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der Universität Tübingen**

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Evidence for a role of protein kinase C alpha in urine concentration

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1. Abbreviations

| | |
|--------------------|---|
| AC | Adenylyl cyclase |
| aPKC | Atypical protein kinase C subfamily |
| APS | Ammonium peroxodisulfate |
| AQP-2 | Aquaporin-2 |
| BSA | Bovine serum albumin |
| cPKC | Conventional protein kinase C subfamily |
| ddH ₂ O | Double distilled H ₂ O |
| EDTA | Ethylendiaminetetraacetic acid |
| GFR | Glomerular filtration rate |
| HEPES | 4-(2-Hydroxyethyl)-piperazin-1-ethansulfon acid |
| Hkt | Hematocrit |
| IgG | Immunoglobulin |
| MAP | Mean arterial blood pressure |
| nPKC | Novel protein kinase C subfamily |
| PFA | Paraformaldehyde |
| PKC | Protein kinase C |
| RT | Room temperature |

| | |
|-------|---|
| SDS | Sodium dodecyl sulfate |
| TEMED | N, N, N', 'N-Tetramethylethylenediamine |
| UNaV | Urinary sodium excretion |
| UOsmo | Urinary Osmolality |
| UV | Urinary flow rate |
| -/- | Knock out |
| +/+ | Wild type |

2. Introduction

2.1 Protein kinase C

Protein kinase C (PKC) is a serine/threonine kinase, which means that it is an enzyme capable of transferring a phosphate group, from ATP to an OH-group of a specific threonine or serine amino acid residue in target proteins. It is presumably present in all cells and thus has been implicated in a multitude of physiological processes ranging from cell growth, cell proliferation, cell differentiation to more organismal functions, such as memory (9, 34, 35, 46, 49). And as a consequence PKC is interesting from a therapeutic view: defects in human PKC isoenzymes have been associated with the development of various diseases like cancer (2, 27), Alzheimer's disease (11, 17) or vascular complications in diabetes mellitus (21, 22). Therefore, PKC isoenzymes may present new targets for treating these diseases or complications.

2.1.1 The superfamily of PKC

Up to 12 distinct members of the PKC family have been discovered in mammalian cells. They are grouped into three subfamilies based on their

primary structure. Conventional PKCs (cPKC) are, like the originally described kinase activity, dependent upon Ca^{2+} and phospholipids. This group includes PKC alpha, beta I, beta II and gamma. The second group, including the novel PKCs (nPKC), does not require Ca^{2+} but is phospholipid dependent, and includes PKC delta, epsilon, eta, and theta. The third, more recently identified group includes PKC zeta and lambda/iota. They are structurally similar, and are named atypical PKCs (aPKC) since they require neither phospholipid nor Ca^{2+} for activation. Figure 1 gives the general organization of the PKC molecule.

Major differences exist in the developmental expression, tissue

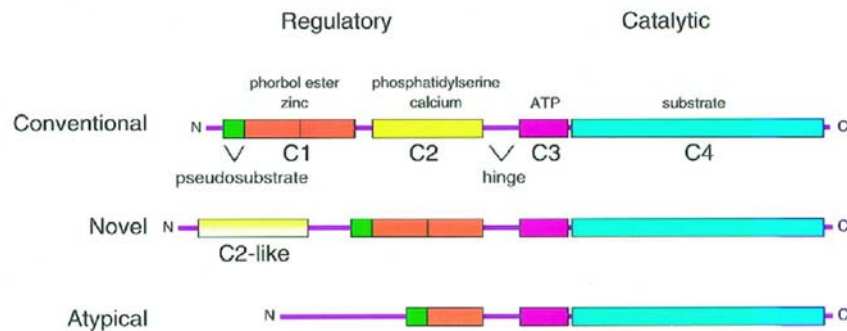


Fig 1: Schematic representation of the primary structure of conventional PKCs, novel PKCs and atypical PKCs. Shown are the pseudosubstrate domain (green), C1 domain comprising one or two cysteine-rich motifs (orange), C2 domain (yellow) in the regulatory half, and the ATP-binding lobe (C3, pink) and substrate-binding lobe (C4, teal blue) of the catalytic region. PKCs are comprised of an N-terminal regulatory region and a C-terminal catalytic region. They are separated by a hinge region that becomes proteolytically labile when the enzyme is membrane-bound; the proteolytically generated kinase domain, freed of inhibition by the pseudosubstrate, is constitutively active. The C2 domain of novel PKCs lacks amino acids involved in binding calcium but has key conserved residues involved in maintaining the C2 fold (hence its description as "C2-like"). Atypical PKCs have only one Cys-rich motif, and phorbol ester binding has not been detected. Adapted from (37).

distribution, regulatory properties, and susceptibility to activators and inhibitors between PKC isoenzymes (45).

2.1.2 The tissue distribution of PKC superfamily

PKC is presumably present in all cells. PKC alpha and beta I are present in all tissues examined, whereas the PKC beta II isoenzyme appears absent in the lung and the liver; PKC gamma was identified in brain, spinal ganglia and adrenal gland; PKC epsilon isoenzyme is abundantly expressed in spinal ganglia and in the smooth muscle cells of the bronchial wall (4). Antisera to PKC zeta and PKC eta isoenzymes heavily stained liver, kidney, brain, and stomach (4). Thus, PKC alpha, beta I, beta II, zeta, eta and theta are the isoenzymes present in many of the organs investigated.

2.1.2.1 The kidney distribution of PKC superfamily

Expression of various PKC isoenzymes has been reported in the kidney of the rat (15, 23, 39): employing Western blot and in situ hybridization, PKC alpha, beta I, delta, epsilon, and zeta were detected in rat glomeruli and PKC alpha, beta I, beta II, delta, epsilon, zeta, and eta in rat renal tubule. In mouse kidney,

only PKC alpha and beta I were detected in glomeruli and PKC alpha, beta I, beta II, delta, epsilon, zeta, and eta were localized to the renal tubular and collecting duct system (44).

2.1.3 The function of PKC in kidney

2.1.3.1 PKC and kidney development

In mature tissue, PKC is important for homeostatic functions. During development, PKC is involved in the regulation of growth and differentiation. PKC is expressed in an age-dependent pattern during kidney development. Inhibition of PKC disturbs nephron formation, inhibits growth and induces apoptosis in the developing kidney. Thus, PKC plays an important role in regulating normal kidney growth and differentiation (47).

2.1.3.2 PKC and mesangial cells

PKC fulfills at least two major functions in glomerular mesangial cells: it contributes to hormone-induced prostaglandin formation, and it acts as a negative feedback regulator of the inositol lipid signaling cascade (43). Glomerular mesangial cells show marked changes in rates of proliferation and in matrix accumulation when stimulated during pathological conditions, and

these events may be mediated in part by PKC (16, 30, 46, 54).

2.1.3.3 PKC and glomerular perfusion

Control of glomerular filtration is regulated in part by regulation of blood flow, accomplished by the balance between the tone of the afferent and efferent arterioles. The basal PKC activity directly modulates voltage-gated potassium channel activity, thereby indirectly affecting myogenic reactivity and the vasoconstriction of the afferent arteriole (28).

2.1.3.4 PKC and renal tubular function

In renal proximal tubule (PT) cells, PKC affects transport of bicarbonate, sodium, chloride and glucose by altering processes at both the basolateral and luminal membranes (5, 6). PKC may participate in as much as one third of the transport stimulation such as bicarbonate and water transport induced by angiotensin II (ANG II) (33). In luminal membrane vesicles (LMV) isolated from cortical tubules of rat kidney, PKC modulated Na^+/H^+ exchanger in different manner: phorbol myristate acetate (PMA) acutely increases the Na^+/H^+ activity, by translocation of PKC isoenzymes alpha, delta, and epsilon into LMV, whereas low-dose ANG II stimulated Na^+/H^+ activity via selective translocation of PKC-zeta; high-dose ANG II inhibiting Na^+/H^+ exchanger activity in LMV was

not PKC mediated (24). Recent studies have shown that the renal organic anion transport is negatively correlated with PKC activity which directly or indirectly controls the basolateral step in transport (19, 36). In cultured cortical collecting duct, the activation of PKCs can inhibit vasopressin-stimulated cAMP production which then restrain vasopressin-stimulated water transport (13). Furthermore, evidence show that the water channel AQP₄ function is regulated by PKC-induced phosphorylation, which means PKC may contribute to the regulation of water reabsorption in the kidney (45).

In summary, PKC has been proposed to play a central role in mammalian kidney function, where it appears to contribute to the control of renal hemodynamics, tubular transport, kidney growth and differentiation. Little is still known about which PKC isoenzymes mediate the respective functions.

2.2 PKC alpha and kidney

PKC alpha is a member of the cPKC subfamily and appears to be the predominant isoenzyme in the kidney (14, 15, 39, 41). Employing immunohistochemistry and confocal laser scanning microscopy in the SV129 and C57BL/6 mouse kidney, PKC alpha was localized to glomeruli, intercalated

cells of cortical collecting duct as well as medullary collecting duct (44). These expression and localization experiments suggest a contribution of PKC alpha to kidney function. Numerous studies have used different PKC inhibitors such as staurosporine, H-7, and GF109203X to examine the role of PKC, but long-term studies involving PKC inhibitors have not been possible because of their toxicities that are the result of their nonspecificity for the isoenzymes and their actions on other kinases (22, 32). Gene knockout can be a powerful approach to delineate respective gene function and therefore, using homologous recombination, Michael Leitges recently generated a mouse which is deficient for PKC alpha (31). Despite the fact that PKC alpha is presumed to be the most ubiquitously expressed PKC isoenzyme, the PKC alpha knockout mouse appears normal with regard to external characteristics, viability, and fertility. A closer look revealed that insulin signaling to insulin receptor substrate 1-dependent phosphoinositide 3-kinase (PI3K), protein kinase B, and PKC lambda, and downstream processes like glucose transport and activation of extracellular signal-regulated kinase (ERK) is enhanced in skeletal muscles and adipocytes from PKC alpha knockout mice. Serum glucose or insulin levels, however, were not significantly different in fed PKC alpha knockout mice as compared with fed control PKC alpha wild type mice (31).

2.3 Aim of thesis

Despite the broad comprehension of the functional contribution of PKC on the kidney, little is known about physiologically relevant functions assigned to PKC alpha. Here PKC alpha knockout mice were used to gain insights into the role of PKC alpha in kidney function.

3. Chemicals and Materials

3.1 Chemicals

3.1.1 Normal chemicals

| | |
|-----------------------------------|-----------------------|
| Acrylamid/bisacrylamid (30 : 0.8) | Roth, Karlsruhe |
| NH ₄ Cl | Merck, Darmstadt |
| APS, p.a. | Roth, Karlsruhe |
| Complete-protease inhibitor | Roche, Mannheim |
| Dako-Pen | Dako, Danemark |
| EDTA | Sigma, Deisenhofen |
| Glycin | Roth, Karlsruhe |
| Glycerol | Sigma, Deisenhofen |
| Hepes | Sigma, Deisenhofen |
| Liquemin (Heparin solution) | B. Braun, Melsungen |
| Inactin | Sigma, Deisenhofen |
| Ketamine | CuraMed GmbH, Germany |

| | |
|---|----------------------------------|
| Xylazine | Bayer Vital, Leverkusen, Germany |
| KCl | Merck, Darmstadt |
| KH_2PO_4 | Merck, Darmstadt |
| Mager milk powder | Merck, Darmstadt |
| $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, p.a. | Merck, Darmstadt |
| 2-Propanol | Merck, Darmstadt |
| Methanol | Merck, Darmstadt |
| 2-Methylbutan (Isopentan) | Merck, Darmstadt |
| Methylsulfoxide | Fluka, neu-Ulm |
| NaOH | Merck, Darmstadt |
| NaCl, p.a. | Merck, Darmstadt |
| $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ | Merck, Darmstadt |
| $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ | Merck, Darmstadt |
| NaN_3 | Sigma, Deisenhofen |
| PFA | Merck, Darmstadt |
| Procaine-HCl | Merck, Darmstadt |

| | |
|--|--|
| Roti-Load 1 | Roth, Karlsruhe |
| SDS, ultra pure | Roth, Karlsruhe |
| D-Saccharose, p.a. | Roth, Karlsruhe |
| TEMED, p.a. | Roth, Karlsruhe |
| Tissue-Tek® | Sakura, Holland |
| Tris-base, p.a. | Sigma, Deisenhofen |
| Triton X-100 | Fluka, neu-Ulm |
| Tween-20 | Merck, Darmstadt |
| BenchMark Prestained protein ladder (6.7-179.3 Kda) | Invitrogen, Carlsbad, CA, USA |
| CDP-Star Ready-to-use | Tropix, Bedford, MA |
| PVDF membrane | Millipore Corporation, USA |
| Paraffin liquid | Merck, Darmstadt |
| Bovine serum albumin | Sigma, Deisenhofen |
| V2-receptor antagonist SR121463 | Kindly provided by C. Serradeil-Le Gal, Sanofi-Recherche, France |

FluorSave™ Reagent

Calbiochem, USA

3.1.2 Antibodies

anti-AQP2 (goat)

Santa Cruz, Heidelberg, Germany

Donkey anti-goat IgG(alkaline phosphatase) Santa Cruz, Heidelberg, Germany

AlexaFluor 488®-conjugated donkey anti-goat antibody

MoBiTec, Göttingen, Germany

3.1.3 Kits for analysis

Albumin ELISA kit

Cell Trend GmbH, Luckenwalde, Germany

Vasopressin RIA kit

IBL, Hamburg, Germany

3.1.4 Diets

Normal diet (g/kg: 2.5 Na⁺, 9.5 K⁺, 4.8 Cl⁻, 10.7 Ca²⁺, 7.0 Pi)

Altromin GmbH, Germany

NaCl deficient diet (g/kg: 0 Na⁺, 0.1 K⁺, 0 Cl⁻, 0.2 Ca²⁺, 0.3 Pi)

ICN Biomedicals, Ohio, USA

3.1.5 Solutions

3.1.5.1 Normal solutions

- Acrylamid/bisacrylamid stocking solution (30 : 0.8)

- 10% APS stocking solution, 5ml:

| | | |
|-----|------|-----------------------|
| APS | 0.5g | 60µl portions (-20°C) |
|-----|------|-----------------------|

- 0.5M EDTA / NaOH (pH 8.0), 100ml:

| | | |
|------|---------|--|
| EDTA | 18.612g | |
|------|---------|--|

- 1M Hepes, 10ml:

| | | |
|-------|--------|----------------------|
| Hepes | 2.383g | 1ml portions (-20°C) |
|-------|--------|----------------------|

- Complete inhibitor

| | |
|----------------------------------|-----------------------|
| 1 Table + 2ml ddH ₂ O | 60µl portions (-80°C) |
|----------------------------------|-----------------------|

- 5N NaOH (RT), 100ml:

| | | |
|------|-----|--|
| NaOH | 20g | |
|------|-----|--|

- 5X PBS (pH7.4, RT), 1000ml:

| | | |
|-------|------|--------|
| 40.0g | NaCl | 0.685M |
|-------|------|--------|

| | | |
|------|-----|--------|
| 1.0g | KCl | 13.5mM |
|------|-----|--------|

| | | |
|------|----------------------------------|------|
| 7.1g | Na ₂ HPO ₄ | 50mM |
|------|----------------------------------|------|

| | | |
|-------|---------------------------------|------|
| 1.35g | KH ₂ PO ₄ | 10mM |
|-------|---------------------------------|------|

- 10% SDS (RT), filter sterilize:

SDS 5g

ddH₂O 50ml

3.1.5.2 Solutions for western blot

- Lysis buffer (pH7.4):

1.25ml 1M HEPES 25mM

4.28g Saccharose 0.25M

0.5g Triton X-100 1%

0.5ml 0.5M EDTA 5mM

Add 50ml ddH₂O

Briefly before use per 5ml lysis buffer add

200µl complete protease inhibitor cocktail

- 10X Electrophoresis buffer (pH 8.3, 4°C)

30g Tris-base

144g Glycin

10g SDS

Add 600ml ddH₂O

- 8X Upper gel stacking solution (stacking gel, pH6.8), 100 ml:

0.5M Tris-base 6.06g

10% SDS 4ml

- 4X Lower gel stocking solution (separating gel, pH8.8), 200 ml:

1.5M Tris-base 36.34g

10% SDS 8ml

- 10X Transfer buffer

Tris base 30g

Glycin 144g

Add ddH₂O to 1,000ml

1X Transfer buffer: 100ml 10X Transfer buffer

200ml Methanol

Add ddH₂O to 1,000ml (4°C)

- 10X TBS

24.2g Tris Base 200mM

80g NaCl 1.35M

Adjust PH to 7.6, Add ddH₂O to 1,000ml

- 10X Assay buffer (PH = 9.5)

24.2g Tris Base 2M

2.03g MgCl₂.6H₂O 100mM

Adjust PH to 9.5, Add ddH₂O to 100ml

- TBS/T

100ml 10X TBS

1ml Tween-20 (0.1%)

200mg NaN₃ (0.02%)

Add ddH₂O to 1,000ml

- Blocking solution

5% milk powder

1X TBST buffer

- 12% Lower gel (16 ml):

4ml Lower gel stocking solution

6.4ml Acrylamide stocking solution

5.6ml ddH₂O

32μl APS stocking solution

32μl TEMED

- Upper gel (3% , 4 ml):

1ml Upper gel stocking solution

0.4ml Acrylamide stocking solution

2.6ml ddH₂O

14µl APS stocking solution

5µl TEMED

3.1.5.3 Solutions for immunohistochemistry

- Fixation solution 100 ml

2g PFA

3g Saccharose

Add 60ml ddH₂O and 3 drop of 5N NaOH then heating to 60 °C

1.068g Na₂HPO₄·2H₂O

0.828g NaH₂PO₄·H₂O

Add 30ml ddH₂O

Together adjust PH to 7.4 with 5N HCl

Add ddH₂O to 100ml

- PBS (PH = 7.4) 1,000ml:

7.0128g NaCl 120mM

2.8478g Na₂HPO₄·2H₂O 16mM

0.3945g KH₂PO₄ 2.9mM

- 30% Saccharose solution

Biofuge 28RS Haraeus Instrument

Micro 12-24 centrifuge Hettich, Germany

Beckman L-70 Type CU-Optimal-L Beckman Instrument, München

3.2.1.4 PH Meter

Digital pH Meter 646 Carl Zeiss, Oberkochen

3.2.1.5 Shaker

IKA®MSI Minishaker IKA-Labortechnik

3.2.1.6 Water bath

Thermostatic water bath-WBS Fried Electric, Israel

3.2.1.7 MAP measurement

Pressure transducer P23dB GouldStatham, Oxnard, CA, USA

3.2.1.8 Apparatus for surgical experiment

Wild M3C Microscope Heerbrugg, Switzerland

SP210IW syringe pump WPI, USA

KL 1500 electronic lamp Schott, Germany

3.2.1.9 Apparatus for ion measurement

Gonotec Osmomat 030 Genotec, Germany

Flame photometer ELEX 6361, Eppendorf, Hamburg, Germany

3.2.1.10 Apparatus for protein assay

Dynatech MR 5000 Dynatech

3.2.1.11 Apparatus for radioactivity measurement

Gamma counter COBRA auto-gamma , Merden, USA

³H counter Tri-Carb Liquid Scintillation Analyzer, PACKARD, USA

3.2.1.12 Metabolic cages Tecniplast, Hohenpeissenberg, Germany

3.2.1.13 Apparatus for immunohistochemistry

SuperFrost^R Plus R. Langenbrinck, Germany

Confocal laser scanning microscope Zeiss LSM 410 Invert, Jena, Germany

3.2.2. Software for western blot

CSC1.1 and TINA program Raytest, Straubenhardt, Germany

3.2.3 PKC alpha knockout and wild type mice

Dr. M. Leitges, Max-Planck-Institute for Exp. Endocrinology, Hannover,
Germany

3.2.4. Materials for collecting plasma

Microhematocritcapillary (NH4-heparin) Roland Vetter, Germany

End to end mincaps Hirschmann, Germany

4. Methods

4.1 Targeted disruption of the PKC alpha gene.

The generation of a targeted PKC alpha mutation in mouse by homologous recombination was described before by Michael Leitges (31). Briefly, a targeting vector harboring a neomycin cassette(neo) insertion was constructed within the second exon of the PKC alpha gene, thereby disrupting the transcription of the gene after homologous recombination. The targeting vector was electroporated into embryonic stem (ES) cells, and G418-resistant colonies were analyzed by southern blot analysis for homologous recombination at the PKC alpha locus. Two ES cell clones with correct integration of the targeting vector were used for the generation of mutant mice through microinjection of PKC alpha +/- ES cells into NMRI albino blastocysts to generate chimeras. Both ES cell lines resulted in germ line-transmission. Male chimeras were crossed to 129/SV females and gave rise to F1 heterozygous offspring on a pure 129 background. Intercrosses of such were used to establish a homozygote PKC alpha-deficient mouse line. Male mice from this line were compared with age matched male 129/SV PKC alpha control (+/+) mice. Despite the fact that PKC alpha is presumed to be the most ubiquitously expressed PKC isoenzyme, the PKC alpha-deficient mouse

appeared normal with regard to external characteristics, viability, and fertility.

4.2 Metabolic cage experiments in conscious mice.

A) To assess basal renal function, 6 wild type and 6 knockout mice were put into metabolic cages with free access to tap water and a standard diet (2.5g Na⁺/kg). After adaptation to metabolic cages over a period of 3 days, a 24h urine collection was performed and the urinary excretion of fluid, sodium and potassium as well as urine osmolality were assessed. To assure quantitative urine collection, metabolic cages were siliconized and urine was collected under water-saturated oil. Concentration of Na⁺ and K⁺ in urine was determined using a flame photometer and osmolality by freezing-point depression. The concentrations of albumin and vasopressin were determined using a commercial ELISA kit for mouse albumin or a commercial RIA kit for vasopressin, respectively. After finishing urine collection mice were anesthetized with ether and blood was drawn from retrobulbar plexus for determination of hematocrit and plasma osmolality.

B) In a separate set of experiments, 24h urine collections were performed during an adaptation period of three days under standard sodium diet (2.5g

Na⁺/kg) and subsequently in response to a NaCl-deficient diet (0g Na⁺/kg) for 6 days.

C) In another set of experiments, urinary flow rate and urine osmolality were determined in conscious mice in response to

- i) 36h water deprivation with urine collection being performed over the last 14h of water deprivation.
- ii) Application of vasopressin V2-receptor antagonist SR121463 (1mg/kg i.p.) which in the rat induces diuresis through internalization of aquaporin-2 from the luminal membrane and subsequent inhibition of water transport in medullary collecting duct (37).
- And iii) acute water loading (1ml/16g bodyweight) using a gastric tube. Four hours before, access to food had been withdrawn. To prevent contamination with the concentrated urine being present in the bladder before administration of SR121463 or water loading (20, 27), osmolality was measured in the second portion of spontaneously voided urine after drug application and water loading, respectively.

4.3 Blood pressure measurement and clearance experiments in anesthetized mice.

As described before (50-53), mice were anesthetized with 100 mg/kg body weight inactin intraperitoneal (i.p.) and 100 mg/kg body weight ketamine i.m., and were placed on a thermostatically controlled surgical table to keep rectal temperature at 37°C. A tracheostomy (PE 200) was performed to facilitate free breathing. The left femoral artery was cannulated with a PE-50 catheter to obtain blood samples and monitor arterial blood pressure. The jugular vein was cannulated for continuous maintenance infusion of 2.25 g/dl BSA in 0.9% NaCl at a rate of 0.4 ml/hr. For assessment of glomerular filtration rate, [³H]-inulin was added to deliver 20 microCi/h. After surgery, the mice were allowed to stabilize for 60 min. Then a timed urine collection was performed for 60 min using a bladder catheter. Blood was withdrawn at the beginning and at the end of the collection period to determine [³H]-inulin. Urine was analyzed for [³H]-inulin. At the end of the clearance experiment, kidneys were excised and decapsulated and the wet weight were determined. After drying the kidneys over night at 50 °C the dry weight was determined.

4.4 Preparation of kidney samples.

PKC alpha +/+ and -/- mice were anesthetized by intraperitoneal injection of ketamine (60 mg/kg bodyweight) and xylazine (9 mg/kg bodyweight). After

opening of the abdominal cavity, the left renal artery was clamped, the left kidney excised, and the inner medulla dissected and immediately immersed into liquid nitrogen for later Western blot analysis. Subsequently the left cardiac ventricle was exposed. The tip of the perfusion system was inserted into the left ventricle, and the arterial system was perfused for 1 min with 3-5 ml PBS to clear the blood of the kidney, and subsequently for 10 min with 10-15 ml of a fixation solution. Both solutions were at room temperature. Then the right kidney was removed for immunohistochemical and morphological examination.

4.5 Western blot analysis.

As described before (12), total cellular proteins of inner medulla were obtained by pulverizing the tissue and dissolving the powder in lysis buffer. Homogenization was followed by centrifugation (1.000 x g, 10 min, 4°C). Protein content was determined as described by Bradford (8) using a commercial protein assay and bovine serum albumin as a standard. Samples were diluted 1:3 with Roti-Load sample buffer and boiled for 10 min at 65°C. The following steps were performed at room temperature. Samples of 20 µg of protein were subjected to SDS gel electrophoresis using 12% acrylamide gels in a Mini-PROTEAN II Electrophoresis Cell. For determination of molecular

weight, a prestained protein ladder was used. After gel electrophoresis (60 mA/gel, 70 min), proteins were transferred to PVDF membrane of 0,45- μ m pore size. Membranes were blocked for 90 min with blocking buffer and rinsed twice with TBS containing 0.1% Tween 20 (TBST). Thereafter, membranes were incubated overnight with antibody for aquaporin-2 (AQP2) (0.5 μ g/ml) in TBST. The secondary alkaline phosphate-conjugated antibody was incubated at a concentration of 0.1 μ g/ml for 2 hours, RT in TBST. Blots were rinsed twice with TBST and washed three times for 15 min with TBST, then 2 times in assay buffer at RT. Development was done with CDP-Star Ready-to-use solution. Immunoreactive bands were visualized by a chemiluminescence system with CSC1.1 program software and were then quantified by TINA program.

4.6 Immunohistochemical and morphological studies.

The right kidney was cut into slices (3mm in thickness) displaying cortex and outer and inner medulla, and was incubated for 3 hours in the fixation at 4°C. After rinsing in PBS for 15 min, kidney slices were dehydrated in 30% sucrose in PBS overnight at 4°C. Thereafter, kidney slices were frozen in isopentane precooled by liquid nitrogen and stored at -80°C until further use. Cryosections of approximately 10 μ m were made at -20°C and transferred onto gelatine-coated glass slides. After washed in PBS containing 0.1% Triton X-100 for 3

min, PBS for 5 min, sections were preincubated for 20 min in PBS containing 1% BSA. Then the first AQP2 antibody (2 µg/ml) was incubated at 4°C overnight. After washed in PBS 3 times for 5 min, the secondary donkey anti-goat IgG conjugated with AlexaFluro488® antibody (2 µg/ml) was incubated for 1 hour at RT. Sections were then washed twice for 10 min in PBS and mounted in FluorSave™ as fading retardant. The results were studied by confocal laser scanning microscope. Cortex and inner medulla thickness were measured in the maximal cross sectional area of the frontal sections. A total of 9 sections were evaluated on each animal (26).

4.7 Statistical Methods.

Results are presented as mean ± SE. Statistical significance of differences between PKC alpha -/- and PKC alpha +/+ mice was assessed by Student's T test.

5. Results

5.1 Basal kidney function in metabolic cage experiments in conscious mice.

When provided free access to water and standard diet, PKC alpha $-/-$ mice showed similar food intake (Fig 2) and urinary albumin excretion (Fig 3) but higher urinary flow rate (Fig 4) and reduced urine osmolality (Fig 5) compared with PKC alpha $+/+$ mice. These changes in PKC alpha $-/-$ mice were associated with an enhanced urinary vasopressin to creatinine ratio (Fig 6). Arterial hematocrit under these conditions was not different and plasma osmolality tended to be higher in PKC alpha $-/-$ vs. $+/+$ mice (Fig 7).

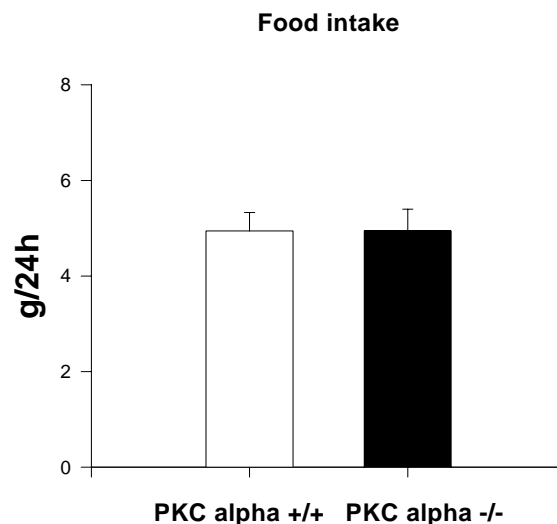


Fig 2: When provided free access to water and standard diet, PKC alpha $-/-$ mice showed no significant differences in food intake versus PKC alpha $+/+$ mice (n=6 mice/group).

Urinary albumine excretion

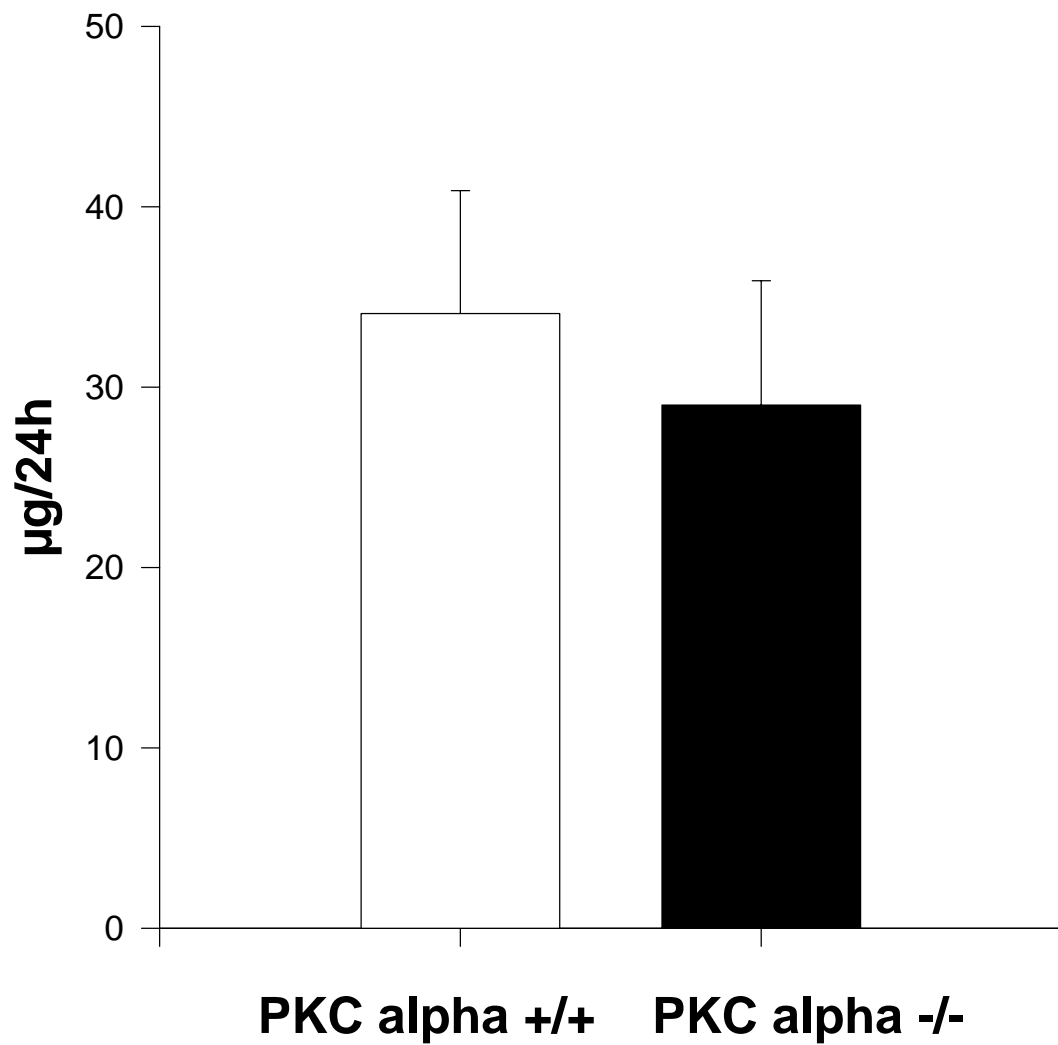
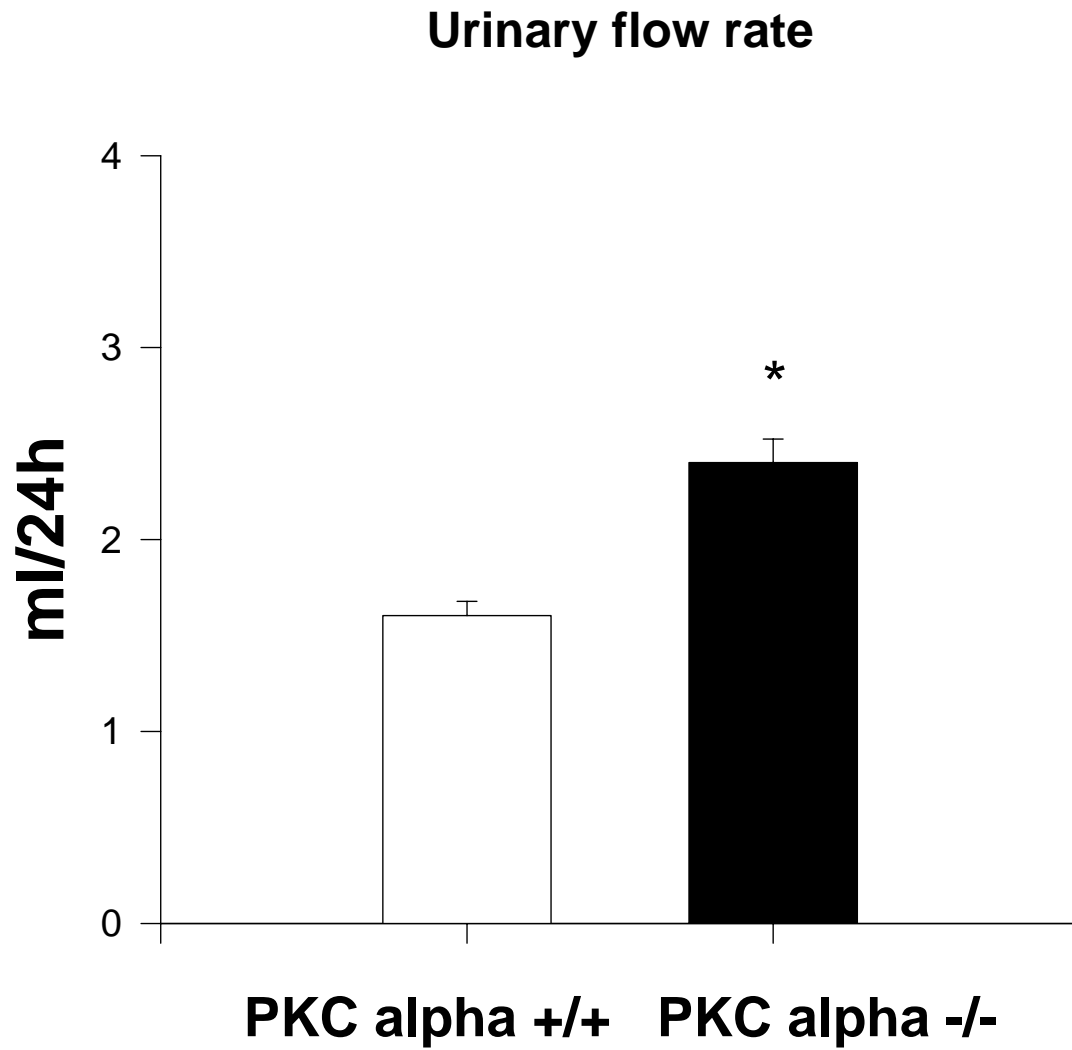
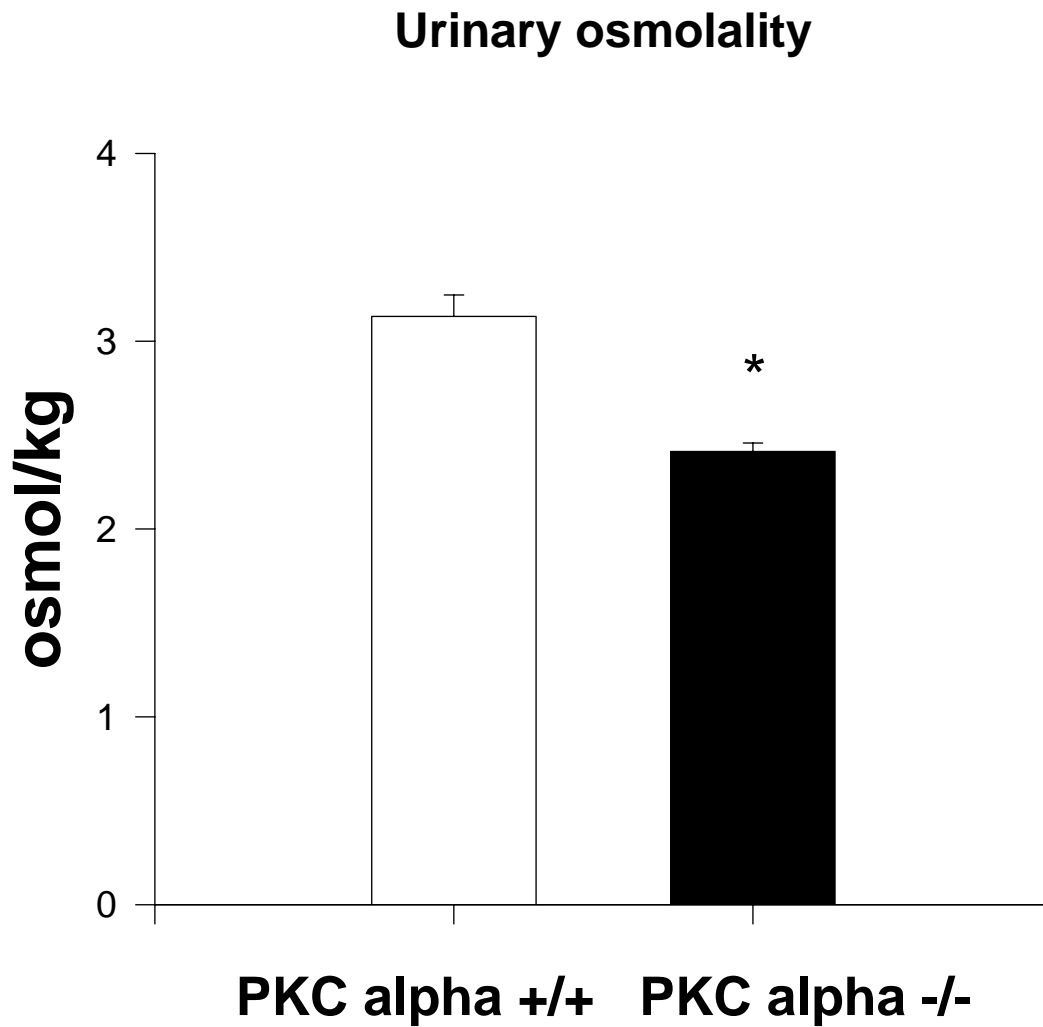


Fig 3: When provided free access to water and standard diet, the urinary albumin excretion was not significantly different between PKC alpha -/- mice and PKC alpha +/+ mice (n=6 mice/ group).



* $P < 0.05$

Fig 4: When provided free access to water and standard diet, PKC alpha -/- mice showed significantly higher urinary flow rate than PKC alpha +/+ mice (n=6 mice/group).



* **P<0.05**

Fig 5: When provided free access to water and standard diet, PKC alpha -/- mice showed significantly lower urinary osmolality than PKC alpha +/+ mice (n=6 mice/group).

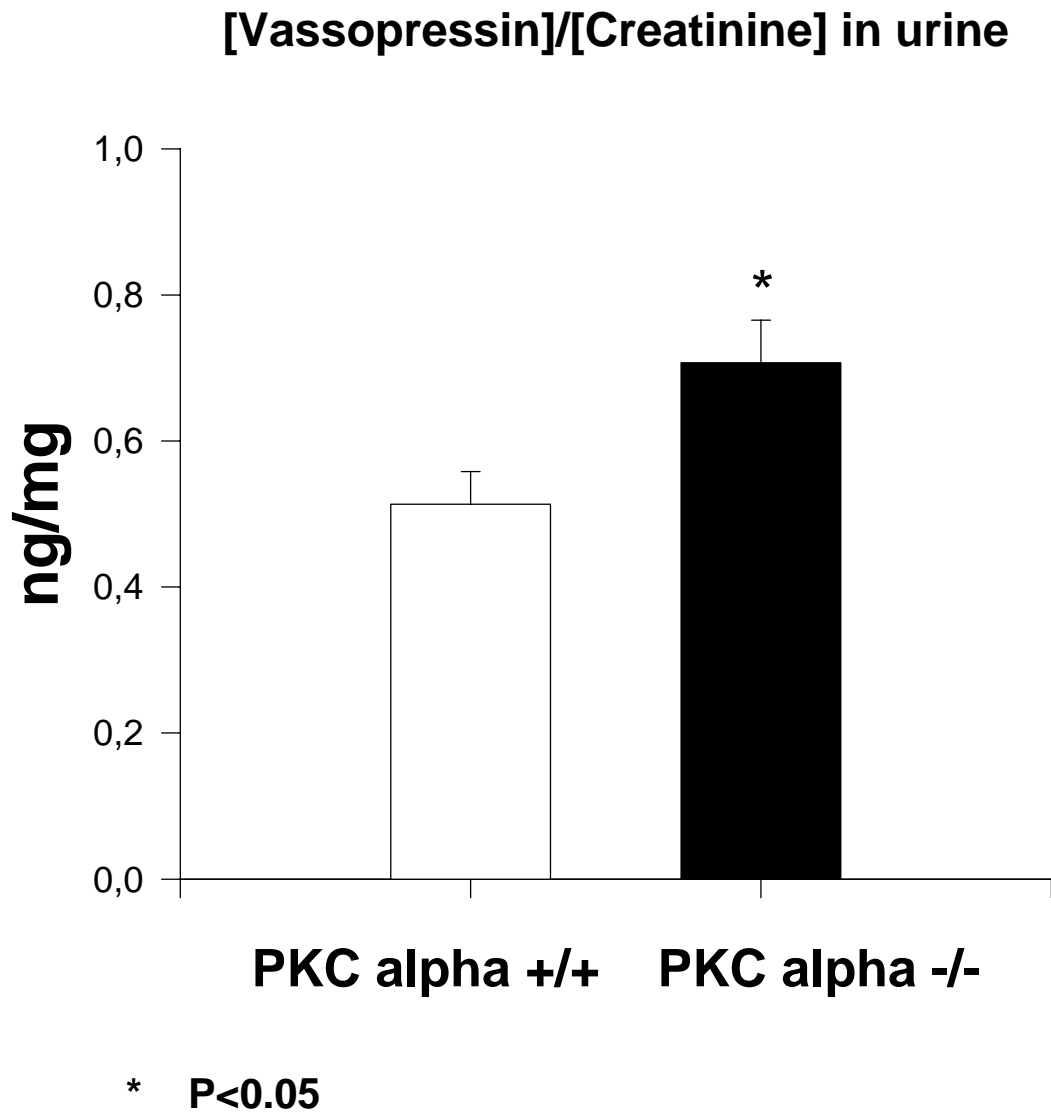


Fig 6: When provided free access to water and standard diet, PKC alpha -/- mice showed a significantly higher urinary vasopressin to creatinine ratio versus PKC alpha +/+ mice (n=6 mice/ group).

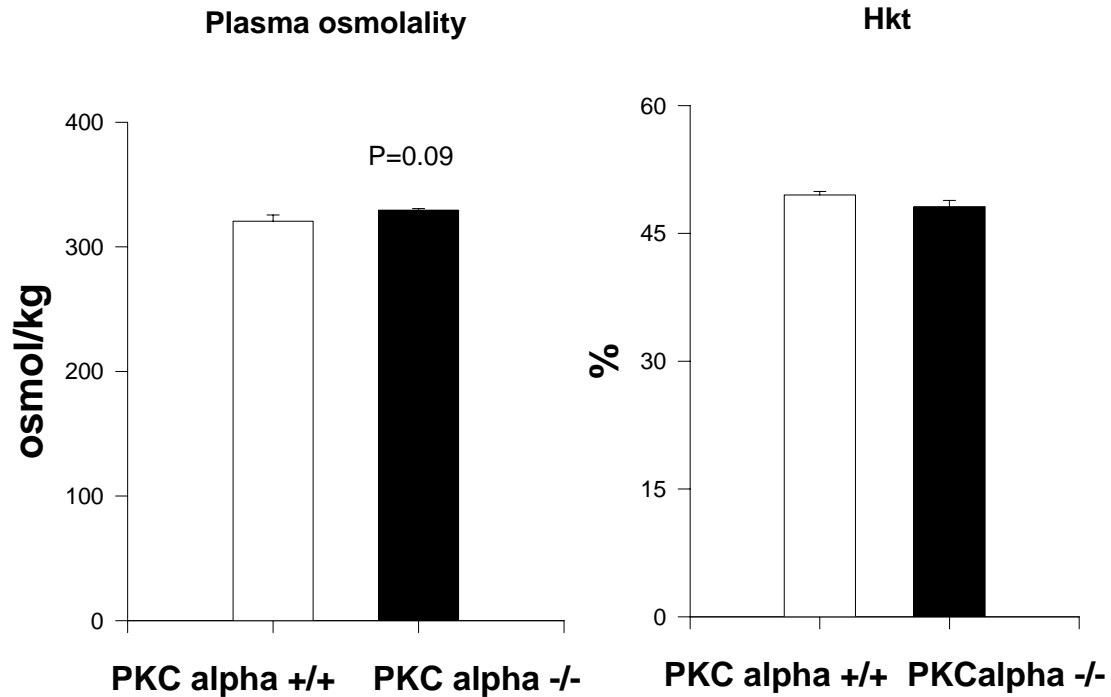


Fig 7: When provided free access to water and standard diet, the hematocrit was not different between PKC alpha +/+ and -/- mice, but the plasma osmolality tended to be higher in PKC alpha -/- mice versus +/+ mice (n=6 mice/group).

5.2 Basal kidney function in clearance experiments in anesthetized mice.

Mean arterial blood pressure and heart rate (Fig 8) were not different between PKC alpha -/- and +/+ mice. PKC alpha deficient mice, however, exhibited a modestly lower GFR which was associated with an enhanced absolute

fractional fluid excretion (see Fig 9) and a modestly lower kidney wet weight and kidney dry weight (see Fig 10).

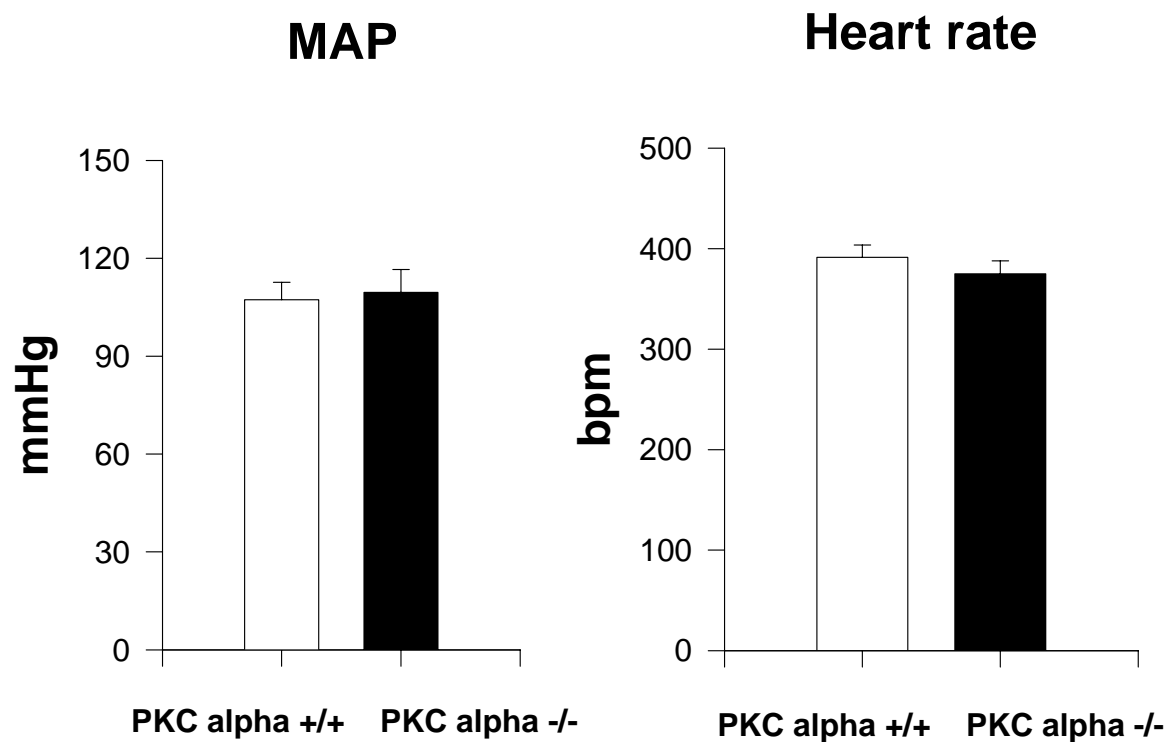


Fig 8: Under anesthesia, the mean arterial blood pressure (MAP) and heart rate were not different between PKC alpha +/+ mice and PKC alpha -/- mice (n=8 mice/group).

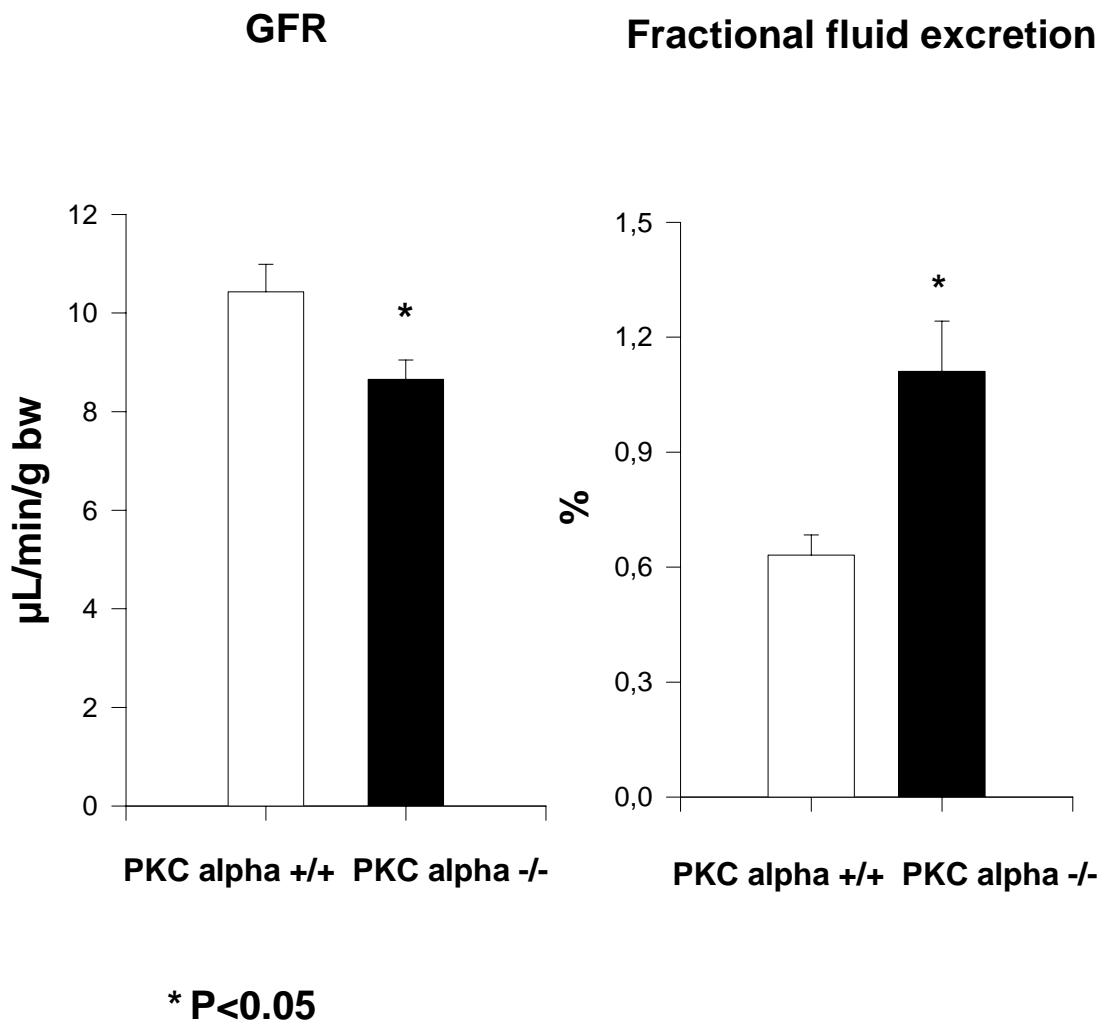


Fig 9: Under anesthesia, PKC alpha -/- mice exhibited a modestly lower GFR which was associated with an enhanced fractional fluid excretion (n=8 mice/group).

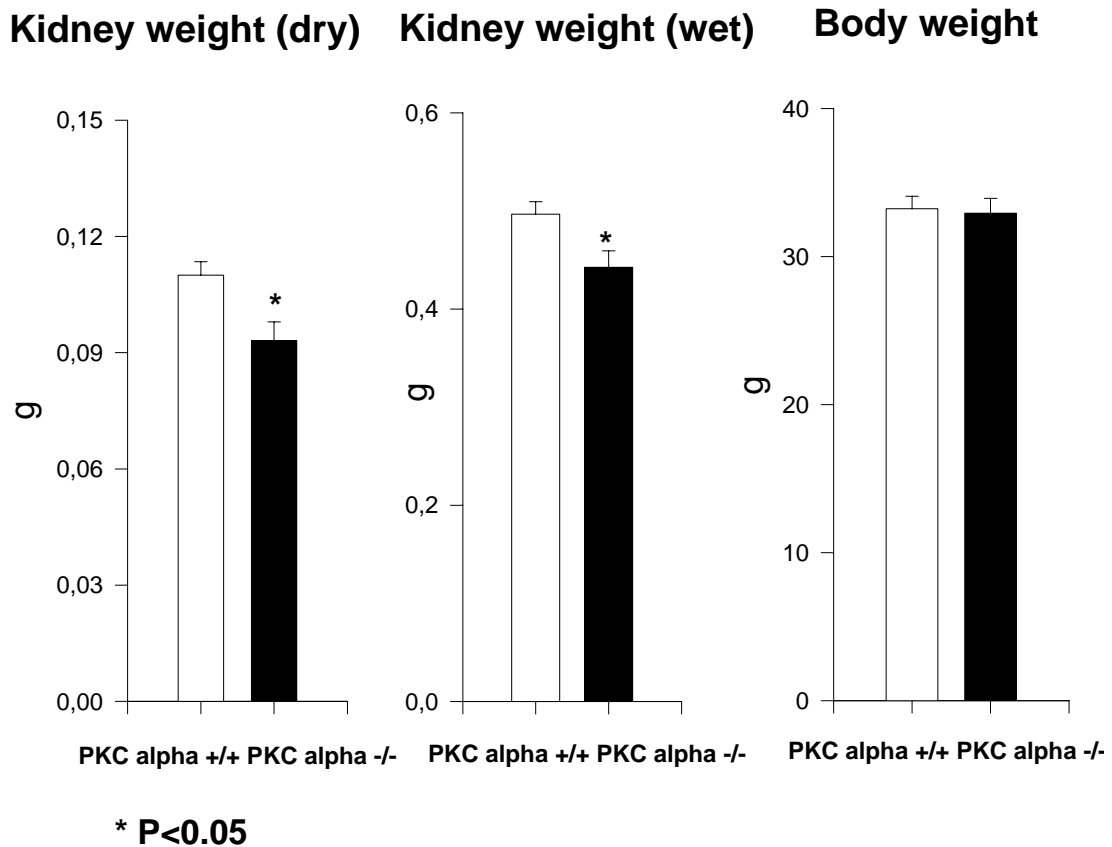


Fig 10: PKC alpha -/- mice showed a lower kidney wet and dry weight than PKC alpha +/+ mice, whereas body weight was not different (n=8 mice/group).

5.3 Response to a sodium-deficient diet.

As illustrated in Figure 11, urinary sodium excretion was not different under standard diet (day 0). Dietary sodium restriction for 6 days also did not reveal

significant differences in urinary sodium excretion or body weight change between groups. The greater urine flow rate and lower urine osmolality persisted in PKC alpha $-/-$ mice over the 6 days sodium restriction (Fig 12).

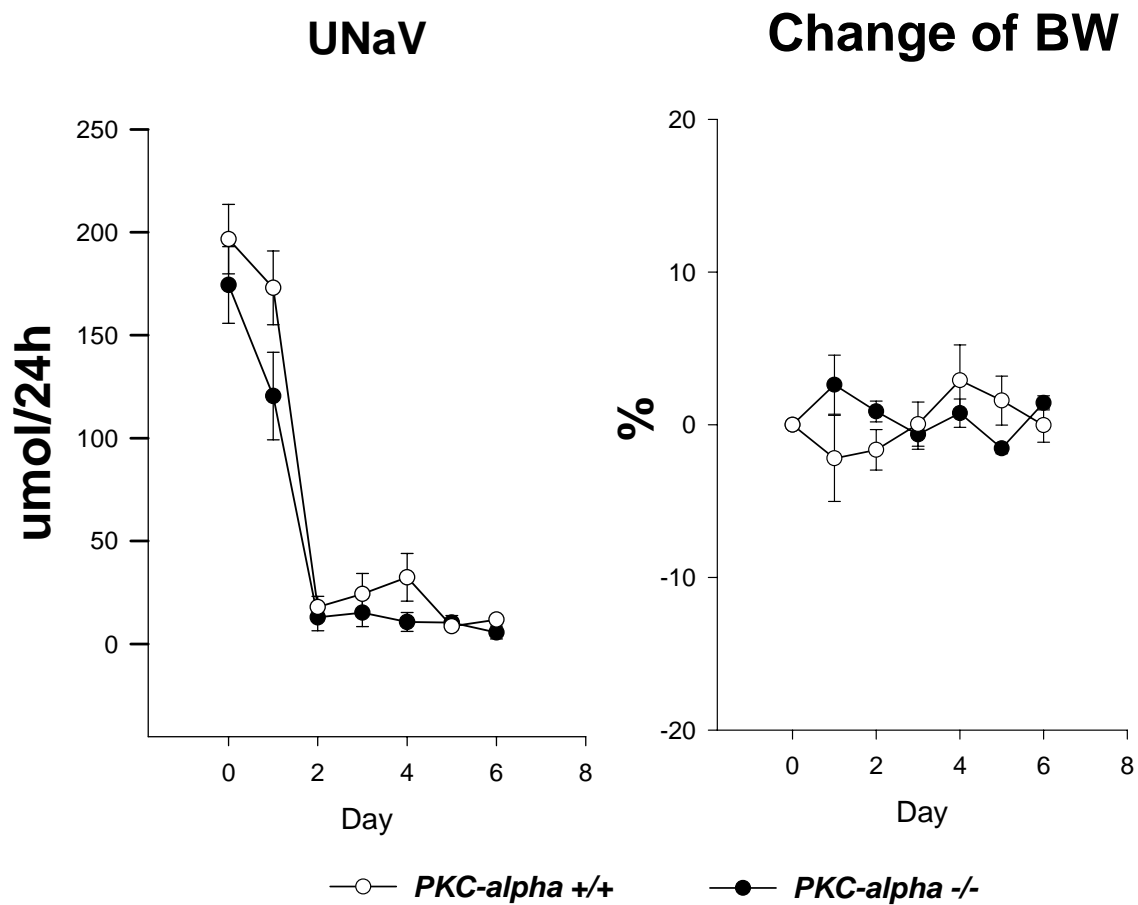


Fig 11: Starting on a zero-NaCl diet after Day 0 for 6 days, PKC alpha $-/-$ mice showed no different response in urinary sodium excretion (UNaV) and body weight (BW) change versus PKC alpha $+/+$ mice ($n=6$ mice/group).

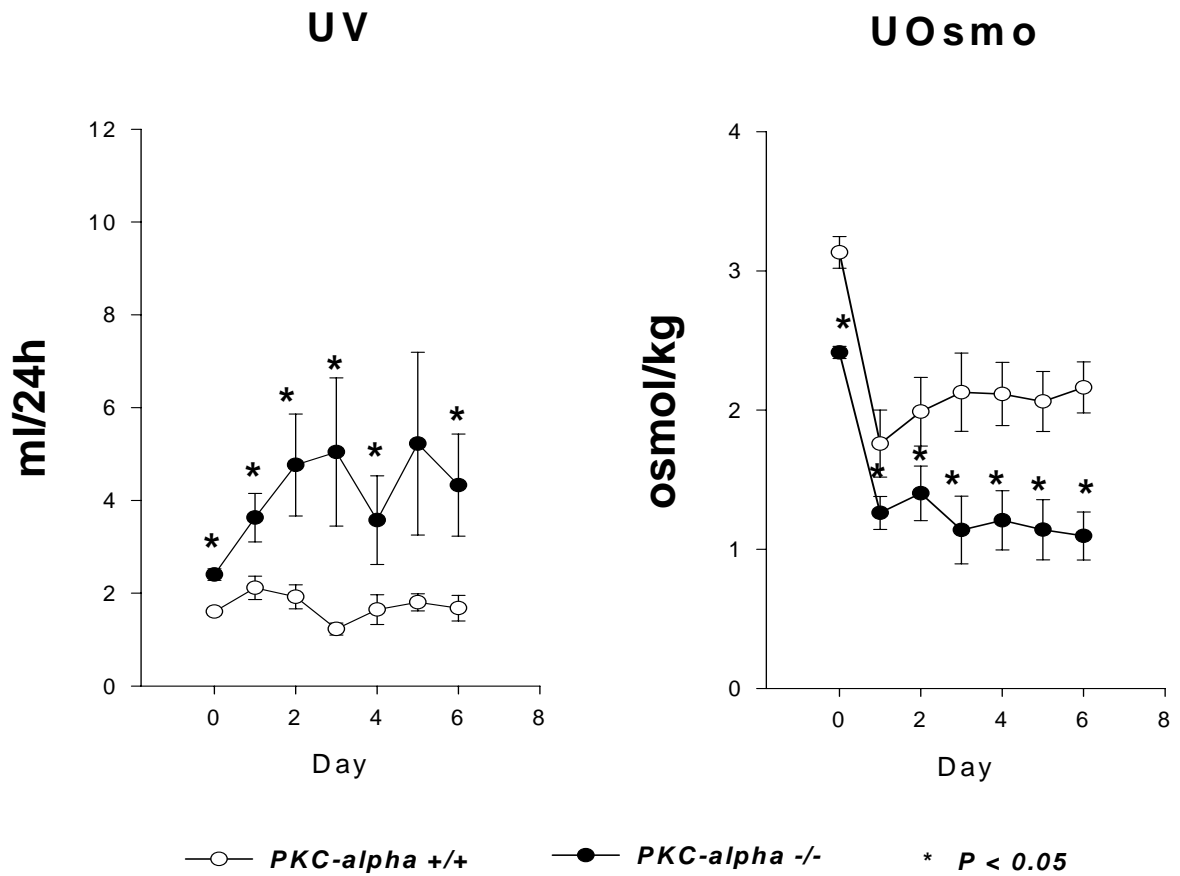


Fig 12: Under standard diet (day 0), *PKC alpha -/-* mice showed a higher urinary flow rate (UV) and lower urinary osmolality versus *PKC alpha +/+* mice; and these differences persisted under 6 days of zero-NaCl diet (n=6 mice/group).

5.4 Response to water deprivation, water loading and vasopressin V2-

receptor blockade.

Withdrawal of water for 36h increased urinary osmolality in both groups but osmolality remained lower in PKC alpha $-/-$ mice compared with PKC alpha $+/+$ mice (see Fig 13). In comparison, acute water loading or application of the V2 receptor antagonist SR121463 lowered urinary osmolality to comparable hypotonic levels in PKC alpha $-/-$ and $+/+$ mice.

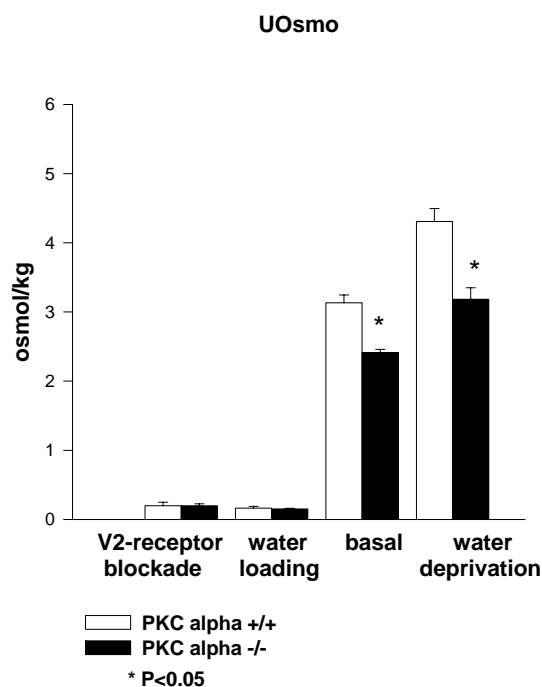


Fig 13: Under conditions of free access to food and water (basal), PKC alpha $-/-$ mice exhibited modestly lower urine osmolality compared with PKC alpha $+/+$ mice. The difference persisted in response to water deprivation but was absent during acute water loading or application of the vasopressin V2 receptor antagonist SR121463 which induced hypotonic urine in both groups (n= 6-8 mice/group).

5.5 Expression of AQP2 protein in inner medulla.

The expression of AQP2 proteins was determined in the kidney inner medulla. The anti-AQP2 antibody recognizes 29 kD and 35 to 50-kD bands corresponding to unglycosylated and glycosylated AQP2, respectively (29, 48). No significant decrease of AQP2 protein expression was noted in PKC alpha $-/-$ mice (Fig 14).

A

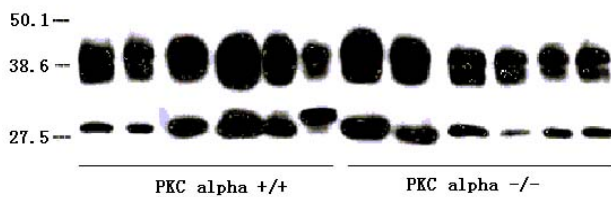
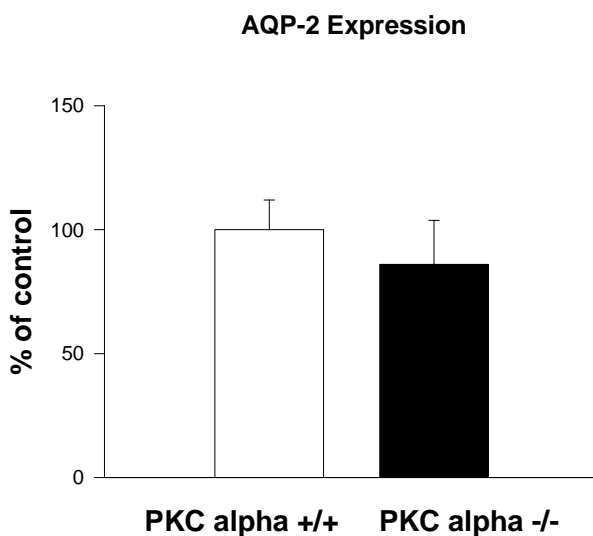


Fig 14: AQP2 in the inner medulla. A: Representative Western blot of AQP2 protein from inner medulla of PKC alpha $+/+$ and $-/-$ mice probed with an antibody to AQP2. The 29-kD represents AQP2 and the 36- to 50- kD band represents AQP2 in glycosylated form. The observed molecular weights were in agreement with the literature. B: summary of densitometric analysis of AQP2 in inner medulla expressed as % of $+/+$ control value. There was no significant difference in the abundance of AQP2 protein in PKC alpha $+/+$ mice compared with PKC alpha $-/-$ mice (n= 6 mice/group).

B



5.6 Immunohistochemical and morphological studies.

Immunohistochemical studies were carried out using 10 μm sections of the kidney. The immunohistochemistry showed comparable abundance of AQP2 labeling in inner medulla of both PKC alpha +/+ and -/- mice (Fig 15). The thickness ratio of inner medulla to the cortex was not different between PKC alpha +/+ and -/- mice (Fig 16).

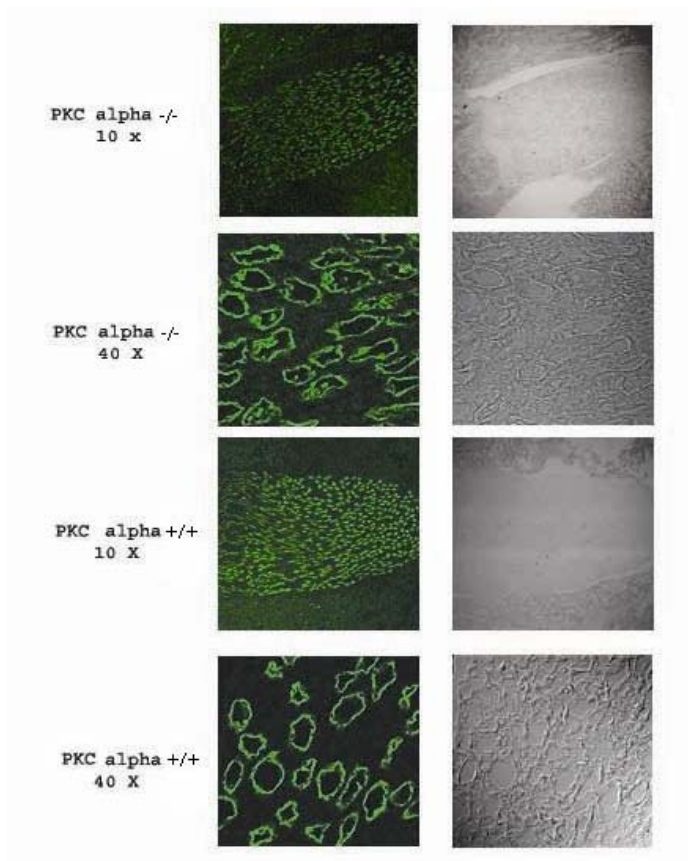


Fig 15: Immunohistochemical examination showed that both PKC alpha +/+ and -/- mice have similar abundance of AQP2 protein expression in kidney inner medulla. Displayed are representative magnifications of 10x and 40x (n=9 mice/group).

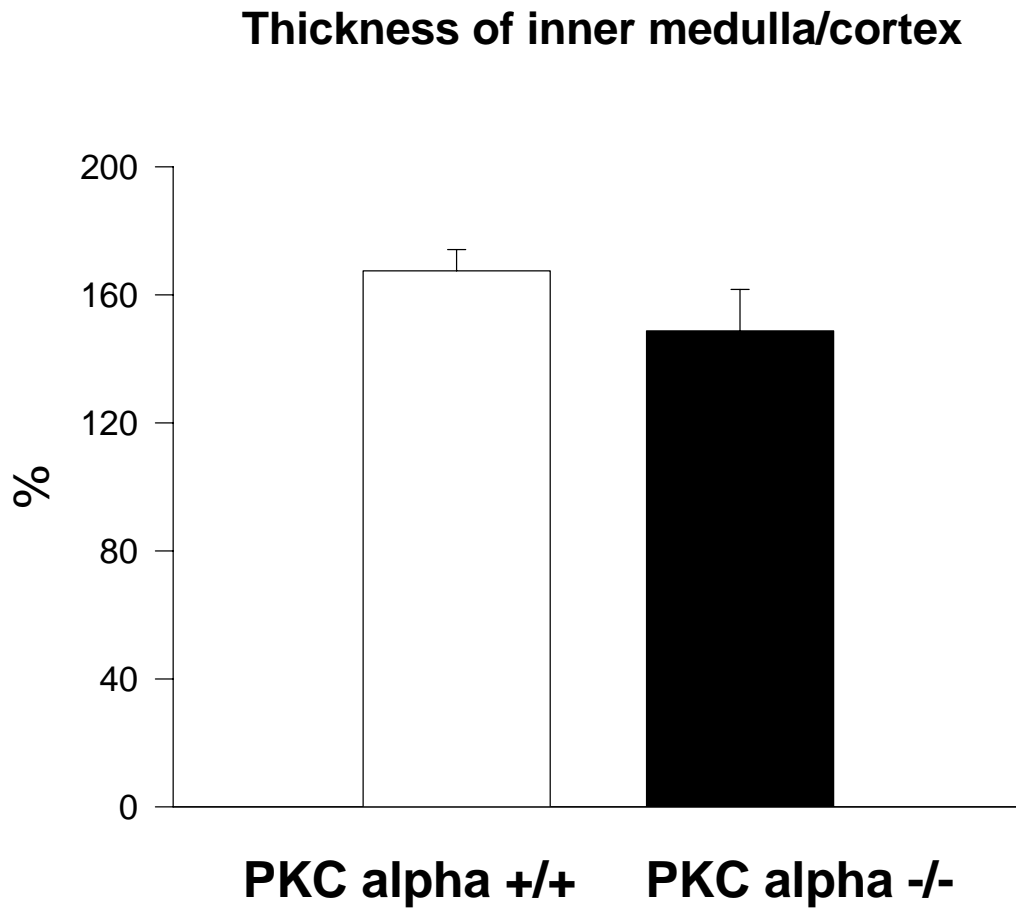


Fig 16: Morphological examination showed that the ratio of the thickness of inner medulla to cortex was not significantly different between PKC alpha -/- and +/+ mice (n=9 mice/group).

6. Discussion

The present experiments show that mice deficient for PKC alpha exhibit modestly greater urinary flow rate and modestly lower urine osmolality under basal conditions, i.e. under conditions of free access to water. The notion that this phenotype is due to intrarenal alterations and particularly impaired water reabsorption in medullary collecting duct is supported by further findings of the present study which are discussed in the following.

The greater urinary flow rate and lower osmolality observed under basal conditions was associated with a tendency for greater plasma osmolality and a greater urinary vasopressin to creatinine ratio as determined from 24 hours urine collection in metabolic cages. This index had been used before as a surrogate parameter for mean plasma vasopressin concentration in mice (1) and was also used in the present study because precise measurements of plasma vasopressin in mice were not possible due to the relatively large volume of plasma which is required for commercial assays and which could not be obtained by decapitation of the mice. A tendency for greater plasma osmolality and a greater urinary vasopressin to creatinine ratio, however, are not compatible with a greater intake of water as the primary cause of greater renal

water excretion. In accordance, lower urinary osmolality persisted in PKC alpha knockout mice during 36h water deprivation. This shows that the phenotype is independent of water intake and also indicates a lower urinary concentrating ability in PKC alpha knockout mice.

Clearance experiments established that the modestly greater urine flow rate in PKC alpha knockout mice was in fact the consequence of reduced fluid reabsorption in the kidney. The question remained which segments of the tubular and collecting duct system contributed to this phenotype? Notably, the dilution of urine was not impaired in PKC alpha $-/-$ mice as indicated by experiments with acute water loading and application of a vasopressin V2 receptor antagonist. Because intact function of the thick ascending limb is required for urine concentration but also hypotonic dilution, these findings argue against a primary defect in PKC alpha knockout mice in this nephron segment. Furthermore, the ability of the kidney to retain sodium in response to dietary sodium restriction was unaffected in PKC alpha knockout mice arguing against a significant defect in the major sodium retaining segments of the nephron including proximal tubule, thick ascending limb, distal tubule and cortical collecting duct. This leaves the medullary collecting duct as the most likely candidate for the defective nephron segment in PKC alpha knockout mice and

this notion is perfectly matched by the renal localization of the isoenzyme in wild type mice: PKC alpha is highly expressed in the medullary collecting duct whereas it is not detectable in proximal tubule, thick ascending limb, distal tubule or principal cells of cortical collecting duct.

The observed modestly lower GFR in PKC alpha knockout mice may be part of a water conserving compensating response in PKC alpha knockout mice. Alternatively, Bankir and colleagues proposed before that vasopressin-mediated urinary concentrating mechanisms are related to GFR with high concentrating activity being related to high GFR and vice versa (7). Thus, the lower GFR observed in PKC alpha knockout mice may be related to an impaired urinary concentrating ability independent of body fluid loss. The modestly lower kidney wet and dry weight, on the other hand, may be the consequence of the lower GFR which requires less absolute salt reabsorption and is in accordance with the preserved GFR to kidney weight ratio.

The morphological examination indicates that the high urine flow rate observed in PKC alpha knockouts is not due to structural changes of the inner medulla. Since AQP2 plays a dominant role in vasopressin-regulated collecting duct water permeability (38), we determined the expression of AQP2 protein in inner

medulla on PKC alpha wild type and knockout mice by semi-quantitative immunoblotting and in addition by immunohistochemistry. These studies provided no evidence for significant differences between PKC alpha knockout and wild type mice.

The mechanism how PKC alpha can potentially contribute to urinary concentration in medullary collecting duct, i.e. what are the target molecules that are phosphorylated and what activates PKC alpha remains to be determined. Interestingly, there are reports from rabbit cortical collecting duct that PKC in this nephron segment can mediate an inhibitory influence on water channels and transport (3,18), i.e. an influence which is opposite to the one proposed here for PKC alpha in mouse medullary collecting duct. On the other hand, a study by Kato et al. in rat terminal inner medullary collecting duct showed that angiotensin II increases vasopressin-stimulated facilitated urea permeability via a PKC-mediated signaling pathway, and the authors proposed that this may play a physiological role in the urinary concentrating mechanism by augmenting the maximal response to vasopressin (25). It remains to be determined whether vasopressin-stimulated facilitated urea permeability is affected in PKC alpha $-/-$ mice.

Notably, neither PKC alpha, beta I, beta II, delta nor epsilon were detected in principal cells of cortical collecting duct in mice and, therefore, the identification and functional characterization of PKC isoenzymes expressed at this site will be of significant interest. Furthermore, it is of interest that beside PKC alpha, the isoenzymes PKC beta I and II, which also belong to the group of cPKC isoenzymes, are likewise expressed in the medullary collecting duct of the mouse. The fact that PKC alpha knockout mice show a phenotype that presumably is localized to the medullary collecting duct indicates a limited ability of cPKC isoenzymes to compensate functionally for each other in vivo even though they are expressed within the same cell.

Beside the medullary collecting duct, PKC alpha in mouse kidney is expressed in intercalated cells of cortical collecting duct and in glomeruli. In the present studies we did not follow up on the potential role of PKC alpha in intercalated cells. Because PKC alpha expression is enhanced in mesangial cells by high glucose and is high in whole rat kidney in response to streptozotocin-induced diabetes mellitus and because there is evidence for a link between glomerular PKC and albuminuria (10, 23, 42), we determined urinary albumin excretion under basal conditions, but could not detect differences between PKC alpha knockout and wild type mice. Whether glomerular PKC alpha plays a role for

albuminuria under pathophysiologic conditions remains to be determined.

In summary, evidence is provided that PKC alpha which is expressed in medullary collecting duct is involved in urinary concentration in mice. The observed phenotype of PKC alpha knockout mice also indicates that the ability of classical PKC isoenzymes expressed in the same nephron segment, like PKC alpha and beta I and beta II in medullary collecting duct, to compensate functionally for each other is limited.

7. Summary

PKC is a family of serine-threonine kinases, which plays an important role in kidney function. It can influence tubular transport, renal hemodynamics, cell growth and differentiation. One of the most abundantly expressed isoenzymes in rat kidney is PKC alpha. In mouse kidney, the classical PKC isoenzyme alpha is expressed in glomeruli, cortical collecting duct (intercalated cells only) and medullary collecting duct. To assess the role of PKC alpha in kidney function, PKC alpha knockout mice (-/-) were studied which had been generated before by Michael Leitges.

First, PKC alpha -/- mice and littermate wild type mice (+/+) were housed in metabolic cages for 24-hour urine collection with free access to water and comparable food intake. PKC alpha -/- mice showed a higher urine output (mean±SE: 2.4±0.1 vs 1.6±0.1 ml/day, n=6 mice/group, P<0.001) and reduced urine osmolality (2.41±0.04 vs 3.13± 0.11 osmol/kg, P<0.001) than PKC alpha +/+ mice despite a greater urinary vasopressin to creatinine ratio in PKC alpha +/+ mice. Under the same conditions, neither hematocrit (52.9±1.0 vs 51.4±1.2 %) and plasma osmolality (322±5 vs 329±1 mosmol/kg) nor urinary sodium excretion (197±17 vs 174±19 µmol/day) or albumin excretion were different

between PKC alpha $+/+$ and $-/-$ mice. Dietary NaCl deprivation for 6 days did not reveal significant differences in body weight or urinary sodium excretion between the two groups although polyuria and lower urine osmolality persisted in PKC alpha $-/-$ mice.

Second, clearance experiments were performed on inactin/ketamine anesthetized mice. PKC alpha $-/-$ mice showed modestly lower glomerular filtration rate and reduced fractional renal fluid reabsorption, accompanied with a lower kidney weight versus PKC alpha $+/+$ mice.

Third, PKC alpha $-/-$ and $+/+$ mice were i.p. injected with the vasopressin V_2 -receptor antagonist SR121463 (1 mg/kg) or orally water-loaded (1 ml/16g body weight). The acute diuresis and the fall in urinary osmolality in response to these two maneuvers were not different between PKC alpha $-/-$ and $+/+$ mice. In comparison, the lower urinary osmolality observed in PKC alpha $-/-$ mice vs. $+/+$ mice under basal conditions persisted during water restriction for 36h.

At last, immunohistochemical and morphological studies were applied on PKC alpha $+/+$ and $-/-$ mice. The expression of aquaporin-2 in inner medulla was not different between these two groups. Morphological examination revealed no

difference in the thickness ratio of inner medulla to cortex between PKC alpha -/- and +/+ mice.

In conclusion PKC alpha appears not to play a major role in renal sodium transport, but contributes to urinary concentrating ability which is consistent with its expression in the medullary collecting duct.

8. References

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