fractional SR Ca release due to the RyR sensitization). That is precisely consistent with the effects of low caffeine concentration on SR Ca load and CaT (Trafford et al., 2000). The fact that this effect was independent of PKD1, suggests a PKD-independent AngII induced effect. This could potentially be mediated by CaMKII which is known to be activated by AngII (Erickson et al., 2011) and to sensitize RyR2 (Uchinoumi et al., 2010; van Oort et al., 2010).

The chronic pathophysiological AngII-induced effects in the heart are better described than the acute effects, but the chronic effects relate more to remodeling and heart failure than Ca handling. Here we demonstrated that chronic exposure to AngII has no significant effect on CaT amplitude, but slowed twitch $[Ca]_i$ decline (longer tau), but only in the PKD1 cKO myocytes when compared to WT. We infer from this that endogenous PKD1 may help to maintain normal CaT decline during chronic AngII treatment, an effect that is lost when PKD1 is ablated. Overexpression of AT_1R in mice caused cardiac hypertrophy, reduced I_{CaL} and CaT amplitude, and prolonged CaT duration (CaTD90) (Rivard et al., 2011). However, we detected similar changes only when PKD1 was ablated. These findings suggest that in physiological conditions, chronic exposure to AngII, PKD1 is necessary to preserve CaT amplitude and decay that is similar to the control conditions (saline).

In contrast to the acute AngII exposure, chronic AngII stimulation did not reduce SR Ca load in either WT or PKD1 cKO myocytes, but CaSp frequency was significantly reduced in the WT myocytes. This suggest that chronic AngII-induced activation of PKD1 may suppress rather than promote RyR2 sensitization. PKD1 has been demonstrated to modulate L-type Ca channel vesicle trafficking and membrane exposure, and phosphorylation with changes in open probability (Aita et al., 2011; Jhun et al., 2012; Maturana et al., 2008). An additional acute AngII treatment on chronic exposed myocytes did not alter the previous described results. Reinforcing that short incubation times do not cause alterations of CaT parameters. Another factor to be considered, is that in the absence of PKD1 other kinases like PKC might help to compensate and preserve the functions for which PKD1 is involved.

In conclusion, we demonstrated here that PKD1 is necessary for full cardiac remodeling induced by chronic AngII exposure (hypertrophy and reduced ejection fraction) and to preserve normal CaT kinetics and SR Ca load in myocytes of the same hearts. Additionally, we showed that acute treatment with AngII does not alter CaT amplitude or kinetics, but reduces SR Ca load and sensitized SR Ca release, but those effects were similar in the PKD1 cKO myocytes. These findings suggest that PKD1 can modulate the effects of AngII on Ca handling but that these effects are time dependent. Further studies are necessary to investigate the mechanisms by which this modulation occurs.

AFFILIATIONS

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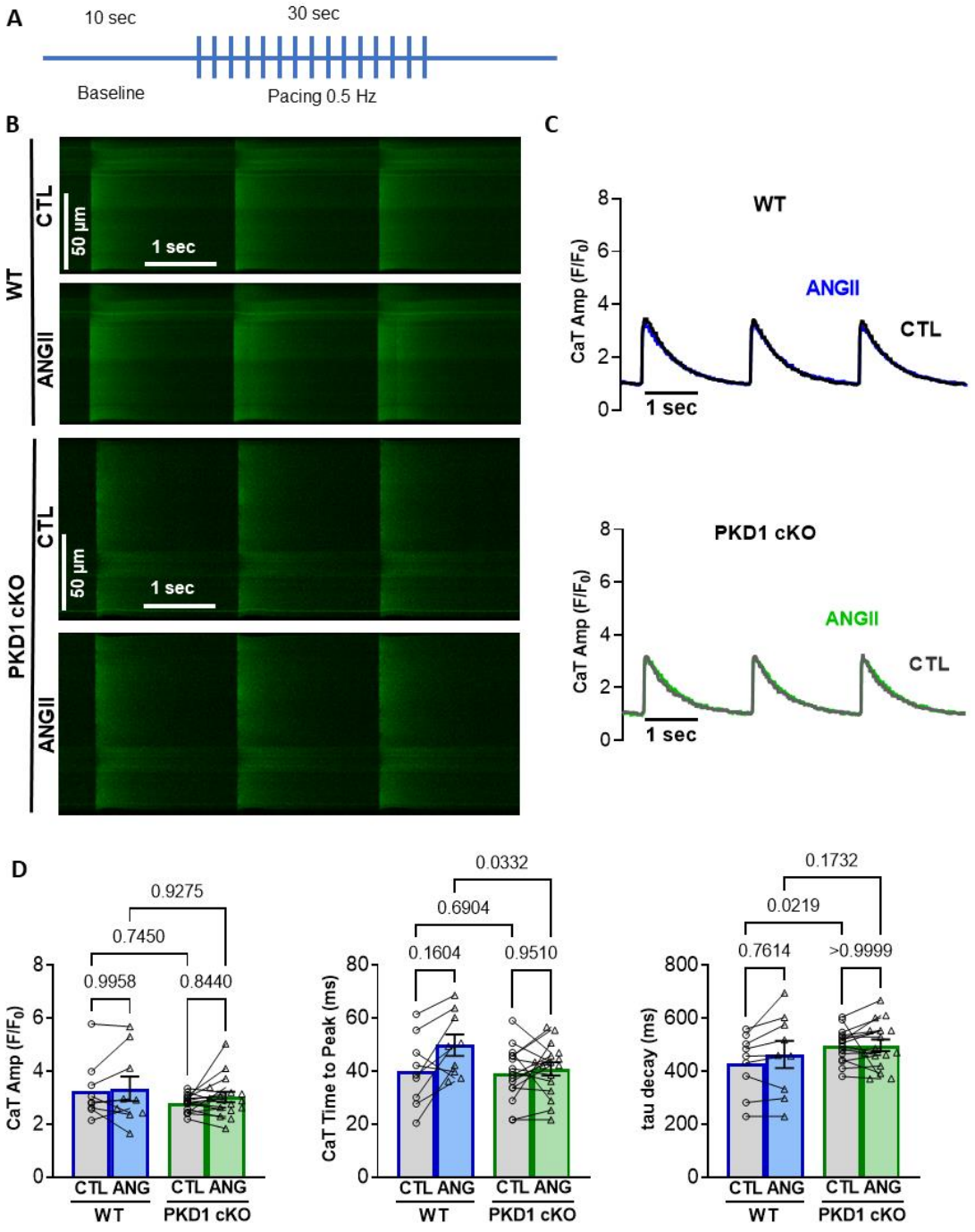

FIGURE 1. Upon angiotensin II (AngII) stimulation, PKD1 cKO and WT cardiomyocytes showed similar Ca^{2+} transient (CaT) amplitudes and kinetics. A, Experimental protocol for Ca^{2+} imaging. **B,** Representative confocal images and **C,** representative traces of intracellular Ca^{2+} transients (CaT) in both WT and PKD1 cKO Fluo-4 AM loaded myocytes at baseline and after 5-minute incubation with 100 nM AngII. **D,** No significant changes in CaT amplitude, tau decay or CaT time to peak after AngII treatment were observed in both WT and PKD1 cKO. Data points represent cells (WT n=9, KO n=16). Mice: WT N=5, KO N=6. Ordinary two-way ANOVA, followed by Tukey's multiple comparisons test.

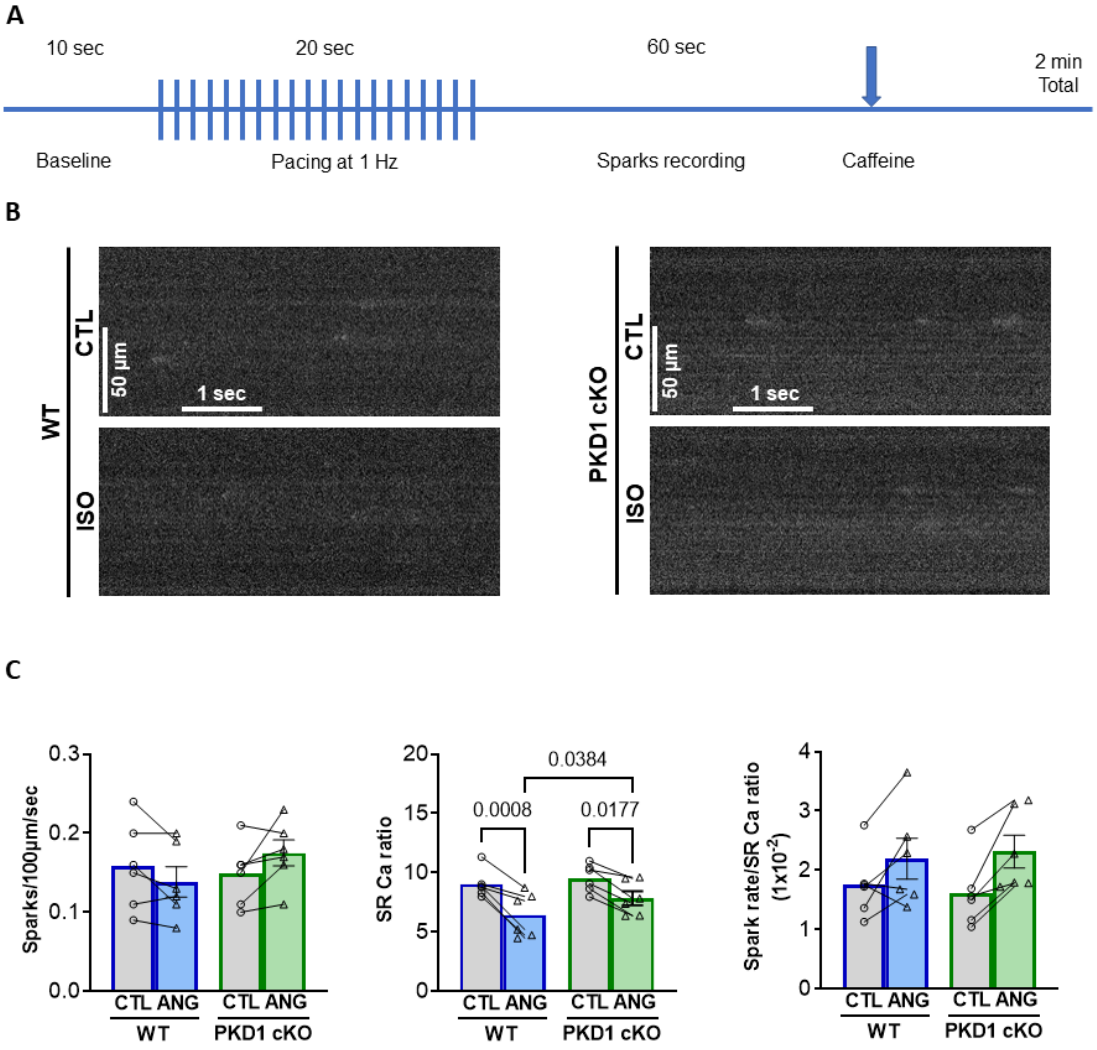

FIGURE 2. Spontaneous Ca release represented as spark rate remains similar after AngII treatment in both WT and PKD1 cKO cardiomyocytes. **A,** Experimental protocol for diastolic Ca^{2+} imaging (sparks). **B,** Representative confocal images of diastolic Ca^{2+} sparks in both WT and PKD1 cKO myocytes at baseline and after 5-minute incubation with 100 nM AngII. **C,** Diastolic Ca^{2+} sparks rate is similar in both WT and PKD1 cKO after AngII treatment. SR Ca^{2+} load reduction is larger in WT ventricular myocytes compared to PKD1 cKO. Normalization of spark rate to SR Ca^{2+} load shows a similar non-statistically significant increase in both genotypes. Data points represent cells (WT n=6, KO n=6). Mice: WT N=4, KO N=4. Ordinary two-way ANOVA, followed by Tukey's multiple comparisons test.

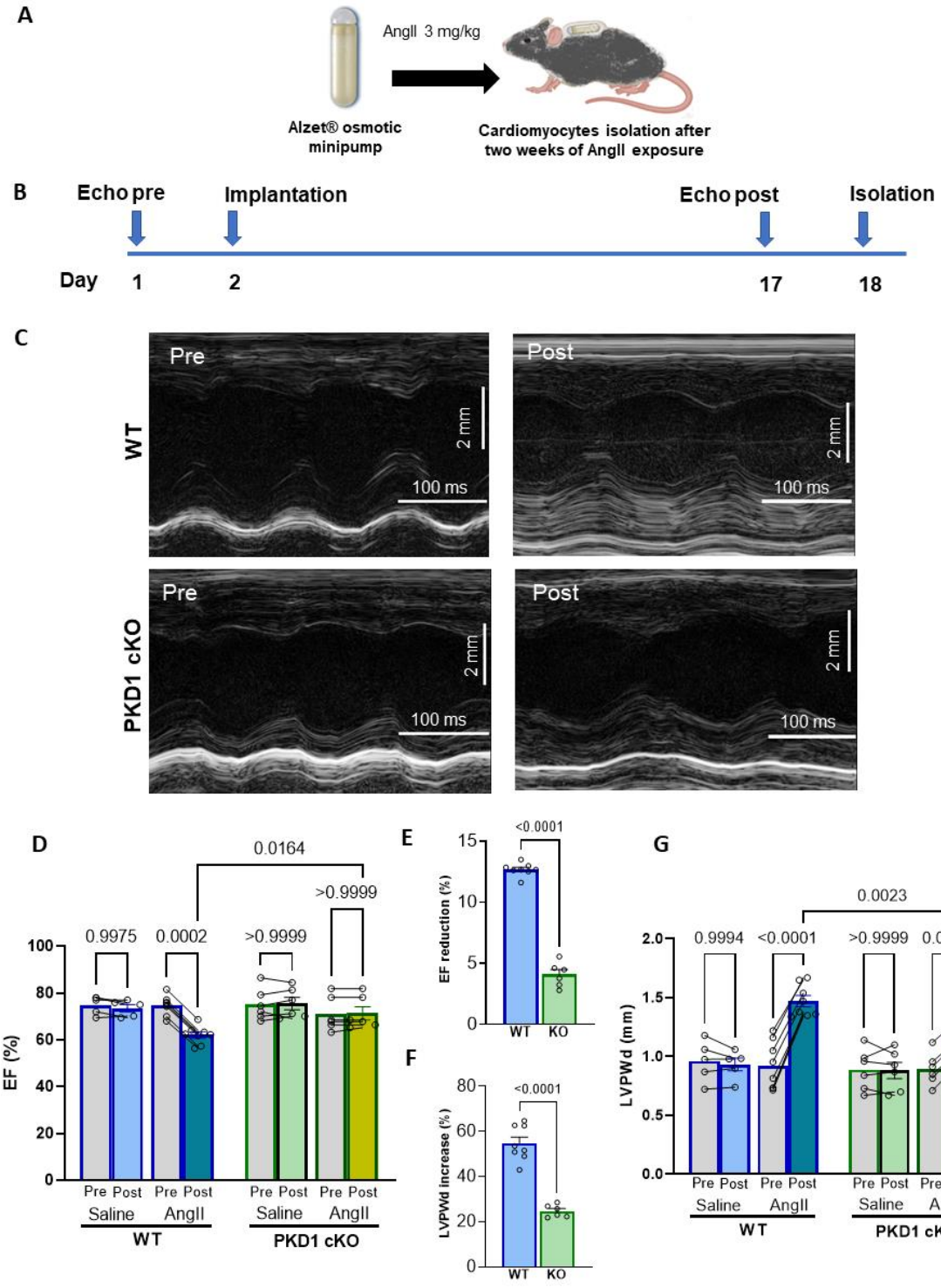

FIGURE 3. PKD1 cKO mice were more resistant to chronic AngII-induced hypertrophy. **A,** Osmotic minipump graphical representation. **B,** Minipump chronological protocol and echocardiographic time points. **C,** Representative M-mode echocardiographic traces in WT and PKD1 cKO mice, before and after AngII-minipump implantation. **D,** Ejection fraction (EF) in WT and PKD1 cKO with saline or AngII pump (One-way ANOVA). **E,** EF reduction in WT vs. KO with AngII pump (unpaired t-test). **F,** LVPW increase in WT vs. KO (unpaired t-test). **G,** Left posterior ventricular wall (LPVW) WT and PKD1 cKO mice with saline or AngII pump (One-way ANOVA). Data points represent mice (WT saline N=6, AngII N=8; KO saline N=6, AngII N=7).

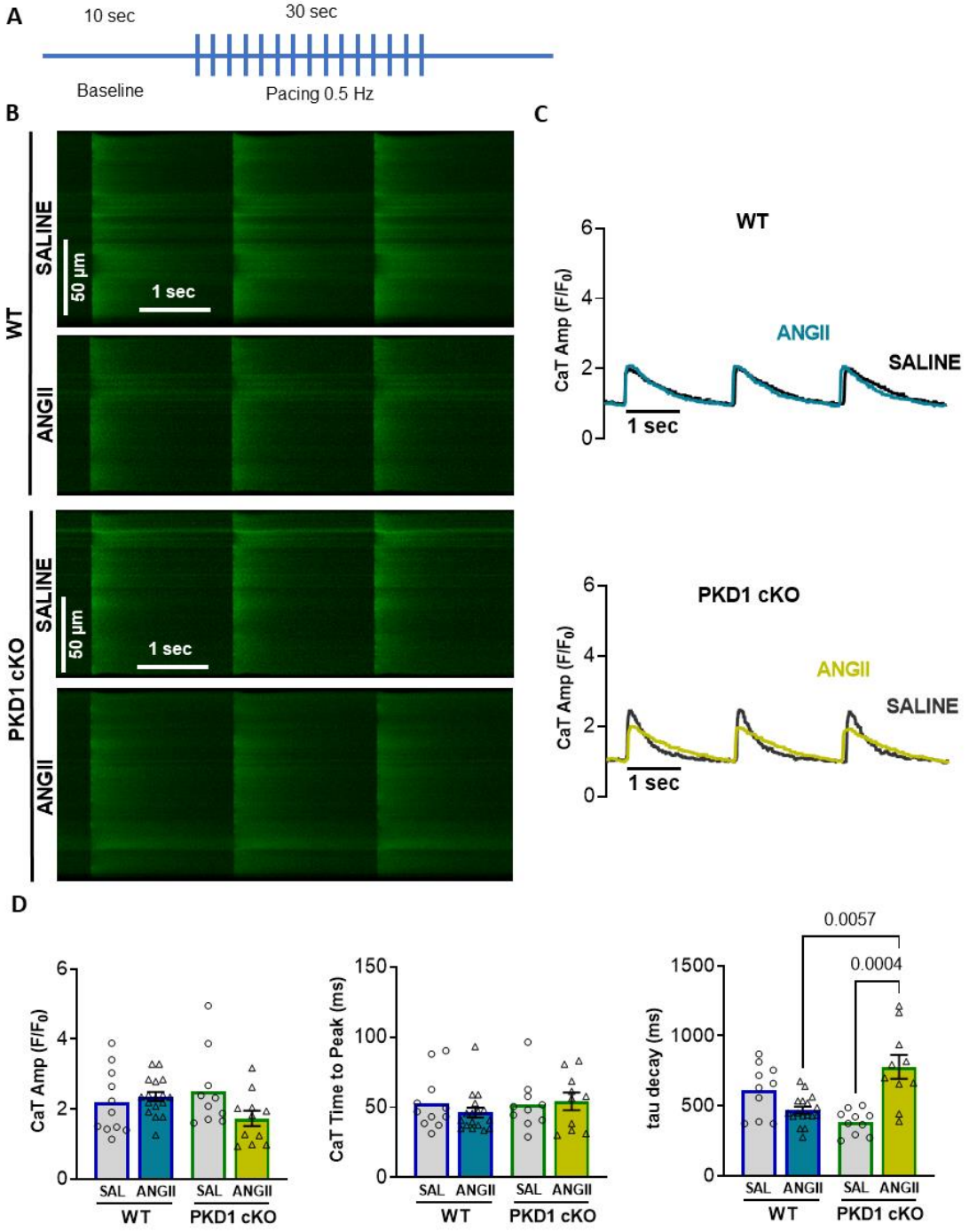

FIGURE 4. In PKD1 cKO cardiomyocytes chronically exposed to AngII kinetics were slower compared to WT. A, Experimental protocol for Ca^{2+} imaging. **B,** Representative confocal images and **C,** representative traces of intracellular Ca^{2+} transients (CaT) in both WT and PKD1 cKO Fluo-4 AM loaded myocytes at baseline and after 2 weeks treatment with 3 mg/kg/day of AngII or saline. **D,** CaT amplitude and CaT time to peak was similar in both genotypes but tau decay was significantly slower in the PKD1 cKO. Data points represent cells (WT saline n=11, AngII n=18; KO saline n=10, AngII n=10). Mice: WT saline N=6, AngII N=8; KO saline N=6, AngII N=7. Ordinary two-way ANOVA, followed by Tukey's multiple comparisons test.

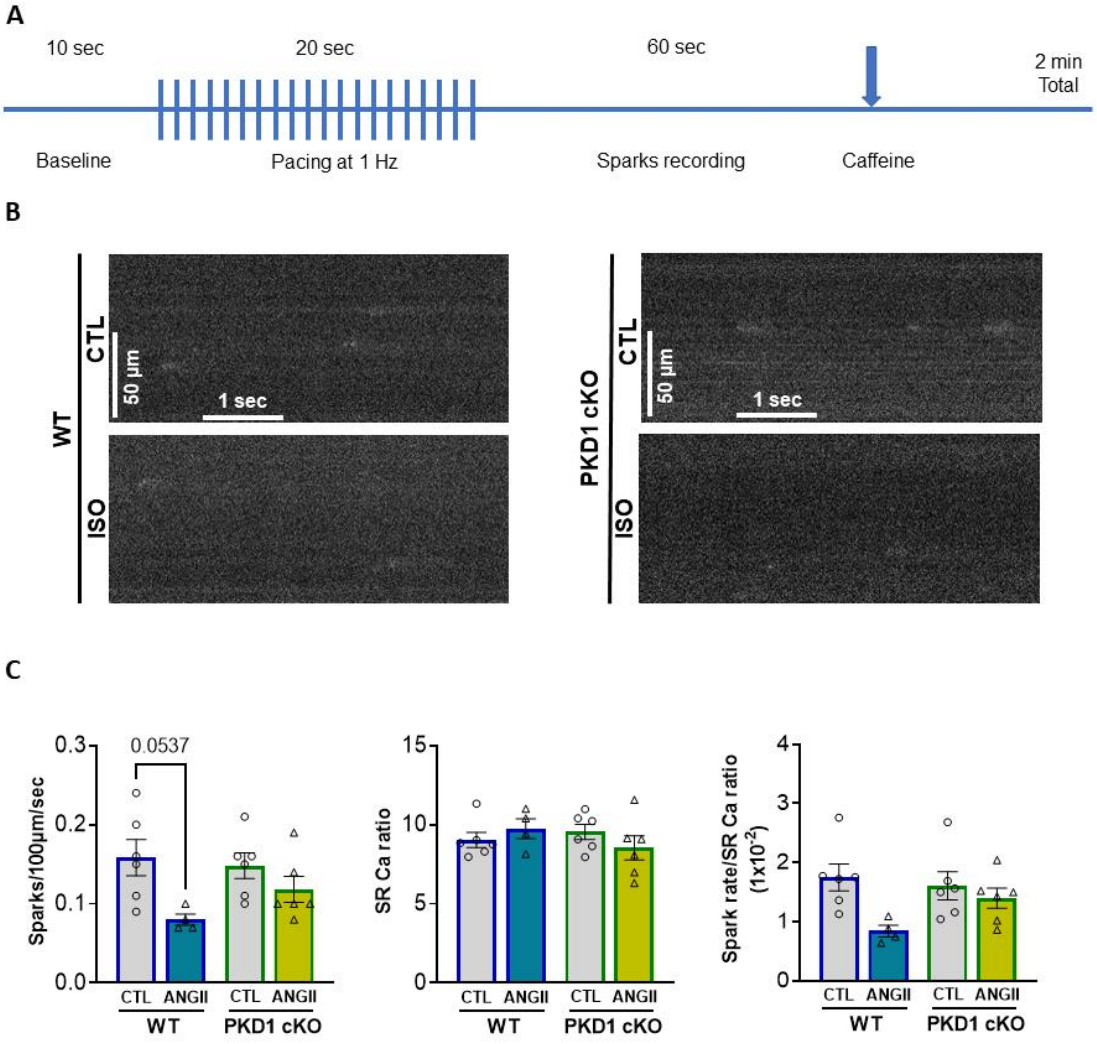

FIGURE 5. Spontaneous Ca release after chronic AngII exposure was reduced in WT but not PKD1 cKO mice. A, Experimental protocol for diastolic Ca^{2+} imaging (sparks). **B,** Representative confocal images of diastolic Ca^{2+} sparks in both WT and PKD1 cKO myocytes in control and after chronic AngII exposure (3mg/kg/day). **C,** Diastolic Ca^{2+} sparks rate was significantly lower in the WT but not the PKD1 cKO cells. SR Ca^{2+} load was similar in both genotypes. Normalization of spark rate to SR Ca^{2+} load shows a similar non-statistically significant results as non-normalized spark rate. Data points represent cells (WT CTL n=6, AngII n=4; KO CTL n=6, AngII n=6). Mice: WT CTL N=4, AngII N=2; KO CTL N=4, AngII N=4 Ordinary two-way ANOVA , followed by Tukey's multiple comparisons test.

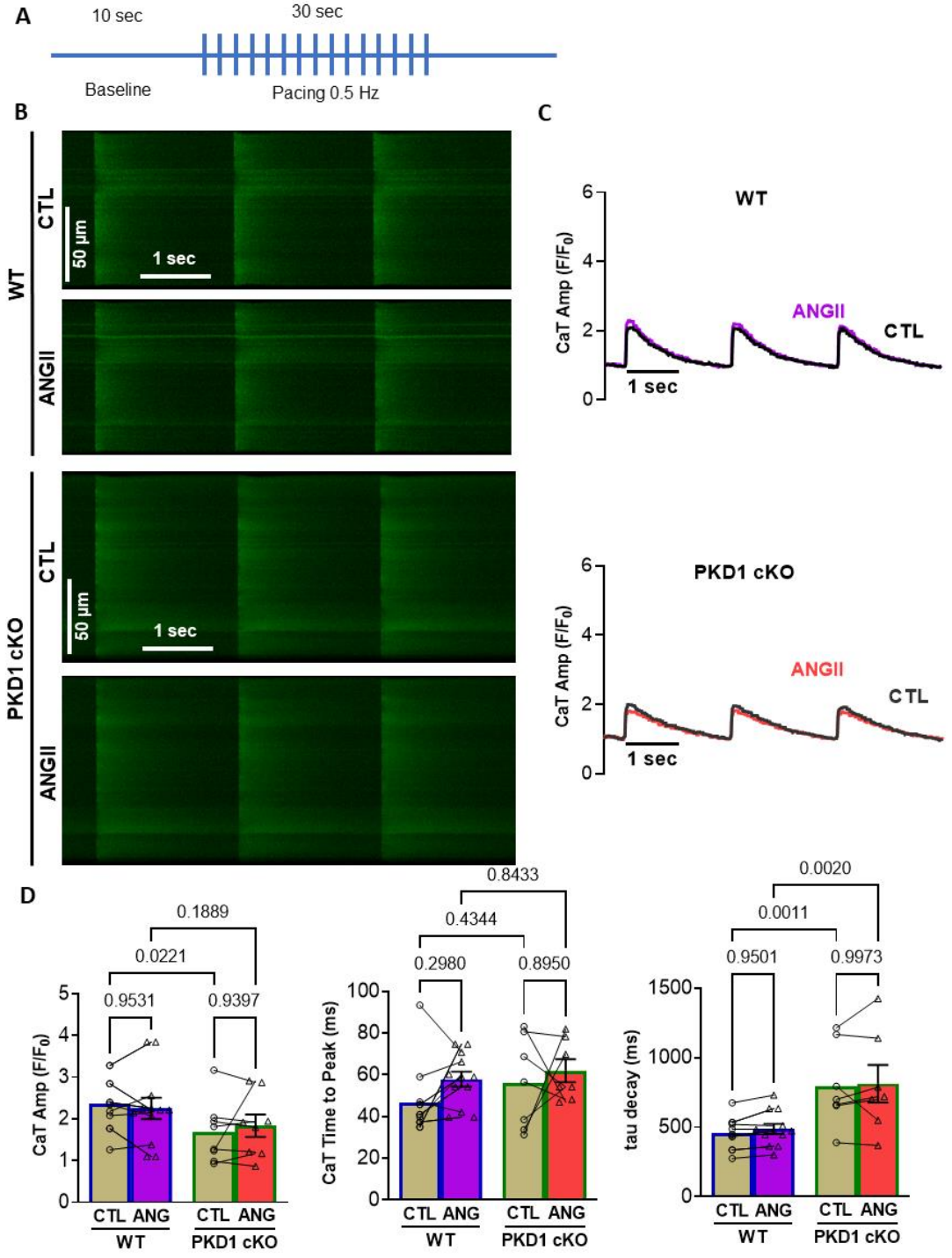

FIGURE 6. In chronically AngII-exposed myocytes and additional treatment with acute AngII did not cause significant changes of CaT parameters in both PKD1 cKO and WT. A, Experimental protocol for Ca^{2+} imaging. **B,** Representative confocal images and **C,** representative traces of intracellular Ca^{2+} transients (CaT) in both WT and PKD1 cKO (chronically AngII-exposed) Fluo-4 AM loaded myocytes at baseline and after 5-minute incubation with 100 nM AngII. **D,** No changes on CaT amplitude, CaT time to peak or tau decay were observed after acute AngII treatment. The same slower tau decay previously observed in the PKD1 cKO was preserved after acute AngII treatment. Data points represent cells (WT n=11, KO n=8). Mice: WT N=6, KO N=5. Ordinary two-way ANOVA, followed by Tukey's multiple comparisons test.

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Chapter 4: Conclusions and future directions

Cardiovascular diseases represent the number one cause of death in the United States and are the leading cause of death in the world. Preventing and treating these diseases have been for decades a priority for medicine and research. This led to the extensive investigation of heart and vessel physiology and pathophysiology, at the organ, cellular and molecular level. After decades of results, we are still surrounded by infinite unknowns. Such is the case of the frame of targets and effects of protein kinases. This is the case of Protein Kinase D, a well-known multifunctional kinase that is key in the treatment of cancer, but that in the heart is less studied compared to other kinases with more robust effects in cardiomyocytes. This study presented here, demonstrated for the first time, the PKD modulation of EC-coupling and Ca handling during acute β-AR and chronic AngII stimulation, beyond the well-studied effects on cardiac remodeling or myofilament Ca sensitivity.

Here we showed that PKD1 ablation in cardiomyocytes reduces the response to acute β-AR stimulation, exhibiting smaller CaT amplitude, slower kinetics and less spontaneous Ca release from the SR. These effects may be mediated by RyR desensitization and slower SERCA pump rate, and they are independent of changes in I_{CaL}, ECC protein expression or contractility. The specific mechanism by which these effects are caused remain to be elucidated. The β-AR signaling pathway involves a wide variety of proteins that can be altered by interactions with several other proteins, making this pathway complex and multifactorial. In addition to this, for β-AR and some other kinases signaling (like PKD) appears to be compartmentalized, making the dissection of

the specific molecular mechanisms even more complex. Future studies are necessary with targeted fluorescent sensors and other transgenic mice that allow the assessment of PKD interaction with other kinases in myocytes. Despite having made some progress, it will be important to evaluate the treatment with PKD inhibitors in disease models.

We also demonstrated that PKD is necessary for full cardiac remodeling induced by chronic AngII exposure (hypertrophy and reduced ejection fraction) and for the preservation of normal CaT kinetics and SR Ca load. In addition, acute treatment with AngII does not alter CaT amplitude or kinetics, but reduces SR Ca load and sensitizes SR Ca release, similarly in both WT and PKD1 cKO myocytes. This suggests that AngII effects on Ca handling may be modulated by PKD1, but that these effects are time dependent. The AngII effect in EC-coupling experiments have been controversial and this may come from the variation in models (animal species, cell vs. whole heart, in vitro vs. in vivo, etc) and the experimental design (dose, incubation time, purity of the compound, etc). Therefore, these experiments need to be carefully analyzed in the context of the model and experimental design used. Additionally, other kinases (like CaMKII) that can be activated by AngII may compensate in the absence of PKD1 and complicate the interpretation of the results.

As mentioned before, protein kinases have a wide variety of intracellular distributions (compartments or nanodomains) and functions in cardiomyocytes that have been recognized, but we still have many unknowns, and it is difficult to accurately state that a specific kinase does not have a specific function, location, or target until it is directly tested experimentally. This is why, studies like the ones presented here help feed the

infinite map of protein kinase functions and targets and pave the road for new studies that keep advancing our knowledge in the cardiovascular system and with this help the development of new therapeutic solutions to the several diseases of this system.