Protein kinase D (PKD) exists as a family of structurally related enzymes that are activated through similar phosphorylation-dependent mechanisms involving protein kinase C (PKC). While individual PKD isoforms could in theory mediate distinct biological functions, previous studies identify a high level of functional redundancy for PKD1 and PKD2 in various cellular contexts. This study shows that PKD1 and PKD2 are activated in a stimulus-specific manner in neonatal cardiomyocytes. The α1-adrenergic receptor agonist norepinephrine selectively activates PKD1, thrombin and PDGF selectively activate PKD2, and endothelin-1 and PMA activate both PKD1 and PKD2. PKC activity is implicated in the α1-adrenergic receptor pathway that activates PKD1 and the thrombin- and PDGF-dependent pathways that activate PKD2. Endothelin-1 activates PKD via both rapid PKC-dependent and more sustained PKC-independent mechanisms. The functional consequences of PKD activation were assessed by tracking phosphorylation of CREB and cardiac troponin I (cTnI), two physiologically relevant PKD substrates in cardiomyocytes. We show that overexpression of an activated PKD1-S744E/S748E transgene increases CREB-Ser133 and cTnI-Ser23/Ser24 phosphorylation, but agonist-dependent pathways that activate native PKD1 or PKD2 selectively increase CREB-Ser133 phosphorylation; there is no associated increase in cTnI-Ser23/Ser24 phosphorylation. Gene silencing studies provide unanticipated evidence that PKD1 down-regulation leads to a compensatory increase in PKD2 activity and that down-regulation of PKD1 (alone or in combination with PKD2) leads to an increase in CREB-Ser133 phosphorylation. Collectively, these studies identify distinct roles for native PKD1 and PKD2 enzymes in stress-dependent pathways that influence cardiac remodeling and the progression of heart failure.

Protein kinase D (PKD)² consists of a family of three structurally related serine-threonine kinases (termed PKD1 (or PKDμ), PKD2 and PKD3 (or PKDν)) that play key roles in cell growth, differentiation, migration, and apoptosis (1). PKD isoforms share a common domain structure, consisting of a C-terminal kinase domain and an N-terminal regulatory domain. The regulatory domain contains a C1 domain that anchors full-length PKD to diacylglycerol/phorbol ester-containing membranes and a pleckstrin homology (PH) domain that participates in intramolecular autoinhibitory interactions. PKD activation is generally attributed to a phosphorylation-dependent mechanism involving PKC. Receptors that activate phospholipase C and promote diacylglycerol accumulation co-localize PKD with allosterically activated PKC at lipid membranes. PKD is then activated as a result of phosphorylation at Ser744 and Ser748, a pair of highly conserved serine residues in the activation loop of the kinase domain (nomenclature based upon rodent PKD1). Once activated, PKD1 and PKD2 execute autophosphorylation reactions at a residue in PKD consensus phosphorylation motif at the extreme C terminus (Ser916 in PKD1 and Ser876 in PKD2); PKD3 lacks a C-terminal autophosphorylation site and is not regulated in this manner.

PKD has recently emerged as an important component of signaling pathways that induce structural and functional features of cardiac hypertrophy (2). Several substrates that mediate PKD cardiac actions have been identified. PKD phosphorylates the class II histone deacetylase HDAC5, creating a docking site for 14-3-3 proteins that escort HDAC5 from the nucleus to the cytosol, neutralizing the repressive effects of class II HDACs on MEF2-dependent transcription and effectively inhibiting HDAC5 antihypertrophic actions (3). PKD also phosphorylates cardiac troponin I (cTnI), the inhibitory subunit of the troponin complex (4). cTnI contains three phosphorylation clusters (Ser23/Ser24, Ser30/Ser31, and Thr144) that act in a functionally distinct manner to fine-tune the myofilament to hemodynamic load (5, 6). PKD-dependent cTnI phosphorylation has been mapped to Ser23/Ser24 and implicated in the endothelin-1 (ET-1)-dependent mechanism that regulates myofilament Ca²⁺ sensitivity in adult rat cardiomyocytes (4, 7). PKD also is a CREB-Ser133 kinase that links the Goq-PKCβ pathway to CREB-Ser133 phosphorylation, activation of a CRE-responsive promoter, and induction of Bcl-2 (CREB target gene) expression in cardiomyocytes cultures and transgenic mice that overexpress Goq (TgGoq). Of note, cTnI Ser23/Ser24 and CREB Ser133 phosphorylations are generally attributed to protein kinase A (PKA). The relative importance of PKA versus PKD (or individual PKD iso-
forms) as agonist-activated cTnl-Ser23/Ser24 or CREB-Ser133 kinases in vivo in cardiomyocytes has never been examined. PKD1, PKD2, and PKD3 have been detected at the mRNA level in various cardiac preparations (2, 8). While similar studies of PKD isoform protein expression have not been published, antibodies raised against epitopes conserved across PKD family members typically detect multiple molecular forms of PKD with distinct electrophoretic mobilities in cardiomyocytes (9). While this molecular heterogeneity is generally assumed to reflect the co-expression of multiple PKD isoforms with distinct cellular functions, direct experimental evidence that native PKD isoforms are activated in a stimulus-specific manner and/or phosphorylate different target substrates in vivo in a cellular context has never been published. In fact, studies using either RNA interference technology or genetic knock-out strategies generally identify functionally redundant for individual PKD isoforms (2, 10). This study is the first to show that PKD1 and PKD2 are activated in an agonist-specific manner in cardiomyocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies were from the following sources: PKD1-Ser(P)744/Ser(P)748, PKD1-Ser(P)916, PKD1, CREB-Ser(P)133, ERK, and cardiac troponin I-Ser(P)23/Ser(P)24 were from Cell Signaling Technologies. PKD1-Ser(P)742 (numbering based upon human sequence, corresponding to rodent PKD1-Ser748) and PKD2 (Cat. ab57114, a monoclonal antibody raised against residues 1–110 at the N terminus of human PKD2) were from Abcam. Anti-PKD2 antibodies from Calbiochem (Cat. ST1042) and Affinity Bioreagents (Cat. PAI-23446) were not used in this study, since preliminary experiments indicated that these reagents (which were raised against a synthetic peptide corresponding to residues at the extreme C terminus of human PKD2) preferentially recognize the inactive, not phosphorylated, form of the enzyme. Data were replicated in six experiments on separate culture preparations. Panels B and C, day 1 cardiomyocyte cultures were infected with an Ad-PKD1-RNAi that silences PKD1 expression, Ad-PKD2-RNAi that silences PKD2 expression, Ad-β-gal, or Ad-scramble as control as indicated (each at MOI of 20). Three days later, cultures were challenged for 20 min with vehicle or PMA (300 nM) and whole cell extracts were prepared for immunoblotting (IB) with antibodies that recognize PKD-Ser916 phosphorylation (nomenclature based upon rodent PKD1), PKD1 protein, or PKD2 protein according to “Experimental Procedures.”

**Cardiomyocyte Culture**—Cardiomyocytes were isolated from hearts of 2–day-old Wistar rats by a trypsin dispersion procedure that uses a differential attachment procedure followed by irradiation to enrich for cardiomyocytes (11). Cells were plated on prolammine sulfate-coated culture dishes at a density of 5 × 10⁶ cells/100-mm dish and grown in MEM (Invitrogen, BRL) supplemented with 10% fetal calf serum for 4 days and then serum-deprived for 24 h prior to experiments.

**Adenoviral Infections**—Cardiomyocytes were infected with adenoviral constructs that drive expression of HA-tagged PKD1-S744E/S748E (a PKD construct with phosphomimetic substitutions in the activation loop), a PKD1 silencing vector (Ad-PKD1-RNAi, generously provided by the Avkiran laboratory), a PKD2 silencing vector (Ad-PKD2-RNAi, generated using the pSilencer adeno 1.0-CMV kit (Ambion), with 5’-AGATGGGCGAGGCGATATAT-3’ inserted to produce PKD2 siRNA transcripts), a scramble vector (generated in a similar manner with 5’-GAAGACATGCATAGTCATA-3’ inserted as a scramble control), or β-galactosidase (β-gal). In each case, infections were at an MOI of 20 plaque forming units/cell according to methods described previously (12).

**Immunoprecipitation and Immunoblotting Studies**—Immunoblotting was performed on cell extracts according to methods described previously or manufacturer’s instructions (13). In each figure, each panel represents the results from a single gel (exposed for a uniform duration); detection was enhanced chemiluminescence. All results were replicated in at least four experiments on separate culture preparations.

**Luciferase Assays**—pCRE-luciferase (Strategene) and Renilla luciferase (Promega) vectors were introduced into cardiomyocytes using a nucleasevectors (Amaza Inc.) according to manufacturer’s instruction. Cre-luciferase signals were normalized to luminescence from Renilla using a dual-luciferase reporter assay system (Promega).

**RESULTS**

PKD1 and PKD2 Are Activated in a Stimulus-specific Manner in Cardiomyocytes—We recently reported that the phosphorylated/activated form of PKD accumulates as a single molecular species, with an electrophoretic mobility corresponding to PKD1 (115 kDa), in cardiomyocytes treated with norepinephrine (NE), whereas the phosphorylated/activated form of PKD is resolved as doublet (with mobilities corresponding to PKD1 (115 kDa), in cardiomyocytes treated with thrombin or PMA (12). Fig. 1 provides evidence that the phosphorylation of PKD in response to stimulation with thrombin or PMA comes from PKD2, which is activated in response to stimulation with thrombin or PMA. In contrast, the phosphorylation of PKD in response to stimulation with thrombin or PMA comes from PKD2, which is activated in response to stimulation with thrombin or PMA.
Agonist-specific PKD Isoform Activation in Cardiomyocytes

expands upon the analysis to examine the molecular forms of PKD that accumulate in cardiomyocytes treated with endothelin-1 (ET-1) and platelet derived growth factor (PDGF). PKD activation was tracked by immunoblot analysis with three different phosphorylation-site specific antibodies (PSSAs). Activation loop phosphorylation was monitored with two PSSAs that recognize phosphorylation reactions in the conserved activation loop motifs of all three PKD isoforms; recent studies indicate that the anti-PKD-Ser(P)744/ Ser(P)748 PSSA (raised against a peptide phosphorylated on serines equivalent to Ser744 and Ser748 in rodent PKD1) primarily recognizes PKD1 phosphorylation at Ser744, whereas the anti-PKD1-Ser(P)748 PSSA preferentially recognizes PKD1 phosphorylation at Ser748 (14). PKD phosphorylation also was tracked with the anti-PKD-Ser(P)916 PSSA that detects a conserved autophosphorylation site at the C terminus of PKD1 and PKD2, but not PKD3. Immunoblotting for total PKD1 and PKD2 protein was included as a loading control.

Fig. 1A shows that PKM, NE, ET-1, thrombin, and PDGF all increase PKD phosphorylation at the activation loop and the C terminus. However, agonist-dependent differences in the mobility of the major phosphorylated/activated form of PKD were detected; these differences are most prominent in immunoblotting studies with the anti-PKD-Ser(P)916 PSSA. PKD accumulates as a single molecular species, with a relatively slow electrophoretic mobility, in cardiomyocytes treated with NE. This slowly migrating form of Ser916-phosphorylated PKD also accumulates in cardiomyocytes treated with PKM or ET-1; it is considerably less prominent in cardiomyocytes treated with thrombin or PDGF, where a more rapidly migrating form of Ser916-phosphorylated/activated PKD predominates. This more rapidly migrating form of phosphorylated/activated PKD also is detected in cardiomyocytes treated with PKM and ET-1. It is at the limits of detection (i.e. it is detected only in some experiments with relatively long gel exposures) following treatment with NE.

Fig. 1B (left) shows that cardiomyocytes co-express PKD1 and PKD2 and that these PKD isoforms are readily resolved by our electrophoresis conditions. The more slowly migrating form of Ser916-phosphorylated PKD that accumulates in PKM-treated cardiomyocytes co-migrates with PKD1, whereas the faster migrating form of Ser916-phosphorylated PKD co-migrates with PKD2. Fig. 1, B (right) and C show that abundance of the more slowly migrating form of Ser916-phosphorylated PKD is decreased by treatment with a Ad-PKD1-RNAi vector that decreases PKD1 expression; the Ad-PKD1-RNAi vector (which does not decrease PKD2 protein expression) does not alter the abundance of the more rapidly migrating Ser916-phosphorylated form of PKD. Rather, the level of the more rapidly migrating form of Ser916-phosphorylated PKD is decreased by an Ad-PKD2-RNAi vector that decreases PKD2 expression (Fig. 1C). These results indicate that PKM induces the coordinate activation of PKD1 and PKD2, that activated forms of PKD1 and PKD2 migrate with different mobilities in SDS-PAGE, and that immunoblotting studies with an anti-PKD-Ser(P)916 PSSA can distinguish PKD1 and PKD2 activation (even in experiments performed on cell lysates).

In keeping with the observation that treatment with PKM, ET-1, PDGF, or thrombin leads to the appearance of a more rapidly migrating form of Ser916-phosphorylated PKD (that co-migrates with PKD2), Fig. 1A shows that PKM, ET-1, PDGF, and thrombin decrease the electrophoretic mobility of the PKD2 protein; this band shift (which is indicative of protein phosphorylation) is not detected following treatment with NE.

Agonist-stimulated pathways that activate PKD1 or PKD2 also were examined using an immunoprecipitation strategy. Fig. 2A shows that PKD1 is recovered in similar amounts (without detectable co-immunoprecipitation of PKD2) from resting and agonist-activated cardiomyocytes. NE, PKM, and ET-1 increase PKD1-Ser916 phosphorylation and slow the electrophoretic mobility of the PKD1 protein. Thrombin and PDGF do not increase PKD1-Ser916 phosphorylation (or induce a PKD1 protein electrophoretic mobility shift). Of note, the Ser916-phosphorylated form of PKD is resolved as a doublet in cell extracts from PKM-treated cardiomyocytes, but a single molecular form of Ser916-phosphorylated PKD1 is recovered in PKD2 pulldowns (that do not contain PKD2). These results indicate that the native PKD1 enzyme in cardiomyocytes is activated by NE, PKM, or ET-1 (but not thrombin or PDGF).

A similar approach was used to identify the agonist-dependent pathways that activate PKD2. Fig. 2B (left) and Fig. 2C show that PKM, thrombin, PDGF and ET-1 slow the electrophoretic mobility of PKD2 and increase PKD2-Ser916 phosphorylation. In each case, a single molecular form of Ser916-phosphorylated PKD2 was detected in PKD2 pulldowns (that do not contain PKD1). While immunoprecipitation experiments expose a minor effect of NE to increase PKD2-Ser916 phosphorylation, NE treatment does not decrease the electrophoretic mobility of PKD2. Immunoblotting studies also were performed on pre- and post-IP cell lysates. Fig. 2B, right shows that the immunoprecipitation protocol effectively clears the PKD2 protein and the more rapidly migrating form of Ser916-phosphorylated PKD from lysates prepared from thrombin-, PDGF-, or PKM-treated cardiomyocytes; the abundance of the more slowly migrating form of Ser916-phosphorylated PKD that accumulates in cardiomyocytes treated with NE or PKM (and to a considerably lesser extent thrombin and PDGF) is not influenced by immunoprecipitation of PKD2. Collectively, these results indicate that PKD1 and PKD2 are activated in an agonist-specific manner in cardiomyocytes. NE preferentially activates PKD1, thrombin and PDGF preferentially activate PKD2, and PKM and ET-1 exert dual effects to activate both PKD1 and PKD2.

The PKC Dependence and Downstream Targets of Agonist-activated PKD1 and PKD2 Pathways in Cardiomyocytes—The kinetics, PKC-dependence, and downstream substrates of PKD signaling pathways activated by NE, ET-1, thrombin, and PDGF were examined more closely. We focused on these agonists, since: 1) they activate PKD1, PKD2, or both PKD isoforms, 2) they signal through either G protein-coupled receptors (GPCRs, in the case of NE, ET-1, and thrombin) or receptor tyrosine kinases (in the case of PDGF), and 3) they are predicted to activate PKD via either PKC-dependent or PKC-independent mechanisms; a previous study concluded that PKD activation is via a PKC-dependent mechanism in
neonatal rat cardiomyocytes treated with \( \alpha_1 \)-AR agonists and via a PKC-independent mechanisms in cardiomyocytes treated with ET-1 (2).

Fig. 3A shows that NE induces a rapid increase in PKD phosphorylation at the activation loop (detected by both anti-PKD-Ser(P)^747/Ser(P)^748 and anti-PKD-Ser(P)^748 PSSAs) and
Agonist-specific PKD Isoform Activation in Cardiomyocytes

at Ser\textsuperscript{916}; this response is detected at 5 min and sustained for at least another 40 min of continuous agonist treatment. The effect of NE to increase PKD phosphorylation is associated with a modest decrease in the electrophoretic mobility of PKD1 (and no change in the electrophoretic mobility of PKD2, Figs. 1 and 3, A and B). In contrast, thrombin and PDGF induce more transient increases in PKD phosphorylation at the activation loop and Ser\textsuperscript{916} in association with a marked decrease in the electrophoretic mobility of PKD2 (and considerably smaller changes in the electrophoretic mobility of PKD1, Figs. 1 and 3C). The effects of thrombin and PDGF to increase PKD phosphorylation and decrease the electrophoretic mobility of PKD2 wane when stimulation intervals are prolonged to 30–60 min (Fig. 3C).

The kinetics of ET-1-dependent PKD activation follows an intermediate pattern. ET-1 treatment leads to the rapid accumulation of two molecular forms of Ser\textsuperscript{916}-phosphorylated PKD (Fig. 3, B and C). The more rapidly migrating form of Ser\textsuperscript{916}-phosphorylated PKD accumulates transiently in ET-1-treated cardiomyocytes, in association with a transient decrease in the electrophoretic mobility of PKD2. The slower migrating form of Ser\textsuperscript{916}-phosphorylated PKD (which co-migrates with PKD1) accumulates in a more sustained manner; the more slowly migrating form of Ser\textsuperscript{916}-phosphorylated PKD is the major molecular species detected in cells treated with ET-1 for 60 min (Fig. 3, B and C).

Stimulations were performed in the presence of GF109203X (a PKC inhibitor that does not inhibit PKD activity (15, 16)) to examine the PKC requirement for PKD activation. PMA (a direct activator of PKC and PKD isoforms) was included as a control in these experiments. Fig. 3 shows that effects of NE, PMA, thrombin, and PDGF to increase PKD phosphorylation (at all three sites) are markedly inhibited by GF109203X. GF109203X exerts a more nuanced effect on the ET-1-dependent PKD activation pathway (Fig. 3, B and C). GF109203X inhibits the more rapid/transient effects of ET-1 to increase PKD phosphorylation and decrease the electrophoretic mobility of PKD2. The more sustained effects of ET-1 are largely preserved in GF109203X-treated cardiomyocytes. These more sustained effects of ET-1 are blocked when cells are simultaneously treated with GF109203X + Gö6976 (a PKD inhibitor that does not inhibit novel PKCs). These results suggest that PKD activation loop Ser\textsuperscript{744}/Ser\textsuperscript{748} phosphorylation is sustained during the chronic phase of ET-1 receptor activation by a PKC-independent mechanism, either an autocatalytic reaction or a trans-phosphorylation by some other cellular G06976-sensitive kinase. Finally, Fig. 3, B and C shows that Gö6976 treatment does not prevent the ET-1-dependent increase in PKD-Ser\textsuperscript{916} phosphorylation. This result does not necessarily exclude an autocatalytic phosphorylation at the C terminus, since we recently demonstrated that PKD1-Ser\textsuperscript{916} autophosphorylation is an exquisitely efficient reaction that proceeds at very low ATP concentrations that do not support autophosphorylation at the activation loop or trans phosphorylation of target substrates (13). The PKD1-Ser\textsuperscript{916} autophosphorylation reaction is relatively refractory to inhibition by Gö6976 (a compound that inhibits PKD activity by competing with ATP for binding to the enzyme (13)).
PKC and/or PKD activities. Fig. 6 shows that GF109203X and Go6976 act similarly to prevent the NE-dependent increase in CREB-Ser133 phosphorylation, but these inhibitors do not prevent NE-dependent cTnI-Ser23/Ser24 phosphorylation. Rather, the effect of NE to increase cTnI-Ser23/Ser24 phosphorylation is blocked by H89, an inhibitor of PKA activity; isoproterenol-dependent cTnI-Ser23/Ser24 phosphorylation also is inhibited by H89, and not by GF109203X or Go6976 (data not shown). Fig. 6 also shows that GF109203X and Go6976 prevent the thrombin- and PDGF-dependent increases in CREB-Ser133; these agonists do not increase cTnI-Ser23/Ser24 phosphorylation. In contrast, ET-1 promotes CREB-Ser133 phosphorylation via a Go6976-sensitive pathway that is attenuated (but not fully inhibited) by GF109203X; this pharmacologic profile is most consistent with a PKD-dependent pathway with varying requirements for PKC activity. Finally, Fig. 6 shows that PMA increases CREB-Ser133 phosphorylation via a GF109203X-sensitive pathway that also is attenuated (but not fully inhibited) by Go6976; these results are consistent with the known effects of PMA to activate multiple enzymes with CREB-Ser133 kinase activities, including PKD isoforms (that are inhibited in vivo by both GF109203X or Go6976) and novel PKC isoforms (that are inhibited by GF109203X, but not Go6976). Collectively, these results identify agonist-dependent pathways involving PKD1 (following treatment with NE or ET-1) or PKD2 (following treatment with PDGF, ET-1, or thrombin) that increase CREB-Ser133 phosphorylation, with little-to-no associated increase in the phosphorylation of cTnI. Rather, NE increases cTnI-Ser23/Ser24 phosphorylation via a β-AR pathway involving PKA (and not PKD).

We previously demonstrated that PKD1 is an effective in vitro cTnI-Ser23/Ser24 kinase; the in vitro cTnI-Ser23/Ser24 kinase activities of PKD1, PKA, and PKCa, PKCB, and PKCd are quite similar (17). Additional studies showed that cTnI also is phosphorylated by PKD2; the level of cTnI-Ser23/Ser24 phosphorylation is similar in kinase assays performed with human recombinant PKD1 or PKD2 enzymes (under assay conditions calibrated to achieve comparable levels of PKD1 and PKD2 autophosphorylation, data not shown). Hence, the failure to detect PKD-dependent cTnI-Ser23/Ser24 phosphorylation in vivo in agonist-activated cardiomyocytes cannot be attributed to an inherent defect in the cTnI kinase activity of PKD1 or PKD2. Therefore, we performed cell-based studies to determine whether the PKD1-dependent cTnI-Ser23/Ser24 phosphorylation pathway is inherently defective in vivo in the intracellular microenvironment of a cardiomyocytes. Fig. 7 shows that heterologous overexpression of a PKD1-S744E/S748E transgene (an activated form of PKD1, with phospho-mimetic substitutions in the activation loop) leads to increased basal phosphorylation of CREB and cTnI; levels of CREB-Ser133 and cTnI-Ser23/Ser24 phosphorylation increase...
further when Ad-PKD1-S744E/S748E cultures are treated with PMA (consistent with recent evidence that maximal PKD1-S744E/S748E activity in cardiomyocytes and certain other specialized cell types requires the presence of lipid cofactors (12)). The observation that PKD1-S744E/S748E overexpression leads to an increase in cTnI phosphorylation indicates that the PKD1-dependent cTnI-Ser23/Ser24 phosphorylation pathway is not inherently defective in vivo in cardiomyocytes. These results suggest that some cellular process must prevent cTnI phosphorylation by native agonist-activated PKD enzymes.

The Consequences of RNAi-mediated Silencing of PKD1 or PKD2 Expression in Cardiomyocytes—Because α₁-ARs activate PKD1 and increase CREB-Ser133 phosphorylation in cardiomyocytes, we used a gene silencing approach to determine whether PKD1 mediates the α₁-AR-dependent increase in CREB-Ser133 phosphorylation. Fig. 8 shows that an Ad-PKD1-RNAi treatment markedly decreases PKD1 protein expression; the level of the more slowly migrating form of Ser916-phosphorylated PKD (that accumulates following agonist activation) also is decreased by the Ad-PKD1-RNAi treatment. Ad-PKD1-RNAi treatment does not influence the abundance of PKD2. In fact, Fig. 8 provides unanticipated evidence that the Ad-PKD1-RNAi treatment leads to a de-
crease in the electrophoretic mobility of PKD2, and an increase in basal PKD2-Ser<sup>916</sup> phosphorylation. These results suggest that PKD1 down-regulation leads to a compensatory increase in PKD2 activity. This conclusion was validated in immunocomplex kinase assays that directly measured PKD2 activity (with CREB as substrate, according to methods detailed in a previous publication (13)). The in vitro kinase assays showed that PKD2 activity is 2.4 ± 0.4-fold higher in resting Ad-PKD1-RNAi cultures than in cultures treated with vector control (n = 3, p < 0.05).

Fig. 8 (panels A–C) shows that Ad-PKD1-RNAi treatment increases basal CREB-Ser<sup>133</sup> phosphorylation and leads to the transactivation of a cAMP-responsive element (Cre-) driven reporter. We used an Ad-PKD2-RNAi vector that effectively silences PKD2 expression to determine whether the increased basal CREB-Ser<sup>133</sup> phosphorylation in Ad-PKD1-RNAi cultures can be attributed to the compensatory increase in PKD2 activity. Fig. 8, C and D show that the Ad-PKD2-RNAi vector markedly decreases PKD2 expression, without influencing the abundance (or Ser<sup>916</sup> phosphorylation) of PKD1. Fig. 8C also shows that basal CREB-Ser<sup>133</sup> phosphorylation remains elevated in cardiomyocytes treated with the Ad-PKD2-RNAi vector (alone or in combination with the Ad-PKD1-RNAi vector), indicating that the increase in basal CREB-Ser<sup>133</sup> phosphorylation in Ad-PKD1-RNAi cultures cannot be attributed to increased PKD2 activity. The observation that basal CREB-Ser<sup>133</sup> phosphorylation is constitutively increased in cardiomyocytes treated with Ad-PKD1-RNAi and/or Ad-PKD2-RNAi vectors exposes an important limitation of the RNAi approach as an approach to examine the role of individual PKD isoforms in agonist-dependent increases in CREB-Ser<sup>133</sup> phosphorylation in cardiomyocytes.

**DISCUSSION**

PKD isoforms recently have emerged as key signaling enzymes that mediate a range of physiologically important cellular responses. There is considerable evidence that many cell types co-express multiple PKD isoforms that in theory could impart signaling specificity. However, a cellular model in which individual agonist-activated receptors stimulate different PKD isoforms has never been identified. This is likely due to the ease with which PKD activation can be detected by immunoblot analysis with PSSAs that specifically recognize PKD phosphorylation at the activation loop or the extreme C terminus, sites conserved in PKD1, PKD2, and (in the case of the activation loop) PKD3. Because commercially available antiphospho-PKD antibodies are highly sensitive/specific, most studies have tracked PKD phosphorylation in cell lysates, without resorting to an immunoprecipitation step to resolve PKD isoforms from other cellular proteins, or individual PKD isoforms from each other. While distinct molecular forms of Ser<sup>916</sup>-phosphorylated PKD have been identified in some cell types, the significance of this observation has never been examined. This study uses immunoprecipitation and PKD1 knockdown strategies to show that the distinct molecular forms of Ser<sup>916</sup>-phosphorylation PKD identified in agonist-treated cardiomyocytes correspond to PKD1 and PKD2. This experimental approach was then used to show that α<sub>1</sub>-ARs selectively activate PKD1, PAR-1 and PDGFRs selectively activate PKD2, and ET-1 and PMA couple to the dual activation of both PKD1 and PKD2 in neonatal cardiomyocytes (as schematized in Fig. 9). This high level of signaling specificity could underlie some of the confusing results obtained in previous studies that have used siRNA-mediated PKD1 knockdown methods in cardiomyocytes (2). Specifically, Harrison et al. reported that a PKD1 down-regulation abrogates α<sub>1</sub>-AR signaling responses, but leads to only a partial (~50%) inhibition of the ET-1-dependent pathway involving PKD that increases HDAC5 phosphorylation. Our studies showing that ET-1 receptors couple to the dual activation of both PKD1 and PKD2 reconcile this inconsistency; an ET-1 receptor pathway involving PKD2 that contributes to cardiac hypertrophy by regulating the phosphorylation and nucleocytoplasmic shuttling of HDAC5 would not be inhibited by PKD1 down-regulation.

Early studies attributed PKD1 activation loop phosphorylation exclusively to a transphosphorylation mechanism involving novel PKC isoforms. However, there is recent evidence that this transphosphorylation mechanism is most prominent during the early phase of GPCR stimulation, and that an autophosphorylation reaction assumes greater importance at later time points (13, 14, 18). The relative contributions of PKC-de-
involving PKA. The failure to detect a role for PKD1 or PKD2 PKC isoform; studies to resolve the relative importance of PKD2 in neonatal rat cardiomyocytes. In theory, PKD2 activation could be mediated by a different PKC isoform; studies to resolve the relative importance of PKCα and PKCe in thrombin- and PDGF-dependent pathways that activate PKD2 (Fig. 9). We previously implicated PKCα in the α1-AR pathway that activates PKD1 in neonatal rat cardiomyocytes. In theory, PKD2 activation could be mediated by a different PKC isoform; studies to resolve the relative importance of PKCα and PKCe in thrombin- and PDGF-dependent PKD2 activation are ongoing. Studies reported herein also expose a more nuanced role for PKC in ET-1 receptor-dependent PKD activation. We show that ET-1 receptors induce a transient increase in PKD2 activity via a PKC-dependent pathway and a more sustained increase in PKD1 activity that does not require PKC activity (Fig. 9). Of note, a previous study concluded that ET-1-dependent PKD activation is via a PKC-independent pathway in neonatal rat cardiomyocytes (2). This conclusion is completely consistent with our findings, since this previous study examined agonist responses exclusively at a 60 min time point. Finally, our studies identify several agonist-activated receptors that transiently activate a PKC-PKD2 signaling pathway; an agonist-dependent pathway that activates PKD2 in a sustained, PKC-independent manner (akin to the delayed mechanism for PKD1 activation in cardiomyocytes treated for 60 min with ET-1) was not identified. These results could suggest that PKD1 and PKD2 have evolved to subserve distinct regulatory functions the cell, and that PKD1 is uniquely suited to maintain PKD activity during chronic agonist stimulation when PKC isoforms are down-regulated. Studies to resolve the specific cellular functions of PKD1 and PKD2 in cardiomyocytes are ongoing.

Our studies identify agonist-dependent pathways involving both PKD1 and PKD2 that selectively couple to CREB-Ser133 phosphorylation, with little-to-no associated increase in cTnI-Ser23/Ser24 phosphorylation in neonatal rat cardiomyocytes cultures (Fig. 9). The effect of NE to massively increase cTnI-Ser23/Ser24 phosphorylation is attributable to a β-AR pathway involving PKA. The failure to detect a role for PKD1 or PKD2 as receptor-activated cTnI-Ser23/Ser24 kinases was surprising, because PKD1 and PKD2 exhibit similar CREB-Ser133 and cTnI-Ser23/Ser24 kinase activities and PKD1-S744E/S748E overexpression leads to an increase in cTnI-Ser23/Ser24 phosphorylation in neonatal cardiomyocytes. These results emphasize that receptor-dependent pathways that activate native PKD enzymes in differentiated cell models exhibit a high level of signaling specificity (that cannot be predicted from in vitro kinase assays and is not necessarily retained in overexpression studies). It is likely that PKD signaling specificity is achieved through interactions with scaffolding proteins that compartmentalize the enzyme in signaling microdomains (19–21). While our studies show that PKD activation does not lead to cTnI-Ser23/Ser24 phosphorylation in ET-1-treated neonatal cardiomyocytes, a previous study from the Avkiran laboratory implicated PKD in an ET-1-dependent pathway that increases cTnI phosphorylation and modulates contractile function in adult cardiomyocytes (7). These divergent results could suggest that developmental changes in specific components of the signaling machinery lead to functionally important differences in the signaling repertoires of PKD1 and/or PKD2 in the neonatal and adult heart (and that the cTnI-Ser23/Ser24 kinase activity of PKD might be altered in clinically relevant models of cardiac pathology).

Finally, our studies provided unanticipated evidence that an Ad-PKD1-RNAi vector that silences PKD1 expression leads to a compensatory increase in PKD2. While this result initially was surprising, PKD1 knockdown has been linked to a compensatory up-regulation of PKD2 in another cell type (22). This form of cross-regulation between PKD1 and PKD2 represents an important limitation of RNAi experiments which could force a reassessment of certain conclusions of previous publications (23). The mechanism underlying this form of PKD isoform cross-regulation remains uncertain. In theory, PKD1 might regulate PKD2 by forming dimeric complexes that facilitate regulatory transphosphorylations, similar to the intermolecular interactions described for PKD2 and PKD3 (24). Alternatively, cross-regulation could arise through indirect mechanisms, such as decreased phosphorylation of a PKD substrate such as heat shock protein 25 (HSP25 in rodents, or HSP27 in humans) that binds/regulates PKCα activity and could potentially influence PKCα-dependent activation of PKD (25, 26).

Recent studies show that cardiac-specific overexpression of a constitutively active PKD1 transgene leads to a transient hypertrophic response that transitions to a lethal form of cardiac failure (2). These results implicate PKD1 activation in the pathogenesis of cardiac hypertrophy and the evolution of heart failure syndromes. A similar analysis of the functional consequences of cardiac-specific PKD2 overexpression has not been published. The observation that cardiac PKD1 and PKD2 enzymes are recruited by distinct receptor-dependent signaling pathways provides the rationale to consider whether antagonists that selectively inhibit the catalytic activity of either PKD1 or PKD2 have distinct effects on the evolution of cardiac hypertrophy or heart failure syndromes.

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Agonist-specific PKD Isoform Activation in Cardiomyocytes