Modulation of Polycystic Kidney Disease Severity by Phosphodiesterase 1 and 3 Subfamilies

Hong Ye,* Xiaofang Wang,* Caroline R. Sussman,* Katharina Hopp,* Maria V. Irazabal,* Jason L. Bakeberg,† Wells B. LaRiviere,* Vincent C. Manganiello,‡ Charles V. Vorhees,§ Haiqing Zhao,|| Peter C. Harris,* Jan van Deursen,¶ Christopher J. Ward,† and Vicente E. Torres* 

*Division of Nephrology and Hypertension and †Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota; ‡Division of Nephrology and Hypertension, The Kidney Institute, University of Kansas Medical Center, Kansas City, Kansas; §Cardiovascular and Pulmonary Branch, National Heart, Lung and Blood Institute, US National Institutes of Health, Bethesda, Maryland; ¶Department of Pediatrics, Division of Neurology, Cincinnati Children’s Research Foundation and University of Cincinnati, Cincinnati, Ohio; and †Department of Biology, Johns Hopkins University, Baltimore, Maryland

ABSTRACT
Aberrant intracellular calcium levels and increased cAMP signaling contribute to the development of polycystic kidney disease (PKD). cAMP can be hydrolyzed by various phosphodiesterases (PDEs). To examine the role of cAMP hydrolysis and the most relevant PDEs in the pathogenesis of PKD, we examined cyst development in Pdeo- or Pde3-knockout mice on the Pkd2−/WS25 background (WS25 is an unstable Pkd2 allele). These PDEs were selected because of their importance in cross-talk between calcium and cyclic nucleotide signaling (PDE1), control of cell proliferation and cystic fibrosis transmembrane conductance regulator (CFTR)–driven fluid secretion (PDE3), and response to vasopressin V2 receptor activation (both). In Pkd2−/WS25 mice, knockout of Pde1a, Pde1c, or Pde3a but not of Pde1b or Pde3b aggravated the development of PKD and was associated with higher levels of protein kinase A–phosphorylated (Ser133) cAMP–response binding protein (P-CREB), activating transcription factor-1, and CREB–induced CRE modulator proteins in kidney nuclear preparations. Immunostaining also revealed higher expression of P-CREB in Pkd2−/WS25;Pde1a−/−, Pkd2−/WS25;Pde1c−/−, and Pkd2−/WS25;Pde3a−/− kidneys. The cystogenic effect of desmopressin administration was markedly enhanced in Pkd2−/WS25;Pde3a−/− mice, despite PDE3 accounting for only a small fraction of renal cAMP PDE activity. These observations show that calcium- and calmodulin-dependent PDEs (PDE1A and PDE1C) and PDE3A modulate the development of PKD, possibly through the regulation of compartmentalized cAMP pools that control cell proliferation and CFTR–driven fluid secretion. Treatments capable of increasing the expression or activity of these PDEs may, therefore, retard the development of PKD.

Received January 15, 2015. Accepted August 4, 2015.

Correspondence: Dr. Vicente E. Torres, Division of Nephrology and Hypertension, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. Email: torres.vicente@mayo.edu

Copyright © 2016 by the American Society of Nephrology

Autosomal dominant polycystic kidney disease (ADPKD), a common inherited renal cystic disease and the fourth leading cause of ESRD, is caused by mutations to PKD1 encoding polycystin-1 (PC1) or PKD2 encoding PC2.1 PC1 and PC2 interact physically and function as a complex, but they may also have independent subcellular localizations and functions. The mechanisms by which mutations to PKD1 or PKD2 result in PKD remain unclear. PC2 is a nonselective cation channel with high permeability for calcium.2 Both PCs interact with several calcium channel and sensor proteins. Substantial experimental evidence supports the hypothesis that disruption of intracellular calcium homeostasis and upregulation of the cAMP signaling have a central role in the pathogenesis of PKD.3 Strategies to hormonally modulate cAMP signaling using vasopressin V2 receptor antagonists or somatostatin analogs have been successful in animal models4–6 and have led to clinical trials with encouraging results.7,8 Accumulation of cAMP in cystic tissues8 may be caused by enhanced adenyl cyclase activity or inhibition of cAMP degradation by phosphodiesterases (PDEs). Indeed, the knockout of adenyl cyclase 6 attenuates the development of
PKD in a Pkd1 knockout mouse.9 Correspondingly, the knockdown of the calcium/calmodulin-dependent pde1a using morpholinos induces or aggravates the cystic phenotype of wild-type or pkd2 morphant zebrafish embryos, respectively, whereas PDE1a RNA partially rescues the phenotype of pkd2 morphants.10 Because the hydrolytic capacity of PDEs far exceeds the maximum rate of synthesis by adenylyl cyclases,11 cellular levels of cAMP are likely more sensitive to inhibition of PDEs than to activation of adenylyl cyclases.

The superfamily of mammalian PDEs consists of 11 families (PDE1–PDE11) with 21 genes and >50 isoenzymes.12,13 PDE family members hydrolyze exclusively cAMP (PDE4, PDE7, and PDE8), exclusively cGMP (PDE5, PDE6, and PDE9), or both (PDE1, PDE2, PDE3, PDE10, and PDE11). They are regulated at the genetic level as well as by phosphorylation, cGMP or cAMP binding, calcium-calmodulin binding, and protein-protein interactions.

Of the large PDE superfamily, the PDE1 (PDE1A, PDE1B, and PDE1C) and PDE3 (PDE3A and PDE3B) families and subfamilies may be particularly important in PKD.14,15 PDE1 accounts for most of the PDE activity in renal tubules16,17 and is the only PDE activated by calcium 14,15 (which is reduced in PKD cells), and its activity is reduced in cystic kidneys.17 Furthermore, the pool of cAMP generated in response to vasopressin (the main adenylyl cyclase agonist in collecting duct and distal nephron18) is mainly hydrolyzed by PDE1, and the accumulation of cAMP in response to vasopressin is markedly increased when intracellular calcium is reduced, mainly because of lower PDE1 activity.19,20 PDE3 is inhibited by cGMP21 (which is degraded by PDE1), localizes to ER membranes,22 and hydrolyzes cAMP pools that control two major factors in the pathogenesis of PKD: tubular epithelial cell proliferation23,24 and cystic fibrosis transmembrane conductance regulator (CFTR)–driven chloride secretion.25,26

We have used established27–30 and newly generated Pde knockout mice to understand the role of specific PDE1 or PDE3 subfamilies in the development of ADPKD using the Pkd2−/WS25 mouse,31 an orthologous model that is double heterozygote for null and

Figure 1. The cystic disease in Pkd2−/WS25 mice and knockouts of Pde1a, Pde1c, and Pde3a are associated with changes in PDE activities in renal tissues. Total PDE, PDE1, PDE3, and PDE4 activities (picomoles per minute per 1 mg protein) in whole lysates from kidneys of 16-week-old Pde+/+, Pde1a−/−, Pde1b−/−, Pde1c−/−, Pde3a−/−, and Pde3b−/− mice on Pkd2+/+ (left panels) and Pkd2−/WS25 (right panels) genetic backgrounds are shown. P values comparing Pde null genotypes with Pde wild-type genotypes of the same Pkd2 background are *P<0.05 and **P<0.01; P values comparing Pde genotypes on the Pkd2−/WS25 background with the corresponding Pde genotype on the Pkd2 wild-type background are †P<0.05 and ††P<0.01.


www.jasn.org
The severity of the renal cystic disease in Pkd2<sup>−/−WS25</sup> mice is aggravated on Pde1a<sup>−/−</sup>, Pde1c<sup>−/−</sup>, or Pde3a<sup>−/−</sup> backgrounds but not on Pde1b<sup>−/−</sup> or Pde3b<sup>−/−</sup> backgrounds. Representative hematoxylin-eosin-stained kidney sections from male and female Pkd2<sup>−/−WS25</sup> mice on a wild-type, Pde1a<sup>−/−</sup>, Pde1b<sup>−/−</sup>, Pde1c<sup>−/−</sup>, Pde3a<sup>−/−</sup>, or Pde3b<sup>−/−</sup> C57BL/6 genetic background are shown.

Figure 2. The severity of the renal cystic disease in Pkd2<sup>−/−WS25</sup> mice is aggravated on Pde1a<sup>−/−</sup>, Pde1c<sup>−/−</sup>, or Pde3a<sup>−/−</sup> backgrounds but not on Pde1b<sup>−/−</sup> or Pde3b<sup>−/−</sup> backgrounds. Representative hematoxylin-eosin-stained kidney sections from male and female Pkd2<sup>−/−WS25</sup> mice on a wild-type, Pde1a<sup>−/−</sup>, Pde1b<sup>−/−</sup>, Pde1c<sup>−/−</sup>, Pde3a<sup>−/−</sup>, or Pde3b<sup>−/−</sup> C57BL/6 genetic background are shown.

unstable (WS25) Pkd2 alleles and closely resembles human ADPKD. This genetic approach overcomes the limitations of a pharmacologic approach using PDE inhibitors, including lack of specificity and toxicity at the doses required to effectively inhibit PDE activity in target tissues. PDE3 activity trended to be lower without reaching statistical significance. The knockout of Pde1a but not Pde1b was associated with significant reductions in total PDE (by 29.4%) and PDE1 (by 35.6%) activities in Pkd2<sup>−/−WS25</sup> kidneys, whereas the knockout of Pde1c was associated with a slight but significant reduction in PDE1 activity (by 7.3%) but not in total PDE activity. Only the knockout of Pde3a but not that of Pde3b was associated with significant reductions in total PDE (by 13.1%) and PDE3 (by 64.0%) activities in Pkd2<sup>−/−WS25</sup> kidneys. This suggests that Pde1a is the main Pde1 subfamily and Pde3a is the main Pde3 subfamily in renal tissue from mice and explains why Pde1b and Pde3b knockouts did not change total or PDE1 or PDE3 activities, respectively. PDE1 activity was reduced in Pde3a knockout mice, whereas PDE3 activity was increased in the Pde1a and Pde1c knockout mice on both wild-type and Pkd2<sup>−/−WS25</sup> genetic backgrounds, consistent with previous observations of PDE1 and PDE3 inhibition<sup>14,15</sup> and PDE3 activation<sup>21,22</sup> by protein kinase A (PKA)–mediated phosphorylation. However, PDE4 activity was reduced in Pde3a knockout mice (the wild type) but increased in Pde1a (both backgrounds) and Pde1c knockouts (Pkd2<sup>−/−WS25</sup>), the latter consistent with a known negative regulatory feedback mechanism for cAMP/PKA signaling.<sup>32,33</sup> These observations suggested a substantial degree of redundancy and cross-talk among the different Pde1 and Pde3 families and subfamilies.

To test the significance of individual PDE1s, we analyzed the severity of PKD in null Pde1 mice bred into the Pkd2<sup>−/−WS25</sup> model of ADPKD and analyzed the outcomes at 16 weeks. We found increased severity of PKD, which was reflected by kidney weights and cystic indices adjusted for body weight, on a Pde1a or Pde1c null but not on a Pde1b null background (Figures 2 and 3, Supplemental Table 1). Compared with Pkd2<sup>−/−WS25</sup> mice, serum urea levels, liver weights, and cystic indices adjusted for body weights were increased in Pkd2<sup>−/−WS25</sup>; Pde1c<sup>−/−</sup> but not in Pkd2<sup>−/−WS25</sup>; Pde1a<sup>−/−</sup> (Supplemental Table 1). Aggravation of polycystic liver disease by the knockout of Pde1c points to a role for PDE1C in the regulation of cAMP pools important for cystogenesis in cholangiocytes.

We followed a similar approach to test the significance of individual PDE3s. At 16 weeks of age, Pkd2<sup>−/−WS25</sup>;Pde3a<sup>−/−</sup> but not Pkd2<sup>−/−WS25</sup>;Pde3b<sup>−/−</sup> mice had higher kidney weights and higher cystic and fibrotic indices adjusted for body weight compared with Pkd2<sup>−/−WS25</sup> mice (Figures 2 and 3, Supplemental Table 1). Serum urea levels were similar. Compared with Pkd2<sup>−/−WS25</sup> mice, liver weights adjusted for body weights were higher in the Pkd2<sup>−/−WS25</sup>;Pde3a<sup>−/−</sup> mice, but cystic indices adjusted for body...
PKD2/WS25; Pde3b−/− mice had slightly higher liver cystic indices without a significant increase in liver weight adjusted for body weight.

The aggravation of PKD associated with loss of Pde1a, Pde1c, or Pde3a occurred without a significant increase in renal cAMP (Figure 3). This could be because of inadequate sensitivity and specificity of measurements of cAMP in whole-tissue lysates to detect changes of cAMP levels in specific nephron segments or the compartmentalization of cAMP pools. Differences in the subcellular expression pattern of the different PDEs are important for the functional compartmentalization of cAMP-mediated responses. The fact that the knockout of Pde3a aggravated the development of PKD, despite the fact that PDE3 accounted for only a small fraction of the total PDE activity in kidney tissue and renal tubules, likely reflects its localization in subcellular compartments, where it regulates cAMP pools that control cell proliferation and CFTR-driven chloride secretion. These results are consistent with the observation that PDE3 inhibitors but not PDE4 inhibitors stimulate the proliferation of MDCK cells, despite the fact that PDE3 accounts for a very small fraction of total cAMP PDE activity and that the increase in cellular cAMP content induced by a PDE3 inhibitor in MDCK cells is negligible compared with that induced by a PDE4 inhibitor.

To look for evidence of activated cAMP and PKA signaling in double-mutant mice with PKD and Pde knockouts, we measured the levels of phosphorylated (Ser133) cAMP–responsive binding protein (P-CREB), phosphorylated activating transcription factor-1 (P-ATF-1), and CREM proteins in nuclear preparations from renal tissue as indicators of cAMP/PKA signaling (Figure 4). P-CREB, P-ATF-1, and CREMs were increased in nuclear preparations from wild-type, Pkd2−/−, Pde1a−/−, and Pkd2−/−; Pde3a−/− kidneys compared with wild-type, Pkd2−/−; Pde1a−/−, Pkd2−/−; Pde3b−/−, kidneys. Immunoblots of P-CREB, P-ATF-1, and CREM proteins in kidney nuclear fractions and total protein stains used for loading control are shown (n=6–10 per group). WT, wild type. *P<0.05; **P<0.01; compared to wild type. ††P<0.01 compared to Pde2−/−; Pde+/+.
The administration of DDAVP markedly increases body and liver weights compared with untreated or DDAVP–treated Pkd2−/WS25 mice (Figures 6 and 7, Supplemental Table 2). Compared with untreated or DDAVP–treated Pkd2−/WS25 mice, DDAVP–treated Pkd2−/WS25;Pde3a+/−;Pde3a+/−;Pde3b−/−;Pde3b−/− kidneys had slightly higher body and liver weights but no increase in liver cystic or fibrotic indices. These results are consistent with the hypothesis that PDE3 activity limits the cystogenic effect of DDAVP in mouse models of PKD. We believe that a similar enhancement of the cystogenic effect of DDAVP would have been observed on a Pde1a or a Pde1c null background.

In summary, here, we show that calcium- and calmodulin-dependent PDEs (PDE1A and PDE1C) and PDE3A modulate the development of PKD, possibly through the regulation of compartmentalized pools of cAMP that affect cell proliferation and CFTR–driven fluid secretion. Treatments capable of increasing their expression or activity could, therefore, be effective in slowing the development of the disease.37

**CONCISE METHODS**

**Animal Models**

All of the mouse models used in this study are inbred on a C57BL/6 background. The Institutional Animal Care and Utilization Committee approved all experimental protocols for the work described within this manuscript.

Double heterozygote Pkd2−/WS25 mice were generated by crosses of Pkd2+/− mice and Pkd2−/WS25/WS25 mice.31 The Pkd2 null allele was generated by a selectable neocassette that introduces an in–frame stop codon in exon 1. The WS25 mutation is caused by the integration of a selectable neocassette into the first intron of Pkd2 without replacing wild-type exon 1. This causes an increased rate of somatic Pkd2 mutations (intragenic homologous recombinations between tandemly repeated portions of the three groups. Consistent with the working hypothesis, kidney weights and cystic and fibrosis indices adjusted for body weights were higher in the DDAVP–treated Pkd2−/WS25;Pde3a+/− mice but not in the DDAVP–treated Pkd2−/WS25;Pde3b−/− mice compared with DDAVP–treated Pkd2−/WS25 mice (Figures 6 and 7, Supplemental Table 2). Compared with untreated or DDAVP–treated Pkd2−/WS25 mice, DDAVP–treated Pkd2−/WS25;Pde3a+/−;Pde3b−/− mice had slightly higher body and liver weights but no increase in liver cystic or fibrotic indices. These results are consistent with the hypothesis that PDE3 activity limits the cystogenic effect of DDAVP in mouse models of PKD. We believe that a similar enhancement of the cystogenic effect of DDAVP would have been observed on a Pde1a or a Pde1c null background.

In summary, here, we show that calcium- and calmodulin-dependent PDEs (PDE1A and PDE1C) and PDE3A modulate the development of PKD, possibly through the regulation of compartmentalized pools of cAMP that affect cell proliferation and CFTR–driven fluid secretion. Treatments capable of increasing their expression or activity could, therefore, be effective in slowing the development of the disease.37

**CONCISE METHODS**

**Animal Models**

All of the mouse models used in this study are inbred on a C57BL/6 background. The Institutional Animal Care and Utilization Committee approved all experimental protocols for the work described within this manuscript.

Double heterozygote Pkd2−/WS25 mice were generated by crosses of Pkd2+/− mice and Pkd2−/WS25/WS25 mice.31 The Pkd2 null allele was generated by a selectable neocassette that introduces an in–frame stop codon in exon 1. The WS25 mutation is caused by the integration of a selectable neocassette into the first intron of Pkd2 without replacing wild-type exon 1. This causes an increased rate of somatic Pkd2 mutations (intragenic homologous recombinations between tandemly repeated portions of

Figure 5. P-CREB expression was increased in the Pde1a, Pde1c, and Pde3a knockouts. Immunostaining for P-CREB was confined to the nuclei, increased in Pkd2−/WS25;Pkd2−/WS25;Pde3a+/−;Pde3a+/−;Pde3b−/−;Pde3b−/− kidneys, and particularly strong in the cells lining the cysts and dilated tubules. Magnification, ×400.
**Figure 6.** DDAVP administration aggravates the development of renal cystic disease in Pkd2−/WS25 mice on a Pde3a−/− genetic background compared with Pkd2−/WS25 mice on a wild-type or Pde3b−/− genetic background. Representative hematoxylin-eosin–stained kidney sections from male and female Pkd2−/WS25 mice on a wild-type, Pde3a−/−, or Pde3b−/−; C57BL/6 genetic background treated with DDAVP (30 ng/100 g body wt per hour subcutaneously by osmotic minipump) between 4 and 16 weeks of age.

**Breeding Strategy**

Pde null, Pkd2+/−, and Pkd2−/−; Pde3a+/−, Pde3b+/−, and Pde3a−/−; Pde3b−/−; Pkd2+/−; Pkd2−/−; and Pde3a−/−; Pde3b−/−; Pkd2−/−; Pde3a+/−; Pde3b+/−; Pkd2−/−; Pde3a+/−; Pde3b+/−; Pkd2−/−; Pde3a+/−; Pde3b+/−; Pkd2−/−; Pde3a+/−; Pde3b+/−; Pkd2−/−; Pde3a+/−; Pde3b+/−; Pkd2−/−; mice were generated by targeting disruption of exon 1. PDE3 activity is markedly reduced in the liver and adipocytes, where PDE3B is highly expressed. The growth, development, general behavior, and activity levels of Pde3b−/− mice are similar to those of wild-type mice, and both sexes are fertile. Pde3b−/− mice are slightly heavier than their wild-type counterparts, have decreased adipocyte size, and exhibit variations in coat color from white to yellowish brown. Sv129 Pde3b knockout males were mated with C57BL/6 wild-type female mice, and the progeny were backcrossed into a C57BL/6 background for nine generations to generate Pde3b knockout mice in a C57BL/6 background.

**Genotyping**

Tissue samples for genotyping were collected by tail clipping at 2 weeks of age into labeled microfuge tubes. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA). For Pkd2 genotyping, DNA was digested with Apal and analyzed by Southern blot with DNA probes for exons 2 (wild-type locus, 10 kb; mutant locus, 12 kb) and 1 (a doublet at 12–12.5 kb indicating two copies of exon 1 is unique to the WS25 allele). Pde genotyping was performed by PCR using appropriate primers (Supplemental Table 3).

**Experimental Groups**

Approximately 10 male and 10 female Pde null; Pkd2−/−; Pde3a+/−; Pde3b−/−; Pkd2−/−; Pde3a+/−; Pde3b−/−; Pkd2−/−; Pde3a+/−; Pde3b−/−; Pkd2−/−; mice were generated for each PDE1 and PDE3 subfamily. Because no significant differences were detected between the five different Pde+/−; Pde3a+/−; Pde3b−/−; Pkd2−/−; Pde3a+/−; Pde3b−/−; Pkd2−/−; Pde3a+/−; Pde3b−/−; Pkd2−/−; Pde3a+/−; Pde3b−/−; Pkd2−/−; Pde3a+/−; Pde3b−/−; Pkd2−/−; mice were euthanized at 16 weeks of age.

Additional Pde3a−/−; Pde3b−/−; Pkd2−/−; Pde3a−/−; Pde3b−/−; Pkd2−/−; mice were
generated to test the effect of treatment with DDAVP on the development of PKD in the absence of Pde3a or Pde3b. DDAVP was administered subcutaneously between 4 and 16 weeks of age using Alzet 1004 Osmotic Minipumps changed monthly.

**Blood Collections and Tissue Harvesting**
Mice were euthanized at 4 months of age. The animals were weighed and anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Blood was obtained by cardiac puncture for determination of serum BUN levels. The right kidney and part of the liver were placed into preweighed vials containing 10% formaldehyde in phosphate buffer (pH 7.4). These tissues were embedded in paraffin for histologic studies. The left kidney was immediately frozen in liquid nitrogen for determination of cAMP levels and PDE activities.

**Histomorphometric Analyses and Immunohistochemistry**
Four-micrometer transverse tissue sections of the kidney, including cortex, medulla, and papilla, and the liver were stained with hematoxylin-eosin and picrosirius red to measure cystic and fibrotic indices, respectively. Image analysis procedures were performed with Meta-Morph software (Universal Imaging, West Chester, PA). Digital images were acquired using a light microscope with a high-resolution Nikon Digital Camera (Nikon D2X 1200). The observer interactively applied techniques of enhancement for a better definition of interested structures and to exclude fields too damaged to be analyzed. A colored threshold was applied at a level that separated cysts from noncystic tissue and picrosirius red–positive material from background to calculate the indices of renal and hepatic cysts or fibrosis as percentages of body weight. Histomorphometric analyses were performed blindly without knowledge of group assignment.

**Immunohistochemistry**
Immunohistochemistry was performed using the SuperPicture Polymer Detection Kit (Life Technologies, Grand Island, NY) following the manufacturer’s protocol. P-CREB was purchased from Cell Signaling Technology, Inc. (catalog no. 9198; Danvers, MA).

**PDE Activities**
Kidneys were homogenized in ice-cold homogenization buffer containing 50 mM Tris (pH 7.5), 0.25 M sucrose, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol and supplemented with protease inhibitor tablets (Roche). PDE activities were measured using 1 µM cAMP as substrate in buffer containing 50 mM Tris (pH 7.5), 5 mM MgCl₂, 4 mM 2-mercaptoethanol, and 0.1% BSA. 3H-cAMP was included as a tracer for quantitation. PDE activity in aliquots incubated with 2 mmol EGTA without Ca²⁺ and calmodulin was determined as basal activity. To determine the activity in a sample caused by a specific PDE, various activators or inhibitors were included in the assay. PDE3 and PDE4 activities were determined as cAMP-PDE activities inhibited by 10 µM cilostamide or rolipram, respectively. In aliquots to determine the activity of Ca²⁺-calmodulin–dependent PDE1, the reaction mixture also contained 2.01 mM CaCl₂ to obtain 10 µM Ca²⁺ and 10 µg/ml calmodulin. Total PDE activity was determined as the sum of the basal PDE and PDE1 activities. Assays were initiated by the addition of substrate and incubated for 10 minutes at 30°C. The reaction was stopped by incubation for 3 minutes in boiling water. Crotalus atrox snake venom was then added, and after a 15-minute incubation at 30°C, hydrolyzed nucleotides were separated using high–capacity pretreated ion exchange resin (FabGennix, Frisco, TX). Slurries were mixed thoroughly and left to stand for 15 minutes on ice before centrifugation at 12,000g for 3 minutes. The radioactivity in 150-µl aliquots of the resulting supernatants was determined by liquid scintillation counting. Hydrolysis of cAMP was linearly proportional to incubation time and enzyme protein. Specific activities were defined as picomoles of cAMP hydrolyzed per minute per 1 mg protein.
**cAMP Content of Whole Kidneys**

Pieces from the flash-frozen left kidney were ground to fine powder and used for the cAMP assay as previously described following the manufacturer’s protocol (Enzo Life Sciences).

**Preparation of Nuclear Extracts and Western Blots**

Kidney nuclear protein was extracted using the Pierce Subcellular Protein Fractionation Kit (Pierce catalog no. 87790; Thermo Fisher Scientific, Wyman, MA) according to the manufacturer’s protocol. Protein concentrations were determined with the BCA Protein Assay Kit (Pierce Chemical Co.). Kidney nuclear proteins were heated in a sample buffer, electrophoresed on SDS-polyacrylamide gel, and transferred to PVDF membrane. The blots were blocked with 5% milk in TBS-T (0.1% Tween-20) at room temperature for 1 hour and then, incubated with the primary antibody (P-CREB; Cell Signaling and CREM; Santa Cruz Biotechnology) overnight at 4°C. After incubation, the membranes were washed and incubated with secondary antibody for 1 hour at room temperature. Detection was performed using enhanced ECL (Pierce Chemical Co.). Membrane was stained using the Swift Membrane Stain Kit (catalog no. 786-677; Geno Technology Inc., St. Louis, MO) according to the manufacturer’s protocol. Total protein stain was used as a loading control, and multiple proteins of a thin strip from top to bottom were used for quantification.

**Statistical Methods**

Data were expressed as means ± SDs. Comparisons between two groups were performed by two-way ANOVA to analyze the main effect of the Pde genotype and sex and their interaction. Comparisons between groups of parameters not affected by sex were also made using a nonparametric Mann–Whitney test.

**REFERENCES**

24. De Jonge HR, Tilly BC, Hogema BM, Pfau DJ, Kelley CA, Kelley MH, Melita AM, Morris MT,


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2015010057/-/DCSupplemental.