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Angiotensin II-Activated Protein Kinase D Mediates Acute Aldosterone Secretion

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Summary

Dysregulation of the renin-angiotensin II (AngII)-aldosterone system can contribute to cardiovascular disease, such that an understanding of this system is critical. Diacylglycerol-sensitive serine/threonine protein kinase D (PKD) is activated by AngII in several systems, including the human adrenocortical carcinoma cell line NCI H295R, where this enzyme enhances chronic (24 hours) AngII-evoked aldosterone secretion. However, the role of PKD in acute AngII-elicited aldosterone secretion has not been previously examined. In primary cultures of bovine adrenal glomerulosa cells, which secrete detectable quantities of aldosterone in response to secretagogues within minutes, PKD was activated in response to AngII, but not an elevated potassium concentration or adrenocorticotrophic hormone. This activation was time- and dose-dependent and occurred through the AT1, but not the AT2, receptor. Adenovirus-mediated overexpression of constitutively-active PKD resulted in enhanced AngII-induced aldosterone secretion; whereas overexpression of a dominant-negative PKD construct decreased AngII-stimulated aldosterone secretion. Thus, we demonstrate for the first time that PKD mediates acute AngII-induced aldosterone secretion.

Keywords

Adrenal Cortex; Bovine Adrenal Gland; Glomerulosa Cell; Hypertension; Protein kinase C PKC; Signal Transduction

Introduction

The importance of aldosterone in the pathogenesis of cardiovascular diseases, including hypertension, has been well documented. Indeed, it is estimated that 8% of moderate hypertensive and 13% of severe hypertensive patients exhibit primary hyperaldosteronism

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(Calhoun, 2006). This hormone is secreted from cells of the zona glomerulosa of the adrenal cortex and modulates blood volume by regulating sodium excretion from the kidney. Malfunction of this system results in pathophysiology, including hypertension, severe hypokalaemic alkalosis, pre-and post-natal growth failure (Fuller and Lim-Tio, 1996; New and Wilson, 1999), cardiac fibrosis (Delcayre and Swynghedauw, 2002) and congestive heart failure (DiBianco, 1994; Weber et al., 1994; Brilla et al., 1995). Finally, results from the randomized aldactone evaluation study (RALES) and the eplerenone post-acute myocardial infarction heart failure efficacy and survival study (EPHESUS) indicate that aldosterone receptor antagonists, added to standard treatments, are able to reduce mortality in congestive heart failure patients by 30% (Struthers, 2004), suggesting the importance of aldosterone in human disease.

AngII is the most important physiological stimulus for the secretion of aldosterone, mediating both acute and chronic steroidogenesis (Foster, 2004). AngII's mechanism of action in acute steroidogenesis is initiated via its binding to the AngII receptor type 1 (AT1) G-protein coupled receptors. The result of this receptor binding is phosphatidylinositol 4,5-bisphosphate hydrolysis resulting in diacylglycerol (DAG) and inositol 1,4,5-trisphosphate production, with resultant activation of protein kinase C (PKC) and calcium (Ca^{2+}) release from intracellular stores, respectively. Elevated potassium levels (K^{+}) and adrenocorticotrophic hormone (ACTH) also induce cytosolic Ca^{2+} increases, which can activate Ca^{2+} /calmodulin-dependent protein kinases [reviewed in (Spat and Hunyady, 2004)]. Acutely, these signals act to stimulate the rate-limiting step in aldosterone synthesis: translocation of cholesterol, transported from storage in lipid droplets, from the outer to inner mitochondrial membrane via the activity of steroidogenic acute regulatory (StAR) protein. Chronically, AngII acts to alter the expression of various genes (Romero et al., 2007), including CYP11A1, CYP11B2 (Bassett et al., 2000) and StAR protein (Krug et al., 2007). Because AngII stimulates the formation of DAG in adrenal glomerulosa cells [e.g., (Bollag et al., 1991) and (Hunyady et al., 1990)], the role of DAG-sensitive enzyme signaling cascades in AngII-elicited aldosterone secretion has been intensely studied. DAG-sensitive enzymes, such as PKC and the serine/threonine kinase protein kinase D [PKD; reviewed in (Wang, 2006)], are thought to be important in aldosterone secretion; however, their actual role is as yet controversial. Thus, several laboratories report that these enzymes are activated in order to generate a positive secretory signal whereas others propose a negative feedback regulatory role [reviewed in (Bollag and Xie, 2009)].

In this study we wished to determine whether PKD plays a role in regulating acute aldosterone secretion in primary cultures of bovine adrenal glomerulosa cells, which demonstrate secretagogue-elicited steroidogenesis within minutes. Here we demonstrate that AngII activates PKD in primary bovine adrenal glomerulosa cells in a time- and dose-dependent manner through the angiotensin II type 1 (AT1) receptor. Interestingly, adenovirus-mediated overexpression of dominant-negative PKD caused a reduction in acute AngII-induced aldosterone secretion, and overexpression of a constitutively-active PKD mutant enhanced aldosterone secretion in response to a one-hour AngII stimulation. These data suggest that PKD acts as a positive regulator of acute AngII-elicited aldosterone secretion in primary cultures of primary bovine adrenal glomerulosa cells.

Materials and Methods

Materials

The following were obtained from Sigma (St. Louis, MO): phorbol 12-myristate 13-acetate (PMA), goat anti-rabbit and rabbit anti-goat secondary antibodies conjugated to alkaline phosphatase, AngII and PD123,319. Immobilon-P PVDF membrane and protein-A/G PLUS agarose (SC-2003) were purchased from Millipore (Billerica, MA) and Santa Cruz (Santa Cruz, CA), respectively. The anti-phosphoserine⁹¹⁶-PKD antibody (CS-2051) and the anti-PKD

antibody (CS-2052) were acquired from Cell Signaling Technology, Inc. (Beverly, MA). The ECF substrate and P-81 paper were purchased from Amersham Biosciences (Piscataway, NJ) and Whatman, Inc. (Clifton, NJ), respectively. Candesartan was a kind gift of Dr. Mario Marrero (Vascular Biology Center, Medical College of Georgia, Augusta, GA). All restriction enzymes and associated buffers, as well as T4 ligase, were obtained from New England Biolabs (Ipswich, MA). Miniprep, maxiprep, and DNA purification kits were acquired from Qiagen (Valencia, CA). Lipofectamine was purchased from Invitrogen (Carlsbad, CA). The adenovirus pAdTrack-CMV shuttle vectors were acquired from Bert Vogelstein (Johns Hopkins University, Baltimore, MD). UltroSer G was obtained from BioSeptra (France) under a permit from the US Department of Agriculture. BJ5183 cells, AdEasyVirus purification kits, and XL10Gold Cells were obtained from Stratagene (La Jolla, CA).

Cell Culture

Bovine adrenal glomerulosa cells were isolated and cultured as previously described (Betancourt-Calle et al., 1999). Briefly, near-term fetal adrenal glands were obtained from a local meat-packing plant, and the zona glomerulosa, identified by its yellowish color, was dissected, collagen-digested and mechanically dispersed. The isolated glomerulosa cells were cultured overnight in Falcon Primaria dishes (Becton Dickinson Labware, Lincoln Park, NJ) as previously described (Betancourt-Calle et al., 1999). On the second day of culture, medium was replaced with serum-free medium (Betancourt-Calle et al., 1999), for an additional 20 to 24 hours. For experiments, cells were incubated with bicarbonate-buffered Krebs Ringer solution containing 2.5 mM sodium acetate (KRB^+) and equilibrated for at least 30 minutes in a 5% CO_2 incubator ($_{\text{eq}}\text{KRB}^+$) prior to stimulation with $_{\text{eq}}\text{KRB}^+$ containing the appropriate agents.

H295R cells were cultured as in Bird et al. (Bird et al., 1993). Briefly, cells were grown in DMEM/Ham's F12 (1:1 vol:vol) containing 1% ITS⁺ (BD Biosciences, Palo Alto, CA), 2% UltroSer G, 100U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin and 0.25 $\mu\text{g}/\text{mL}$ fungizone, to approximately 70–75% confluence. The medium was then replaced with serum-free medium (as above but with the serum replaced by 0.01% BSA), and cells were incubated an additional 20 to 24 hours before use as above. Passage numbers 9–13 were used for all experiments.

Aldosterone Secretion

Cultured adrenal glomerulosa cells were incubated with $_{\text{eq}}\text{KRB}^+$ containing the appropriate agents, and the supernatants collected and stored frozen until aldosterone was assayed using a solid-phase radioimmunoassay kit (Diagnostic Products, Los Angeles, CA; 0.0003% cross-reactivity for cortisone, 0.002% cross-reactivity for corticosterone, no detectable cross-reactivity for cortisol). In the case of stimulation with 15 mM K^+ -containing KRB^+ , KCl was substituted iso-osmotically for NaCl, as in (Betancourt-Calle et al., 2001).

Western Blot

Cultured adrenal glomerulosa cells were stimulated with $_{\text{eq}}\text{KRB}^+$ containing the appropriate agents for the indicated times. Cells were solubilized with warm lysis buffer [Tris-HCl (0.175M, pH 8.5), SDS (3% v:v) and EGTA (1.5mM)] and scraped, and buffer containing 30% glycerol (v:v), 15% 2-mercaptoethanol (v:v) and 0.1% bromophenol blue (v:v) was added to constitute loading buffer. Equal volumes of sample were subjected to SDS-PAGE on an 8% gel and transferred to Immobilon-P membrane, which was cut and each half incubated with either anti-phosphoserine⁹¹⁶PKD antibody (Cell Signaling, pPKD^{S910}) or anti-actin antibody (Sigma). Immunoreactivity was visualized using the ECF system (Amersham, Piscataway, NJ) and imaged on a Typhoon scanner (Molecular Dynamics, Sunnyvale, CA). Note that normalization was performed using actin rather than total PKD because in previous reports it

has been shown that several antibodies have differing affinities for PKD when the enzyme is autophosphorylated versus unphosphorylated (Rennecke et al., 1996; Dodd et al., 2005).

In Vitro Kinase Assay

The PKD *in vitro* kinase activity assay was performed essentially as in Dodd et al. (Dodd et al., 2005). Briefly, PKD was immunoprecipitated using the Cell Signaling PKD antibody (1:200) and protein-A/G PLUS agarose (1:250). PKD activity was then monitored as the transfer of radiolabel from [γ - 32 P]ATP to syntide-2. Reactions were terminated and spotted onto P-81 paper, and the radioactivity was quantified using an LS 6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

Adenovirus

The AdEasy adenoviral system was generously provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD), and adenovirus vectors containing recombinant constructs were made as in Vogelstein et al. (He et al., 1998). In brief, PKD constructs, obtained from Dr. Alex Toker (Harvard Medical School, Boston, MA), were placed in the pAdTrack-CMV shuttle vector, which was then electroporated into BJ5183 cells as per the manufacturer's instructions. Successfully recombinant clones were transfected via Lipofectamine (using the manufacturer's protocol) into Ad-293 cells expressing viral proteins E1 and E3, allowing for virus packaging. Viral particles were isolated and purified using the Stratagene Adenovirus Purification kit with elution into virus storage buffer [20mM Tris/HCl, 25mM NaCl, 2.5% glycerol (w:v), pH 8.5], storage at -80°C , and determination of titre using fluorescence after a 10 minute incubation at 60°C with 0.1% SDS (2). Alternatively, adenoviruses were purified by CsCl_2 ultracentrifugation and titre determined by measuring OD260. Experiments were optimized by infecting primary bovine adrenal glomerulosa cells with various volumes of adenovirus and observing GFP expression by confocal microscopy. Volumes of virus used in experiments were based on at least 90% of the cells expressing green fluorescent protein (GFP) with less than 5% of those cells floating or blebbing. On the second day of culture, infection was achieved by incubating cells for 4 hours in complete serum-free media containing adenovirus. Mock-infected cells were incubated for 4 hours with serum-free media containing virus storage buffer but no virus. The media was then removed and replaced with serum-free media for 20 hours, after which time aldosterone secretion was monitored upon a one-hour treatment with or without AngII in eqKRB^+ . PKD overexpression was verified by western blotting with the total PKD antibody, equivalent infection levels documented using GFP levels and equal protein loading verified using actin levels.

Statistical Analysis

The significance of differences between mean values was determined by ANOVA with a Newman-Keuls post-hoc test using Prism (GraphPad Software, San Diego, CA).

Results

AngII and PMA Induced Aldosterone Secretion and PKD Activity

Because AngII has been shown to activate PKD in several cell types (Abedi et al., 1998; Chiu and Rozengurt, 2001; Tan et al., 2004; Iwata et al., 2005; Romero et al., 2006), we tested the ability of AngII and other aldosterone secretagogues to activate PKD in primary bovine adrenal glomerulosa cells. Cells were treated with or without 10 nM AngII, 100 nM phorbol 12-myristate 13-acetate (PMA), 15 mM K^+ or 10 nM ACTH for 30 minutes. Lysates were then analyzed by western blotting using an antibody to phosphorylated serine residue 916 of PKD (910 in human), a marker for PKD activation (Matthews et al., 1999), or scraped for an *in vitro* kinase assay. The supernatants were assayed for aldosterone secretion. As previously

shown (Betancourt-Calle et al., 1999), all of the above agents induced aldosterone secretion (AngII, 20 ± 3.7 -fold; PMA, 10 ± 1.9 -fold; K^+ , 7.5 ± 1.6 -fold; and ACTH, 22 ± 2.1 -fold over the control value; Figure 1A). On the other hand, AngII and PMA, but not K^+ or ACTH, induced pPKD^{S910} phosphorylation (Figure 1B and C) and *in vitro* kinase activity (Figure 2). Similar increases in pPKD^{S910} phosphorylation were obtained with AngII and PMA in H295R cells (data not shown), as has been previously reported (Romero et al., 2006). In the primary cells AngII increased pPKD^{Ser910} in a concentration-dependent manner (Figure 3A and B), comparable to its effects on aldosterone secretion (Bollag et al., 1991). In addition, AngII- and PMA-induced PKD activation was observed to be rapid and stable up to 30 minutes (Figure 3C and D). This acute activation of PKD in response to AngII was also observed in H295R cells, with increased Ser⁹¹⁰ phosphorylation at 5, 15 and 30 minutes (data not shown), also as previously reported (Romero et al., 2006). Similarly, PMA increased Ser⁹¹⁰ phosphorylation at 5, 15 and 30 minutes in H295R cells (data not shown).

The AngII AT-1 Receptor Mediated PKD Activation

Also of interest was the mechanism of AngII-stimulated PKD activation. Bovine adrenal glomerulosa cells express both the AngII type I (AT1) and type II (AT2) receptors (Lumbers, 1999). Aldosterone secretion is mediated by the AngII AT1 receptor, whereas the AT2 receptor seems to have little effect on this cellular response. In our hands also, the AT1 antagonist candesartan (10 μ M) inhibited, while the AT2 antagonist PD123,319 (10 μ M) had no effect on, AngII-induced aldosterone secretion (Figure 4A). Similarly, candesartan, but not PD123,319, inhibited AngII-induced PKD Ser⁹¹⁰ phosphorylation (Figure 4B and C).

PKD Enhanced Acute AngII-stimulated Aldosterone Secretion

Given that PKD was activated in response to the aldosterone secretagogue AngII, and this activation could be blocked by an antagonist for the pro-secretory AT1 receptor, the role of PKD in regulating acute AngII-induced aldosterone secretion was examined. To this end we infected primary bovine adrenal glomerulosa cells with adenovirus containing no insert, a constitutively-active or a dominant-negative PKD (Yuan et al., 2001; Qin et al., 2006) construct, in which serines 738 and 742 are mutated to phosphorylation-mimicking glutamates (PKD^{S738/742E}) or unphosphorylatable alanines (PKD^{S738/742A}), respectively. Immunoblotting of cell lysates confirmed overexpression of PKD constructs and demonstrated expression of GFP as a marker of infection (Figures 5A and 6A). Overexpression of dominant-negative PKD^{S738/742A} resulted in decreased aldosterone secretion in response to AngII (Figure 5B). In contrast, cells overexpressing the constitutively-active PKD^{S738/742E} exhibited increased AngII-elicited aldosterone secretion (Figure 6B), suggesting that PKD mediates, at least in part, acute AngII-induced steroidogenesis.

Discussion

AngII elicits PKD activation in various cell types (Abedi et al., 1998; Chiu and Rozengurt, 2001; Tan et al., 2004; Iwata et al., 2005; Romero et al., 2006), including NCI H295R adrenocortical carcinoma cells, in which PKD overexpression increases CYP11B2 expression and chronic (24 hour) cortisol and aldosterone secretion. As AngII is the most important physiological activator of aldosterone secretion, it became of interest to determine if AngII induced PKD activation in the primary adrenal glomerulosa cell system, a model showing an acute aldosterone secretory response. We observed that AngII elicited PKD activation (maximal after 5 minutes and stable for up to 30 minutes). Interestingly, elevated K^+ and ACTH had no effect on PKD activity (to our knowledge no other laboratory has reported this). Also, and in agreement with the results of Tan et al. (Tan et al., 2004) in vascular smooth muscle cells, we report that PKD activation was mediated through AngII binding to the AT1 receptor. Finally, we provide evidence that PKD acts as a positive regulator of acute aldosterone

secretion in response to AngII. Thus, overexpression of dominant-negative PKD^{S738/742A} decreased acute AngII-stimulated aldosterone secretion, whereas overexpression of constitutively-active (Yuan et al., 2001; Qin et al., 2006) PKD^{S738/742E} enhanced AngII-stimulated aldosterone secretion.

On the other hand, if PKD were both necessary and sufficient for aldosterone secretion, the constitutively active PKD should increase aldosterone secretion under basal conditions when overexpressed. Instead, our results suggest that PKD is necessary (since the dominant-negative inhibits AngII-elicited aldosterone secretion) but not sufficient to induce aldosterone secretion (because the constitutively active PKD does not increase secretion basally). Indeed, this finding is consistent with the proposal of Rasmussen and colleagues that aldosterone secretion requires both diacylglycerol and calcium signals [reviewed in (Rasmussen et al., 1995)]. Furthermore, our data allow us to determine that PKD is involved in acute aldosterone secretion, since there is no chronic effect of constitutively active PKD overexpression, i.e., no effect on basal secretion is observed. Therefore, our results are distinct from those of Romero et al. (Romero et al., 2006), who showed stimulated aldosterone secretion chronically (over 24 hours) in the NCI H295R adrenocortical carcinoma cell line.

Our hypothesis, that DAG- and phorbol ester-sensitive PKD is activated by AngII to mediate acute aldosterone secretion, fits well with the observations of some groups but conflicts with others. For example, investigators (Romero et al., 2006; Chang et al., 2007) have shown that PKD overexpression stimulates chronic AngII-induced aldosterone secretion in the human adrenocortical carcinoma cell line, NCI H295R cells. Such chronic AngII effects on steroidogenesis may be related to the ability of PKD to directly phosphorylate and activate cAMP response element binding protein (CREB) to induce transcription (Johannessen et al., 2007), since the expression of several steroidogenic enzymes, e.g., StAR, CYP11A1 and CYP11B2, is CREB-dependent (Bassett et al., 2000). Indeed, overexpression of constitutively active PKD mutants, PKD^{S738/742E} and one in which the autoinhibitory pleckstrin homology (PH) domain was deleted, increased CYP11B2 expression in H295R cells (Romero et al., 2006). Nevertheless, it should be noted that the results of Romero et al. (Romero et al., 2006) showing PKD's regulation of CYP11B2 expression are in conflict with reports that PMA, which activates PKD in H295R cells (Romero et al., 2006), does not alter CYP11B2 expression in these cells (Clyne et al., 1997) or reduces secretagogue-stimulated increases in CYP11B2 promoter activity (LeHoux et al., 2001).

On one hand, the current literature provides evidence that phorbol ester- and DAG-sensitive enzymes, such as PKC and PKD, promote AngII-induced secretion. For instance, the DAG mimetic phorbol 12-myristate 13-acetate (PMA) and synthetic DAGs induce acute aldosterone secretion themselves and act synergistically with agents that increase calcium to trigger steroidogenesis [reviewed in (Bollag and Xie, 2009)]. In addition, PKC ϵ and PKD are activated by AngII and are involved in upregulating AngII-induced aldosterone secretion in the H295R human adrenocortical carcinoma cell line (Romero et al., 2006; Chang et al., 2007). In contrast, other reports indicate that the DAG signal and its effectors oppose aldosterone secretion acutely and chronically. For example, pretreatment of rat glomerulosa with PMA (to downregulate PKC), or with the non-selective PKC/PKD inhibitor staurosporine (Toullec et al., 1991), enhances subsequent AngII-induced aldosterone secretion (Hajnoczky et al., 1992). Similarly, staurosporine (Coulombe et al., 1996; Coulombe et al., 1997), the selective PKC inhibitor bisindolymaleimide I (LeHoux and Lefebvre, 1998) and the selective PKC/PKD inhibitor Gödecke 6976 (LeHoux et al., 2000; LeHoux et al., 2001) enhance hamster CYP11B2 promoter activity. Moreover, ectopic expression of constitutively active PKC α and PKC ϵ constructs attenuate, while the dominant-negative forms of the enzymes increase, AngII-stimulated CYP11B2 promoter activity (LeHoux et al., 2001). Finally, PKC ϵ , reported to be upstream of PKD in H295R cells (Romero et al., 2006), has been shown to negatively regulate CYP11B2

expression through interactions with extracellular signal-regulated kinase-1 and -2 (ERK-1/2) and JunB (LeHoux and Lefebvre, 2006). Indeed, PKC ϵ overexpression decreases, while PKC ϵ shRNA increases, AngII-stimulated aldosterone secretion (Romero et al., 2006). Nevertheless, PKC ϵ has been shown to activate PKD (Waldron and Rozengurt, 2003; Romero et al., 2006), and active PKD increases CYP11B2 promoter activity (Romero et al., 2006). The explanation for this apparent paradox may lie in the presence of multiple phorbol ester- and DAG-activated enzymes [reviewed in (Brose and Rosenmund, 2002)], including the various PKC isoenzymes as well as PKD and their ability to crosstalk [reviewed in (Bollag and Xie, 2009)]. Clearly, additional studies are necessary to resolve the role of the DAG/PKC/PKD signaling pathway in the regulation of aldosterone secretion and glomerulosa cell biology.

PKD may play roles in adrenal glomerulosa cell biology in addition to its ability to increase acute steroidogenesis and aldosterone biosynthetic capacity. As an example, AngII elicits mitogen-activated protein kinase (MAPK) activation and triggers proliferation in bovine and rat adrenal glomerulosa cells (Tian et al., 1995; McNeill et al., 1998) and also increases cell hypertrophy in the rat cells after 3 days (Otis and Gallo-Payet, 2007). PKD mediates proliferation and/or hypertrophy in several systems [reviewed in (Bollag et al., 2004)]. Indeed, human adrenocortical tumor tissue shows increased phosphoserine⁹¹⁰ PKD immunoreactivity compared to surrounding normal tissue in patients with aldosterone-producing adenomas (Chang et al., 2007). In many systems, PKD's proliferative effects are mediated by MAPK signaling: for instance, overexpression of PKD prolongs ERK-1/2 activation in response to mitogens and results in enhanced DNA synthesis in Swiss 3T3 fibroblasts (Sinnott-Smith et al., 2004). In neonatal rat cardiomyocytes, AngII also activates PKD (Iwata et al., 2005), and overexpression of a constitutively active PKD construct increases the size of these cells (Iwata et al., 2005). Perhaps another function of PKD in adrenal glomerulosa cells is to promote cell proliferation or hypertrophy, thereby additionally contributing to enhanced aldosterone production. In addition, ERK-1/2 has been shown to phosphorylate and activate cholesterol ester hydrolase (Cherradi et al., 2003), the enzyme that releases cholesterol from storage as cholesteryl esters in lipid droplets. Thus, a stimulation of ERK-1/2 activation by PKD could result in increased availability of this aldosterone precursor for steroidogenesis.

In conclusion, this study shows that AngII activated PKD in primary bovine adrenal glomerulosa cells. PKD activation was mediated by AT1 receptor, but not AT2, receptor signaling. Finally, PKD activation functioned to enhance acute AngII-evoked aldosterone production. This work is unique, as we show for the first time that AngII increases PKD activity (in addition to serine⁹¹⁶ phosphorylation, an indirect measure of PKD activation status). Furthermore, it represents the first demonstration that PKD is involved in acute aldosterone secretion. More importantly, this research supports a larger hypothesis that PKD is a multifunctional protein that is indispensable for cell health, for secretion and/or for cell growth, as well as other processes. AngII-evoked, PKD-mediated cardiac hypertrophy can promote heart failure (Vega et al., 2004; Iwata et al., 2005; Gupta et al., 2007), while AngII-elicited, PKD-enhanced steroidogenesis (and possibly proliferation of glomerulosa cells) could increase overall serum aldosterone levels over time, inducing primary hypertension and further promoting cardiac fibrosis (Weber et al., 1994; Tsybouleva et al., 2004; Williams, 2005). Therefore, continued research in the area of PKD's involvement in AngII-induced cell signaling is necessary to further define the role of PKD in adrenal glomerulosa biology and human disease.

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Figure 1.

AngII and PMA, but not elevated K⁺ or ACTH, induced PKD activation. Cultured adrenal glomerulosa cells were treated with $_{eq}KRB^{+}$ with or without 10 nM AngII, 15 mM K⁺ (NaCl in the KRB⁺ was replaced isoosmotically with KCl), 100 nM PMA or 10 nM ACTH for 30 minutes. Panel (A) illustrates a representative experiment and (B) shows the quantitation of multiple experiments in which pPKD^{S910} levels were normalized using actin. (C) Aldosterone levels were quantified in the supernatants. Values represent the means $\pm$ SEM from at least 3 experiments; * $p < 0.05$ vs. control.

Figure 2.

AngII and PMA, but not elevated K⁺, stimulated PKD activity. Cultured adrenal glomerulosa cells were treated with e_qKRB^+ with or without 10 nM AngII, 15 mM K⁺, 100 nM PMA or 10 nM ACTH for 30 minutes. Equal amounts of cellular protein were immunoprecipitated and PKD activity was determined using an *in vitro* kinase assay as described in the Methods section. Data represent means $\pm$ SEM from 5 experiments performed in duplicate; * $p < 0.05$ vs. control.

Figure 3.

AngII and PMA induced PKD activation in a time- and a dose-dependent manner. (A) Cultured glomerulosa cells were treated with or without $eqKRB^+$ containing various concentrations of AngII, as indicated, for 30 minutes, and immunoblot analysis was performed using antibody directed towards pPKD^{S910}. Panel (A) illustrates a representative western blot and panel (B) shows the quantitation of multiple experiments in which pPKD^{S910} levels were normalized using actin. Values represent means $\pm$ SEM from 4 experiments performed in duplicate; * $p < 0.05$, ** $p < 0.01$ vs. control. (C and D) Cultured glomerulosa cells were treated with or without $eqKRB^+$ containing (C) 10 nM AngII or (D) 100 nM PMA for 5, 15 or 30 minutes, and immunoblot analysis was performed as above. Values represent means $\pm$ SEM from 4 experiments performed in duplicate; * $p < 0.05$, ** $p < 0.01$ vs. control.

Figure 4.

Candesartan, but not PD123,319, inhibited AngII-induced PKD activation. (A) Cultured glomerulosa cells were pretreated with e_qKRB^+ containing no addition, 10 M candesartan (Cand) or 10 M PD123,319 (PD) for 30 minutes. Cells were then treated with or without 10 nM AngII in the presence or absence of the agents as indicated for another 30 minutes, and aldosterone assays were performed on the supernatants. Data represent means $\pm$ SEM from 3 experiments performed in duplicate; * $p < 0.05$ vs. control, † $p < 0.05$ vs. AngII alone or AngII plus PD123,319. (B and C) Immunoblot analysis was performed on the cell lysates. Panel (B) illustrates a representative experiment and (C) shows the quantitation of multiple experiments in which pPKD^{S910} levels were normalized using actin. Data represent means $\pm$ SEM from at least 3 experiments performed in duplicate; * $p < 0.05$ vs. control, † $p < 0.05$ vs. AngII alone or AngII plus PD123,319.

Figure 5.

Adenovirus-induced overexpression of PKD^{S738/742E} enhanced AngII-induced aldosterone secretion from adrenal glomerulosa cells. Cultured glomerulosa cells were incubated for 4 hours with adenovirus expressing pAdtrackCMV (vector) or PKD^{S738/742E} constructs on the second day of culture. The supernatant was removed, and was replaced with serum-free medium for 20 hours. pAdtrackCMV- (vector-) or PKD^{S738/742E}-infected primary cells were then treated with or without 10 nM AngII for one hour in e_qKRB^+ , and aldosterone secretion was assayed. Panel (A) illustrates a representative experiment showing PKD^{S738/742E} and GFP overexpression in primary adenovirus infected cells 20–24 hours post-infection. Results are representative of a minimum of 3 separate experiments. The samples were separated on the same blot but intervening lanes were removed for clarity. Panel (B) illustrates aldosterone secretion data representing the means $\pm$ SEM from 3 experiments performed in duplicate; the results are expressed relative to the maximal aldosterone secretory response for each experiment. The maximal aldosterone secretory values for the 3 experiments were $1,123 \pm 21$; $1,298 \pm 88$ and $2,248 \pm 6$ (means $\pm$ SEM) pg aldosterone/mL/60 min; * $p < 0.05$ vs. control (vector-infected cells without AngII), † $p < 0.05$ versus the vector-infected control with AngII.

Figure 6.

Adenovirus-induced overexpression of PKD^{S738/742A} inhibits AngII-induced aldosterone secretion from adrenal glomerulosa cells. Cultured glomerulosa cells were incubated for 4 hours with adenovirus expressing pAdtrackCMV (vector) or PKD^{S738/742A} constructs on the second day of culture. The supernatant was removed, and was replaced with serum-free medium for 20 hours. pAdtrackCMV- (vector-) or PKD^{S738/742A}-infected primary cells were then treated with or without 10 nM AngII for one hour in $eqKRB^+$, and aldosterone secretion was assayed. Panel (A) illustrates a representative experiment showing PKD^{S738/742A} and GFP overexpression in primary adenovirus infected cells 20–24 hours post-infection. Results are representative of a minimum of 4 separate experiments. The samples were separated on the same blot but intervening lanes were removed for clarity. Panel (B) illustrates aldosterone secretion data representing the means $\pm$ SEM from 4 experiments performed in duplicate; the results are expressed relative to the maximal aldosterone secretory response for each experiment. The maximal aldosterone secretory values for the 4 experiments were 284 ± 20 ; 984 ± 66 ; $1,695 \pm 116$ and $3,964 \pm 442$ (means $\pm$ SEM) pg aldosterone/mL/60 min; * $p < 0.05$ vs. control (vector-infected cells without AngII), † $p < 0.05$ versus the vector-infected control with AngII.