

Original Article

Effect of calcium-sensing receptor activation in models of autosomal recessive or dominant polycystic kidney disease

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Abstract

Background. Antagonists of relevant Gs protein-coupled and agonists of relevant Gi protein-coupled receptors lower renal cAMP and inhibit growth of renal cysts in animal models of human ARPKD (PCK rat) and/or ADPKD (*Pkd2*^{-/*WS25*} mouse). A calcium-sensing receptor (CaR) is expressed in various tubular segments and couples to Gq, thereby activating phospholipase C γ , InsP3 generation and calcium mobilization from intracellular stores, and Gi proteins. By both mechanisms, CaR activation could lower intracellular cAMP and inhibit renal cyst growth.

Methods. PCK rat and *Pkd2*^{-/*WS25*} mouse littermates were fed rodent chow without or with R-568, a type 2 calcimimetic, at a concentration of 0.05% or 0.1% between 3 and 10 or 16 weeks of age. Histomorphometric analysis was performed with Meta-Morph software. Western analysis and immunohistochemical staining were performed using antibodies for aquaporin-2, urea transporter UT-A1 and CaR. Northern blot hybridization was used to quantify the expression of vasopressin V2 receptor and aquaporin 2 mRNAs. Cyclic AMP was measured using an enzyme immunoassay kit.

Results. R-568 had no effect on kidney weight, cyst volume, plasma BUN concentration or severity of the polycystic liver disease. A significant reduction in renal interstitial fibrosis was detected in PCK rats, but not in *Pkd2*^{-/*WS25*} mice. R-568 administration, as anticipated, resulted in hypocalcemia and hyperphosphatemia, and significant increases in urine output, osmolar clearance, and urinary excretions of sodium, potassium and calcium.

Conclusions. CaR activation had no detectable effect on cystogenesis in models of autosomal recessive or dominant polycystic kidney disease. The lack of protective effect could be due to the absence of CaR in the outer medullary and cortical collecting ducts, the reduction in extracellular calcium and the unaffected levels of renal cAMP and renal expression of cAMP-dependent genes. A possible benefi-

cial effect on interstitial fibrosis deserves further study at more advanced stages of the disease.

Keywords: calcimimetic; cyclic AMP; PCK rat; *Pkd2*^{-/*WS25*} mouse; polycystic kidney disease

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD) are important causes of ESRD, morbidity and mortality in children and adults [1,2]. ADPKD is genetically heterogeneous with two disease loci, *PKD1* and *PKD2*. ARPKD is caused by mutations at a single locus, *PKHD1* [3]. The cysts in ARPKD derive mostly from the collecting ducts. The cysts in ADPKD may derive from any tubular segment, but the distal nephron and collecting ducts are predominantly involved.

The *PKD1*- and *PKD2*-encoded proteins, polycystin-1 and -2, and the *PKHD1*-encoded protein, fibrocystin, are membrane-associated glycoproteins. Polycystin-1 and fibrocystin are receptors for unknown ligands and may undergo regulated intramembrane proteolysis. Polycystin-2 is a calcium permeable cation channel. Polycystin-1 directly and fibrocystin indirectly interact with polycystin-2 and regulate its channel function. Polycystin-1, polycystin-2 and fibrocystin are localized to primary cilia [4–7] and are required for the rise in intracellular calcium triggered by mechanical stimulation of these organelles [6,8,9]. Polycystin-2 is also found in the endoplasmic reticulum, functionally interacts with inositol 1,4,5-triphosphate (InsP3) and ryanodine receptors, and modulates intracellular calcium signaling [10,11].

Alterations in two major interacting second messengers, intracellular calcium and adenosine 3',5'-cyclic monophosphate (cAMP) play a role in the pathogenesis of polycystic kidney disease. Cyclic AMP levels reflect a balance between synthesis by adenylyl cyclases (activated by Gs protein-coupled, GsPCR, and inhibited by Gi protein-coupled, GiPCR, receptors) and degradation by

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phosphodiesterases [12]. Stimulation of calcium-inhibitable adenylyl cyclases and inhibition of calcium-dependent cAMP phosphodiesterases may account for increased renal levels of cAMP and increased expression of cAMP-dependent genes in various animal models of polycystic kidney disease [13–15]. Cyclic AMP stimulates tubular cells beyond the loop of Henle to secrete chloride and fluid [16]. In addition, cAMP stimulates mitogen-activated protein kinase and extracellularly regulated kinase signaling and cell proliferation in renal epithelial cells of patients with polycystic kidney disease, whereas it has an inhibitory effect in wild-type cells [17,18]. This abnormal proliferative response is linked to alterations in intracellular calcium since it can be reproduced in wild-type cells by lowering intracellular calcium [19]. Conversely, calcium ionophores or channel activators rescue the abnormal response of cyst-derived cells [20].

Recently, antagonists of relevant GsPCR (vasopressin V2 receptor antagonists OPC31260 and OPC41061) or agonists of relevant GiPCR (octreotide) have been shown to lower renal cAMP [14,15,21,22] and inhibit the development and progression of the renal cystic disease in animal models orthologous to human ADPKD and/or ARPKD. Type 2 calcimimetic drugs act as selective allosteric modulators of calcium action at the level of the calcium-sensing receptor (CaR) [23,24]. CaR, a member of subfamily C of the GPCR superfamily, is capable of coupling to Gq (activating phospholipase C γ , InsP3 generation and mobilization of calcium from intracellular stores) and Gi proteins, lowering intracellular cAMP and inhibiting cAMP signaling [25]. Since CaR is expressed in many tubular segments (apical membrane of the proximal tubule; basolateral membrane of the medullary thick ascending limb, cortical thick ascending limb and distal convoluted tubules; and apical surface of the inner medullary collecting duct) [26], we decided to ascertain whether activation of CaR by a type 2 calcimimetic drug would attenuate or prevent the development of polycystic kidney disease.

Subjects and methods

Experimental animals and study design

Colonies of PCK rats and of *Pkd2*^{+/-} and *Pkd2*^{WS25/WS25} mice have been maintained in the Animal Facilities of the Department of Veterinary Medicine at the Mayo Clinic, Rochester, MN, since 1999. The PCK rat is a model of human ARPKD caused by a splicing mutation (IVS35–2A→T) that skips exon 36 and leads to a frameshift in the ortholog, *Pkhd1*. The *Pkd2*-null mutation was generated by disrupting the wild-type exon 1 of the *Pkd2* with a selectable neocassette that introduces an in-frame stop codon. The *WS25* mutation was generated by the integration of an exon 1 disrupted by the introduction of a selectable neocassette into the first intron of *Pkd2* without replacing the wild-type exon 1. This causes an increased rate of somatic mutations in the *Pkd2* gene (intragenic homologous recombinations between tandemly repeated portions of the wild-type and mutant exon 1). We crossed *Pkd2*^{+/-} and *Pkd2*^{WS25/WS25} mice to generate *Pkd2*^{-/WS25} mice. We

used these double heterozygote mice because, unlike other *Pkd1*^{+/-} or *Pkd2*^{+/-} models, they reliably develop a slowly progressive renal cystic disease that greatly resembles human ADPKD. At the time of weaning or 3 weeks of age, PCK rat and *Pkd2*^{-/WS25} mouse littermates were divided into three groups of 10 male and 10 female animals each receiving a standard ground rodent chow (Teklad 7017, Madison WI, USA) or the same chow containing R-568 at a concentration of 0.1% and 0.05 %, estimated to provide a daily dose of 50 and 25 mg/kg body weight, respectively. Rats were euthanized at 10 weeks and mice at 16 weeks of age, times by which, in our experience, the disease has developed consistently in the absence of treatment. Tail-cuff blood pressures and 24-h urine collections in metabolic cages for determination of urine output, osmolality and urinary excretions of sodium, potassium, calcium and phosphorus were obtained weekly on three consecutive weeks before killing at 10 or 16 weeks of age. At killing, the animals were weighed and anesthetized with ketamine (50 mg/kg, rats; 60 mg/kg, mice) and xylazine (10 mg/kg), intraperitoneally. Blood was obtained by cardiac puncture for determination of plasma BUN, electrolytes, osmolality, calcium and phosphorus. The right kidney and part of the liver were placed into pre-weighed vials containing 10% formaldehyde in a phosphate buffer (pH 7.4). These tissues were embedded in paraffin for histological studies. The left kidney and part of the liver were immediately frozen in liquid nitrogen for determination of cyclic AMP and Western and Northern blot analysis.

Histomorphometric analysis

Whole 4 μ m transverse tissue sections stained with hematoxylin-eosin and with picosirius red were used to measure cyst volumes and fibrosis, respectively. Image analysis procedures were performed with Meta-Morph software (Universal Imaging, West Chester, PA, USA). The Meta-Morph software system includes a light microscope with a color digital camera (Nikon DXM 1200) and a Pentium IBM-compatible computer (Dell OptiPlex). Stained sections are visualized under a Nikon microscope and digital images are acquired using a high-resolution Nikon Digital camera and displayed on the monitor. The observer interactively applies techniques of enhancement for a better definition of interested structures and to exclude fields too damaged to be analyzed. A colored threshold is applied at a level that separates cysts from non-cystic tissue in order to calculate cyst volumes. Areas of interest are expressed as a percentage of total tissue. All the histomorphometric analyses were performed blindly, without knowledge of group assignment.

Western blot analysis and immunohistochemistry

Western analysis and immunohistochemical staining were performed using antibodies for aquaporin-2 (Santa Cruz Biotechnology, Santa Cruz, California), urea transporter UT-A1 (gift from Dr Jeff M. Sands, Emory University School of Medicine, Atlanta, GA, USA) and CaR (Abcam Inc., Cambridge, MA, USA).

Table 1. Effect of R-568 on the development of polycystic kidney disease in PCK rats

	Male			Female			P value	
	Control (n = 10)	0.05% (n = 10)	0.1% (n = 10)	Control (n = 10)	0.05% (n = 10)	0.1% (n = 10)	Gender	R-568
Body weight (g)	340 ± 19	330 ± 20	299 ± 27*	234 ± 10	221 ± 15*	218 ± 10*	< 0.001	< 0.001
Kidney weight (% BW)	1.97 ± 0.30	1.95 ± 0.20	1.85 ± 0.21	1.57 ± 0.26	1.61 ± 0.20	1.63 ± 0.20	< 0.001	NS
Kidney cyst volume (%)	22.7 ± 5.4	20.2 ± 5.5	20.9 ± 2.3	21.1 ± 5.6	19.3 ± 6.3	18.9 ± 4.0	NS	NS
Kidney fibrosis volume (%)	4.53 ± 1.71	2.17 ± 0.92*	1.58 ± 0.41*	2.94 ± 1.17	1.28 ± 0.57*	1.03 ± 0.41*	< 0.001	< 0.001
Kidney cAMP (pmol/mg protein)	25.2 ± 8.9	36.6 ± 12.1	33.9 ± 16.7	20.8 ± 10.0	19.3 ± 5.6	17.0 ± 2.7	< 0.001	NS
Plasma BUN (mg/dL)	16.1 ± 1.7	16.7 ± 2.0	20.3 ± 4.6*	16.4 ± 0.8	16.9 ± 2.2	16.3 ± 1.6	NS	0.031
Plasma osmolality (mosm/L)	310 ± 10	310 ± 14	306 ± 16	318 ± 13	309 ± 10	310 ± 8	NS	NS
Plasma phosphorus (mg/dL)	8.4 ± 1.0	10.4 ± 1.5*	12.5 ± 1.4*	7.4 ± 0.7	9.9 ± 1.3*	11.1 ± 0.9*	0.003	< 0.001
Plasma calcium (mg/dL)	10.3 ± 0.4	8.4 ± 0.4*	8.1 ± 0.5*	10.6 ± 0.2	8.6 ± 0.4*	8.1 ± 0.2*	0.025	< 0.001
Urine volume (μL/min)	8.3 ± 2.4	14.2 ± 2.0*	13.5 ± 1.6*	4.0 ± 0.5	10.0 ± 2.0*	10.1 ± 1.5*	< 0.001	< 0.001
Urine osmolality (mosm/L)	869 ± 391	863 ± 413	1064 ± 420	1785 ± 577	1795 ± 523	1840 ± 352	< 0.001	NS
Osmolar clearance (μL/min)	21.3 ± 7.9	38.7 ± 17.3*	48.1 ± 21.6*	22.3 ± 7.9	55.6 ± 11.4*	60.0 ± 14.5*	0.009	< 0.001
Free water clearance (μL/min)	-13.0 ± 9.4	-24.4 ± 17.9	-34.6 ± 20.5*	-18.3 ± 7.7	-45.6 ± 12.2*	-49.9 ± 13.8*	< 0.001	< 0.001
Urine sodium (mEq/24 h)	0.56 ± 0.22	3.03 ± 0.37*	2.54 ± 0.88*	0.42 ± 0.15	2.02 ± 0.48*	2.22 ± 0.54*	< 0.001	< 0.001
Urine potassium (mEq/24 h)	2.02 ± 0.71	5.44 ± 0.86*	5.01 ± 0.85*	1.43 ± 0.29	3.58 ± 0.93*	4.28 ± 0.99*	< 0.001	< 0.001
Urine phosphorus (mg/24 h)	18.2 ± 8.4	13.5 ± 4.2	8.6 ± 7.7*	13.8 ± 4.2	8.5 ± 4.8*	9.0 ± 1.7*	NS	0.002
Urine calcium (mg/24 h)	1.04 ± 0.63	2.27 ± 0.87*	5.48 ± 2.00*	0.79 ± 0.41	3.02 ± 0.95*	4.30 ± 0.92*	NS	< 0.001

*P < 0.01 versus control.

Table 2. Effect of R-568 on the development of polycystic kidney disease in *Pkd2*^{-/-}*WS25* mice

	Male			Female			P value	
	Control (n = 9)	0.05% (n = 10)	0.1% (n = 10)	Control (n = 7)	0.05% (n = 8)	0.1% (n = 8)	Gender	R-568
Body weight (g)	28.4 ± 2.8	27.1 ± 1.1	26.1 ± 2.0	22.4 ± 1.4	22.0 ± 1.7	21.2 ± 1.5	< 0.001	0.027
Kidney weight (%BW)	1.94 ± 0.76	1.60 ± 0.46	1.77 ± 0.62	1.65 ± 0.32	1.45 ± 0.25	1.82 ± 0.85	NS	NS
Kidney cyst volume (%)	21.3 ± 17.8	11.0 ± 18.6	16.0 ± 17.1	26.0 ± 11.8	17.8 ± 16.8	22.3 ± 16.4	NS	NS
Kidney fibrosis volume (%)	2.44 ± 1.61	1.74 ± 1.68	3.07 ± 3.05	3.13 ± 2.16	1.98 ± 1.91	3.40 ± 3.26	NS	NS
Kidney cAMP (pmol/mg protein)	5.90 ± 2.21	5.75 ± 1.82	5.85 ± 2.20	7.04 ± 1.67	7.24 ± 2.17	6.50 ± 3.71	NS	NS
Plasma BUN (mg/dL)	31.1 ± 3.4	29.0 ± 4.2	29.2 ± 6.6	30.2 ± 7.7	28.9 ± 5.0	28.0 ± 4.0	NS	NS
Plasma osmolality (mosm/L)	317 ± 11	320 ± 10	319 ± 9	319 ± 10	319 ± 10	319 ± 11	NS	NS
Plasma phosphorus (mg/dL)	7.55 ± 0.72	8.25 ± 0.77	8.58 ± 0.95	7.25 ± 1.42	7.63 ± 0.71	7.87 ± 1.02	NS	NS
Plasma calcium (mg/dL)	8.53 ± 0.56	7.95 ± 0.67	7.30 ± 0.89*	7.63 ± 0.74	7.37 ± 0.82	6.68 ± 1.21*	0.009	0.004
Urine volume (μL/min)	0.56 ± 0.48	1.01 ± 0.53	0.72 ± 0.22	0.32 ± 0.22	0.80 ± 0.37*	0.52 ± 0.54	NS	0.013
Urine osmolality (mosm/L)	898 ± 265	1076 ± 339	936 ± 288	1514 ± 752	835 ± 333	885 ± 418	NS	NS
Osmolar clearance (μL/min)	1.60 ± 1.37	3.28 ± 1.84*	2.22 ± 1.26	1.35 ± 0.67	2.20 ± 1.24	1.47 ± 1.46	NS	0.044
Free water clearance (μL/min)	-1.04 ± 0.92	-2.26 ± 1.45*	-1.51 ± 1.08	-1.03 ± 0.59	-1.40 ± 0.97	-0.95 ± 0.96	NS	NS
Urine sodium (mEq/24 h)	0.13 ± 0.07	0.23 ± 0.11*	0.20 ± 0.06*	0.10 ± 0.06	0.25 ± 0.07*	0.22 ± 0.17	NS	0.004
Urine potassium (mEq/24 h)	0.15 ± 0.07	0.32 ± 0.18*	0.23 ± 0.08*	0.11 ± 0.04	0.30 ± 0.08*	0.28 ± 0.22	NS	0.003
Urine phosphorus (mg/24 h)	0.85 ± 0.98	2.32 ± 1.32*	1.17 ± 0.88	0.84 ± 0.49	1.58 ± 0.91	0.96 ± 1.11	NS	0.009
Urine calcium (mg/24 h)	0.07 ± 0.04	0.08 ± 0.03	0.06 ± 0.03	0.03 ± 0.02	0.07 ± 0.04*	0.07 ± 0.05	NS	NS

*P < 0.01 versus control.

Renal expression of vasopressin V2 receptor and aquaporin 2 mRNAs

Total RNA extracted from wild-type and cystic kidney pairs using TRIzol reagent (Invitrogen, Carlsbad, CA) was analyzed by northern blot hybridization using formaldehyde-agarose gel electrophoresis with transfer of RNA to nylon membranes. ³²P-labeled probes to vasopressin V2 receptor and aquaporin 2 were used and the hybridized blots were exposed to X-ray film to obtain autoradiographs. Autoradiographs of hybridized blots were scanned and after background was subtracted and cor-

rected for RNA loading, the pixel density of the band was divided by that of an internal control (L32) providing mRNA expression normalized to L32.

Cyclic AMP content of whole kidneys

The kidneys were ground to fine powder under liquid nitrogen in a stainless steel mortar and homogenized in 10 volumes of cold 5% TCA in a glass-Teflon tissue grinder. After centrifugation at 600 g for 10 min, the supernatants were extracted with three volumes of water-saturated ether.

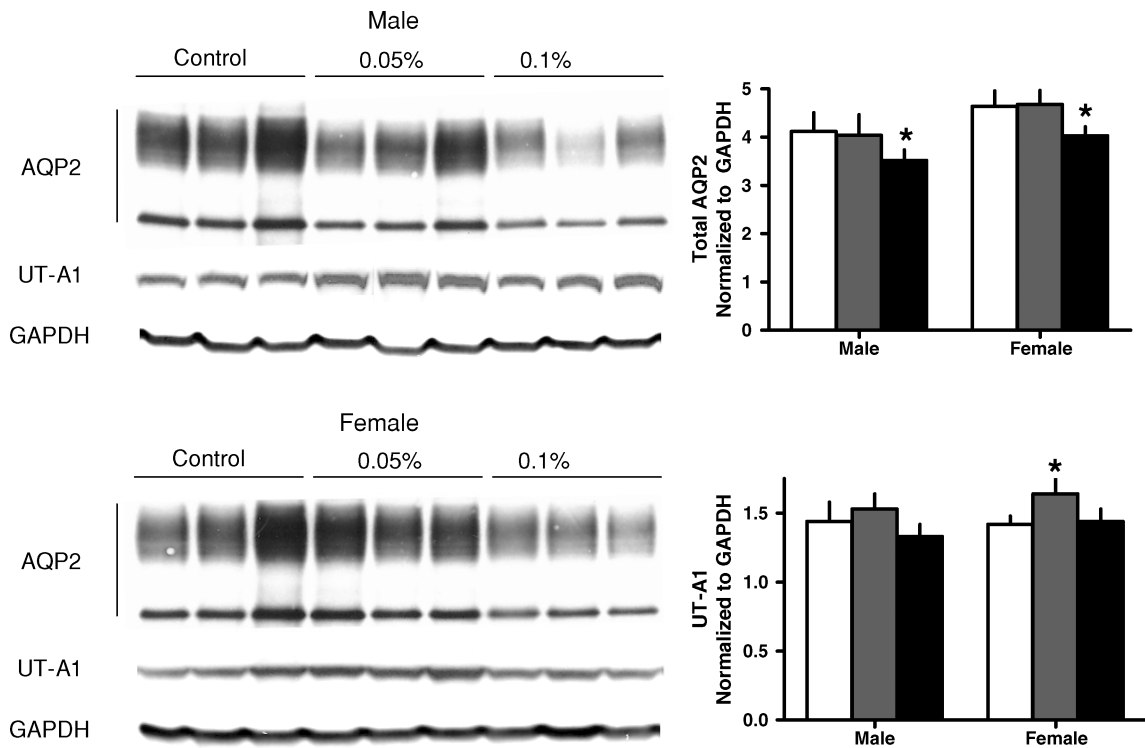


Fig. 1. Renal expression (Western blots) of aquaporin 2 and urea transporter UT-A1 in male and female PCK rats on no treatment (white bars) or treated with 0.05% (gray bars) or 0.1% (black bars) R-568 added to their diet. **P* < 0.05 versus no treatment.

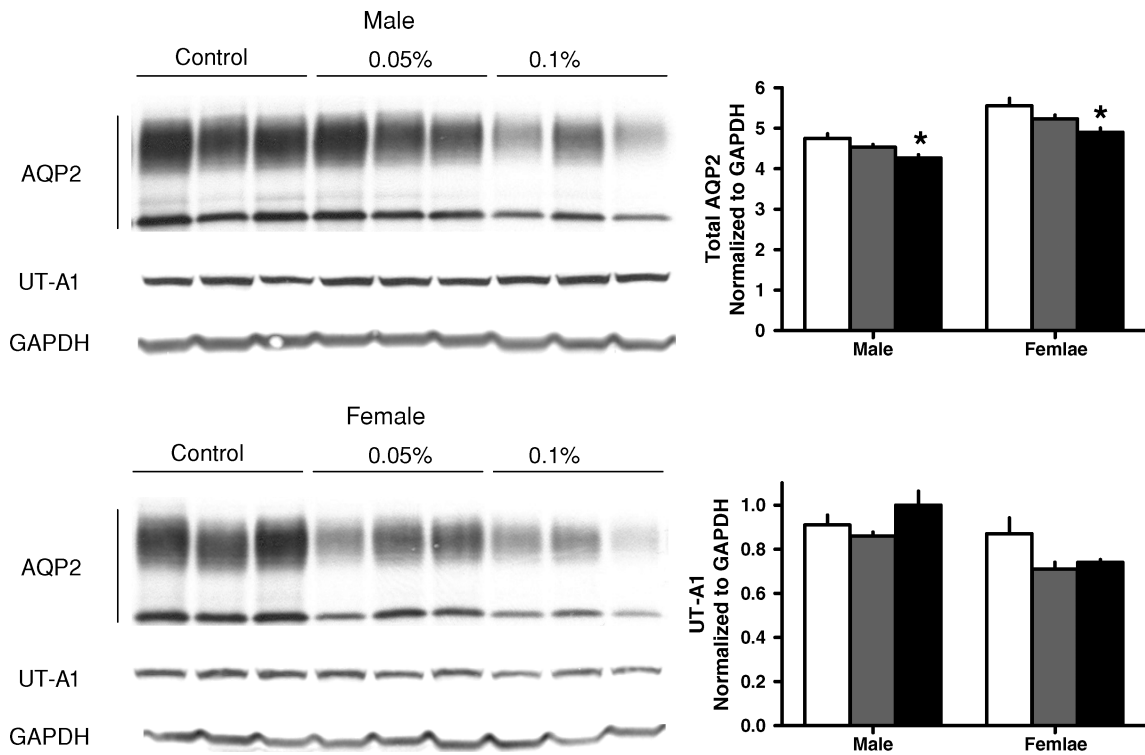


Fig. 2. Renal expression (Western blots) of aquaporin 2 and urea transporter UT-A1 in male and female *Pkd2*^{-/WS25} mice on no treatment (white bars) or treated with 0.05% (gray bars) or 0.1% (black bars) R-568 added to their diet. **P* < 0.05 versus no treatment.

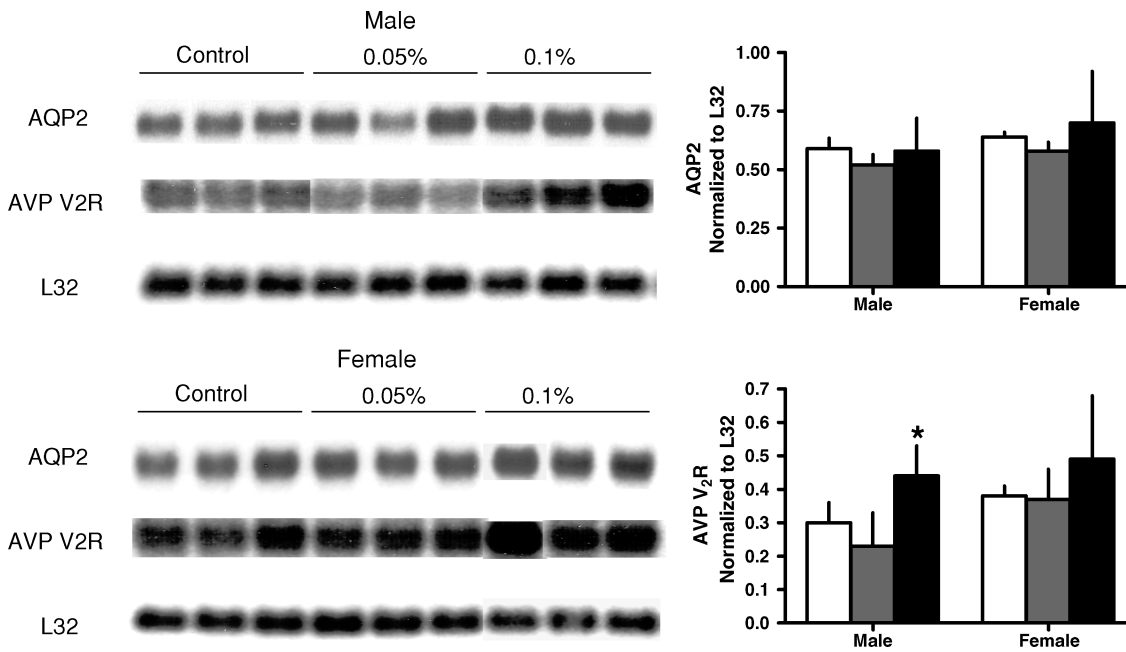


Fig. 3. Renal expression (Northern blots) of aquaporin 2 and vasopressin V2 receptor mRNA in male and female PCK rats on no treatment (white bars) or treated with 0.05% (gray bars) or 0.1% (black bars) R-568 added to their diet. * $P < 0.05$ versus no treatment.

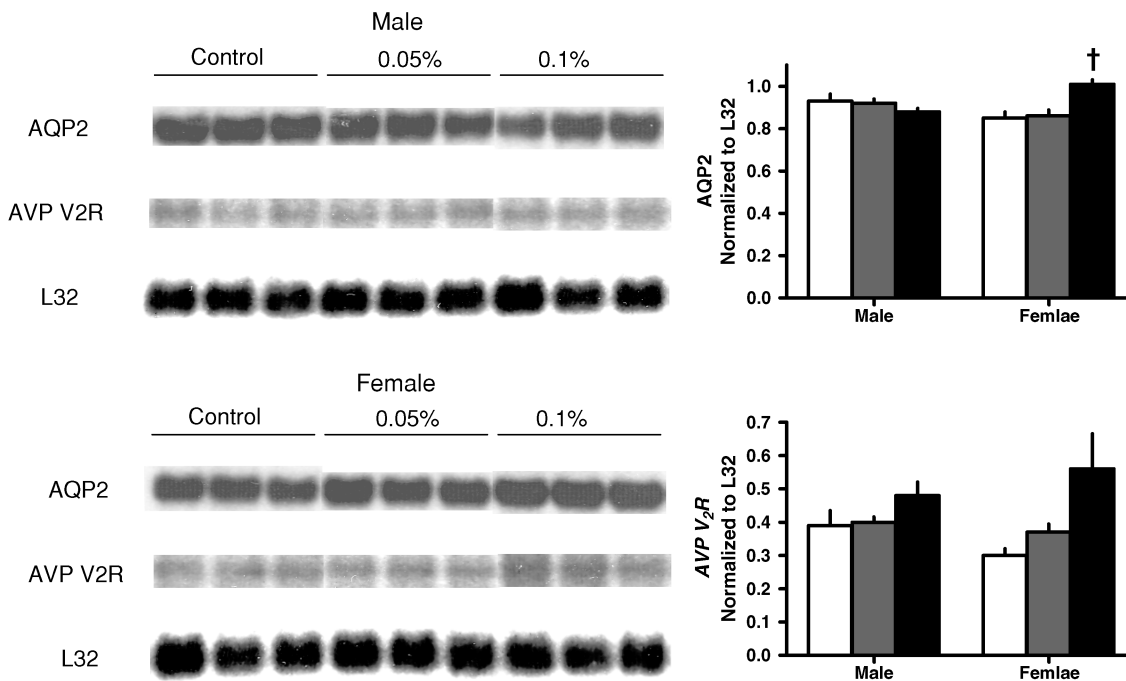


Fig. 4. Renal expression (Northern blots) of aquaporin 2 and vasopressin V2 receptor mRNA in male and female *Pkd2*^{-/-}/*WS25* mice on no treatment (white bars) or treated with 0.05% (gray bars) or 0.1% (black bars) R-568 added to their diet. *† $P < 0.01$ versus no treatment.

After drying the aqueous extracts, the reconstituted samples were processed without acetylation using an enzyme immunoassay kit (Sigma-Aldrich, Inc., St. Louis, MO, USA). The results were expressed in pmol/mg of protein.

Chemical determinations

Plasma blood urea nitrogen (BUN), phosphorus, calcium and electrolytes were measured with a Hitachi Modular

System. Urine phosphorus, calcium and electrolytes were measured using a Hitachi 912 analyzer and Perkin-Elmer Optima 2100 DV instrument, respectively. Plasma and urine osmolalities were measured using a computerized micro-osmometer.

Statistical methods

Comparisons between groups were made using two-way analysis of variance (ANOVA) with least significant

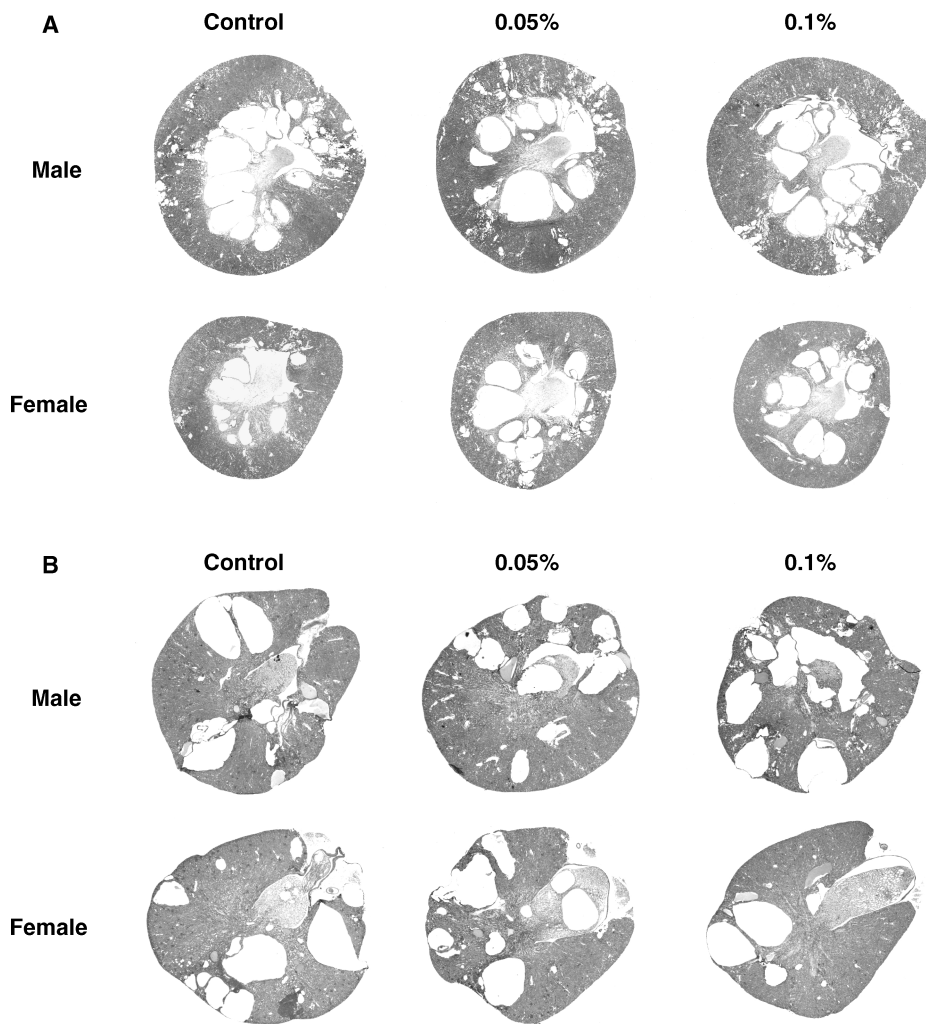


Fig. 5. Representative kidney sections from male and female PCK rats (panel A) and *Pkd2*^{-/WS25} mice (panel B) on no treatment or treated with 0.05% or 0.1% R-568 added to their diet.

difference comparisons of the means. Data are expressed as means \pm SD.

Results

The administration of R-568 to PCK rats (Table 1) and *Pkd2*^{-/WS25} mice (Table 2) was accompanied by a small but significant reduction in body weight, but it was otherwise well tolerated without apparent toxicity. In both animal models, it induced a reduction in serum calcium and an increase in serum phosphorus, which were accompanied in PCK rats by an increase in the urinary excretion of calcium and a reduction in the urinary excretion of phosphorus.

The administration of R-568 also resulted in a significant increase in urine output, osmolar clearance and urinary excretions of sodium and potassium in both rats and mice, without significant changes in plasma or urine osmolality (Tables 1 and 2). The increase in urine output was accompanied by a significant reduction in the renal aquaporin-2 protein in both animal models (Figures 1 and 2). The lev-

els of cAMP (Tables 1 and 2) and mRNA expression of aquaporin-2 were not affected significantly by R-568 administration (Figures 3 and 4). Female PCK rats treated with the lower R-568 dose had a higher expression of the UT-A1 protein than control rats or those treated with the higher dose and male rats treated with the higher R-568 dose had an increased expression of V2R mRNA compared to controls (Figures 1–4).

Despite the expected pharmacologic effects (hypocalcemia and hyperphosphatemia, increased urine output and osmolar clearance), R-568 had no significant effect on the development of polycystic kidney disease in PCK rats and *Pkd2*^{-/WS25} mice as reflected by kidney weights, cyst volumes and plasma BUN concentration (Tables 1 and 2 Figure 5). The majority of cysts in both animal models did not stain with the CaR antibodies (Figure 6). A significant reduction in interstitial fibrosis was detected in PCK rats that appeared to be dose dependent (Table 1); in *Pkd2*^{-/WS25} mice, however, a significant effect on fibrosis was not observed. Liver weights, cyst and fibrosis volumes were also similar in control and treated PCK rats and *Pkd2*^{-/WS25} mice (results not shown).

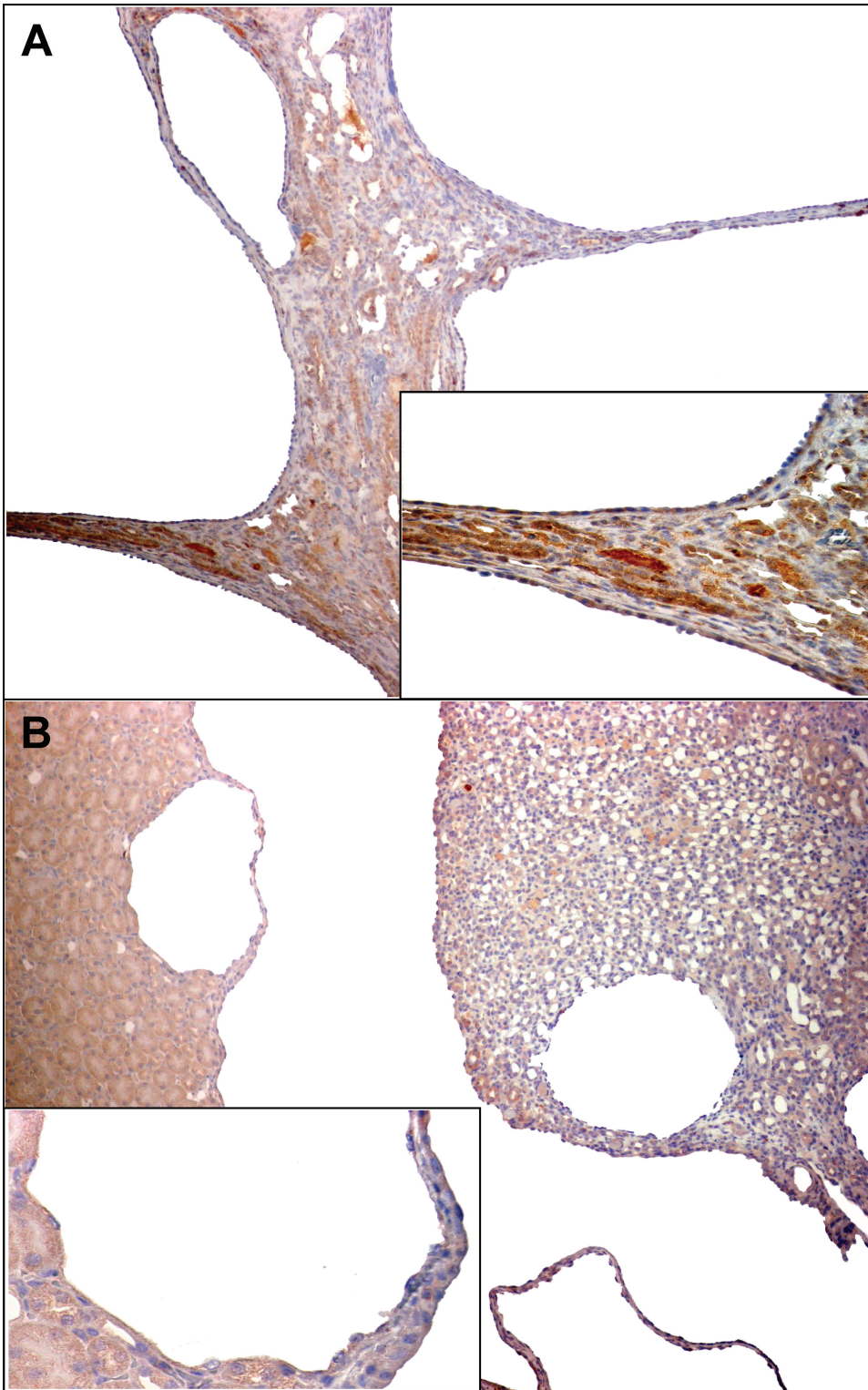


Fig. 6. Immunohistochemical staining of kidney sections from PCK rats (panel A) and *Pkd2*^{-/-}*WS25* mice (panel B) with a CaR antibody. Most cells lining the cysts show no staining. $\times 100$, insets $\times 400$.

Discussion

The administration of R-568 did not have a significant effect on cyst growth in two different models of PKD although it had pharmacologic effects on renal function and mineral metabolism [27]. The hypercalciuric effect of R-568 (Tables 1 and 2) is due to the activation of the CaR in the thick ascending limb of Henle and distal convoluted tubule. In the thick ascending limb of Henle, activation of the CaR inhibits calcium reabsorption by two mechanisms. The first is by eliciting phospholipase A2 (PLA2)-mediated arachidonic acid release and production of 20-hydroxyeicosatetraenoic acid that inhibits the apical potassium channel Kir1.1 (ROMK) [28,29]. ROMK is necessary for the recycling of potassium, sustained reabsorption of sodium chloride via the Na-K-2Cl cotransporter and passive paracellular reabsorption of calcium in the thick ascending limb of Henle. The second mechanism is by inhibiting the vasopressin-dependent stimulation of adenylyl cyclase and downregulation of the Na-K-2Cl cotransporter expression [30]. It should be noted that a marked hypercalciuric effect of R-568 was observed only in the PCK rat (see below). The hypophosphaturic effect in the PCK rat (Table 1) is consistent with the inhibition of parathyroid hormone secretion and with the calcimimetic-induced blunting of the phosphaturic action of parathyroid hormone in the proximal tubule. Most likely, the hyperphosphatemic and hypocalcemic effects of R-568 are directly due to the inhibition of parathyroid hormone secretion and to a large extent independent of the observed hypercalciuric and hypophosphaturic effects (Tables 1 and 2) since they can also be observed in totally nephrectomized animals [31].

Polyuria, as it occurred in this study, is also a known calcimimetic effect. It has been attributed to the disruption of urinary concentration by its action on the basolateral CaR in the thick ascending limb of Henle (inhibition of sodium chloride reabsorption and disruption of countercurrent multiplication mechanism) and on the apical CaR in the inner medullary collecting duct (inhibition of the vasopressin-induced effect on water permeability) [32,33]. Indeed, the administration of R-568 resulted in a significant reduction in the aquaporin-2 protein (Figures 1 and 2). As previously described in chronic hypercalcemic/hypercalciuric rats [32,33], the reduction in the aquaporin-2 protein was likely due to increased degradation rather than reduced synthesis since it occurred without a reduction in aquaporin-2 mRNA (Figures 3, 4). Moreover, renal cAMP levels were not affected by R-568. Female PCK rats treated with the lower R-568 dose had a higher expression of the UT-A1 protein than control rats or those treated with the higher dose, and male rats treated with the higher dose had an increased expression of V2R mRNA. These observations may point to dose-dependent effects that we have not explored fully. The calcimimetic-induced polyuria in our study was accompanied by a significant increase in osmolar clearance and urinary excretion of sodium, potassium and calcium. Therefore reduced expression or apical targeting of aquaporin-2 cannot entirely explain the increase in urine volume.

Despite the observation of the expected pharmacologic effects on renal function and mineral metabolism, R-568 had no significant effect on kidney cyst volume in PCK rats and *Pkd2*^{-/WS25} mice (Tables 1 and 2). There are at least three possible explanations for this lack of effect. First, CaR is not significantly expressed in the outer medullary and cortical collecting ducts [26], the main site of cyst development in the PCK rat and *Pkd2*^{-/WS25} mice [34]; this is consistent with the observation that most cysts did not stain with CaR antibodies. A recent study showing that CaR is expressed in mouse cortical collecting duct cell line (mp-kCCDcl4) [35] makes this explanation less likely. Second, the administration of R-568 resulted in hypocalcemia and therefore it is possible that the effects of R-568 on intracellular calcium and cAMP are negated by the reduction in extracellular calcium. Lowering extracellular calcium has been shown to induce a phenotypic switch from wild type to polycystic [19]. It has also been shown to increase cAMP in rat collecting duct cells [36] and in vascular smooth muscle cells freshly dissected from PKD2WS25/- mice [37]. Third, the inhibition of the vasopressin elicited water permeability induced by the effect of high urinary calcium on the apical CaR in the inner medullary collecting duct may occur despite a fully functional adenylyl cyclase, as suggested by the fact that in models of chronic hypercalcemia vasopressin elicits increases in urea permeability [33] regulated through increases in intracellular cAMP [38]. Consistent with the last two explanations, the renal cAMP levels (Tables 1 and 2) and the expressions of the urea transporter UT-A1 protein and of vasopressin V2 receptor mRNA (Figures 1-4) in the treated PCK rats and *Pkd2*^{-/WS25} mice were similar to or higher than those in the control animals. The administration of R-568 had no significant effect on the development of polycystic liver disease in either animal model. This is consistent with the lack of expression of CaR in the biliary epithelium [39].

The extent of renal interstitial fibrosis in PCK rats and *Pkd2*^{-/WS25} mice at the ages evaluated in our study was modest. The administration of R-568 significantly reduced the extent of the renal fibrosis in the PCK rat, but it had no effect on renal fibrosis in *Pkd2*^{-/WS25} mice or on liver fibrosis in either model. This could reflect that in the rat the renal effects of R-568 are more robust as reflected by its greater effect on urinary calcium excretion. In addition, there may be a species difference since renal fibrosis may be easier to induce in rats than in mice. While the mechanism and significance of the favorable effect on interstitial fibrosis in PCK rats is uncertain, it must be noted that R-568 has been reported to ameliorate the tubulo-interstitial damage in subtotal nephrectomized rats, possibly by reducing the levels of parathyroid hormone [36]. This antifibrotic effect of calcimimetic therapy may have a therapeutic action that may deserve further study at more advanced stages of polycystic kidney disease.

In summary, despite a sound rationale for considering calcimimetics in the treatment of polycystic kidney disease, their administration failed to ameliorate the cystic growth in animal models orthologous to human ARPKD and ADPKD.

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Conflict of interest statement. None declared.

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