

Piezo1-dependent regulation of urinary osmolarity

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Abstract The collecting duct (CD) is the final segment of the kidney involved in the fine regulation of osmotic and ionic balance. During dehydration, arginine vasopressin (AVP) stimulates the expression and trafficking of aquaporin 2 (AQP2) to the apical membrane of CD principal cells, thereby allowing water reabsorption from the primary urine. Conversely, when the secretion of AVP is lowered, as for instance upon water ingestion or as a consequence of *diabetes insipidus*, the CD remains water impermeable leading to enhanced diuresis and urine dilution. In addition, an AVP-independent mechanism of urine dilution is also at play when fasting. Piezo1/2 are recently discovered essential components of the non-selective mechanically activated cationic channels. Using quantitative PCR analysis and taking advantage of a β -galactosidase reporter mouse, we demonstrate that Piezo1 is preferentially expressed in CD principal cells of the inner medulla at the adult stage, unlike Piezo2. Remarkably, siRNAs knock-down or conditional genetic deletion of Piezo1

specifically in renal cells fully suppresses activity of the stretch-activated non-selective cationic channels (SACs). Piezo1 in CD cells is dispensable for urine concentration upon dehydration. However, urinary dilution and decrease in urea concentration following rehydration are both significantly delayed in the absence of Piezo1. Moreover, decreases in urine osmolarity and urea concentration associated with fasting are fully impaired upon Piezo1 deletion in CD cells. Altogether, these findings indicate that Piezo1 is critically required for SAC activity in CD principal cells and is implicated in urinary osmoregulation.

Keywords Ion channels · Mechanotransduction · Kidney · Urine · Urea · Collecting duct

Introduction

During dehydration, increased blood concentration triggers the release of arginine vasopressin (AVP, antidiuretic hormone) from the posterior pituitary gland, causing an increase in water reabsorption from the primary urine [1, 14]. Luminal water enters the collecting duct (CD) principal cells through apical aquaporin 2 (AQP2), traverses the cytosol, and exits at the basal side into the interstitium via aquaporin 3 (AQP3) and aquaporin 4 (AQP4). The trafficking of AQP2 to the apical membrane of CD cells is under the tight regulation of the cAMP/PKA/phosphorylation pathway, downstream of the AVP receptor [1]. Moreover, long-term regulation of AQP2 expression at the messenger RNA (mRNA) and protein levels is also at play in CD principal cells [19]. The passive water movement through the CD is driven by the tubule lumen interstitium osmotic gradient [1, 14]. Conversely, when plasma is diluted, AVP secretion is inhibited, and the CD becomes impermeable to water. As a consequence,

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diuresis and urine dilution occur. Fasting also downregulates AQP2 in the medulla, thereby leading to polyuria [2, 45]. This effect is maintained even in the absence of endogenous AVP, as observed in Brattleboro rats [45].

Recently, Piezo1 and Piezo2 have been shown to be essential components of distinct mechanically activated cationic channels in mammalian cells [10]. In the adult mouse, Piezo1 is highly expressed in the lung, as well as in the bladder and kidney [10, 34]. In contrast, Piezo2 expression is very low in the kidney, while it is remarkably high in sensory dorsal root ganglion neurons [10, 34]. Stretch-activated non-selective cationic channel (SAC) activity (with a reversal potential near 0 mV and single channel conductance of about 35 pS) was observed upon heterologous expression of Piezo1 in response to mechanical stress, including cell poking, membrane stretch, substrate deflexion, or fluid flow [5, 10, 18, 28, 34, 36, 38, 39]. Remarkably, both Piezo isoforms show pronounced inactivation during steady mechanical stimulation [10, 18]. Importantly, bilayer reconstitution experiments demonstrated that purified Piezo1 proteins are pore-forming subunits [12]. Very recently, the 3-D structure of mPiezo1 has been resolved by cryo-electron microscopy [17]. The channel appears to be a trimer, with the extracellular domain resembling three mobile peripheral blades and a central cap. Moreover, recent structure-function analysis indicates that Piezo1 ion channel pore properties are dictated by its carboxy-terminal region [11].

Piezo1 plays an important role during early development, and its constitutive genetic deletion is embryonic lethal [28, 38]. Notably, specific Piezo1 knock-out in the endothelium profoundly alters its sensitivity to shear stress, as well as the vascular architecture [28]. In addition, we have recently demonstrated that in adult mice, Piezo1 opening in smooth muscle cells of resistance arteries is involved in hypertension-dependent arterial remodeling, while it is dispensable for the myogenic response [41]. Gain-of-function mutations in hPiezo1 are associated with xerocytosis, an autosomal dominant hemolytic anemia characterized by dehydration of erythrocytes [3, 4, 21, 46]. These findings suggest that Piezo1 plays an important role in maintaining erythrocyte volume homeostasis and links mechanical forces to red blood cell volume [7]. Conversely, loss-of-function mutations in hPiezo1 lead to the autosomal recessive congenital lymphatic dysplasia, causing persistent lymphoedema [15, 29]. Whether arterial and/or renal dysfunctions are associated with these genetic mutations has not yet been explored.

Kidney epithelial cells respond to both changes in fluid flow and intraluminal pressure [33, 44]. Bending of the primary cilia, at the apical side of tubular epithelial cells, plays a key role in flow sensing [25, 30, 37]. Moreover, rhythmic papillary contractions result in the repetitive stretching of both apical and basolateral membranes [23]. Of note, intraluminal

pressure can also be abnormally elevated in various obstructive kidney diseases, as well as in polycystic kidney disease [33]. Thus, mechanotransduction of tubular epithelial cells is relevant to both physiological and pathological renal conditions and involves shear, as well as pressure stress [33]. In mice, we previously reported the evidence for non-selective SACs, as well as stretch-activated K^+ channels in renal tubular epithelial cells [34, 35]. However, the functional role of mechanosensitive ion channels in the adult kidney remains poorly understood.

In the present study, we demonstrate that Piezo1 is preferentially expressed in the inner medulla of adult mice, where it is critically required for SAC activity in CD cells. Moreover, deletion of Piezo1 in renal epithelial cells impacts on the regulation of urine osmolarity.

Material and methods

IMCD-3 cell culture

The mouse IMCD-3 cell line was cultured in DMEM/F12 medium (Gibco BRL Life Technologies), supplemented with 100 IU/ml penicillin, 100 IU/ml streptomycin and 10 % fetal calf serum (Hyclone) at 37 °C in a humidified 5 % CO_2 atmosphere. Cells were transfected with small interfering RNAs (siRNAs) directed against Piezo1, that have been previously validated (initially called siRNA1 and siRNA3) in [10], using the HiPerFect Transfection Reagent (Qiagen SA, Courtaboeuf, France) according to the manufacturer instructions. After 24 h of transfection, cells were subjected to reverse transcription polymerase chain reaction (RT-PCR) analysis to confirm the invalidation of Piezo1.

Generation of mice with conditional renal epithelium Piezo1 gene deficiency

A transgenic mouse line expressing Cre recombinase in renal epithelial cells under the control of tamoxifen (TAM)-inducible Ksp-cadherin (KspCre*) promoter was generated as previously reported [26]. The mouse with floxed Piezo gene alleles was a gift from Dr. A. Patapoutian at Howard Hughes Medical Institute, La Jolla, CA, USA (see [38, 41] for further information). Crossing KspCre* mice with Piezo1^{lox/lox} mice generates KspCre* Piezo1^{lox/lox} mice with the Piezo1 gene deleted in the CD, but also in the connecting and distal tubules after induction with tamoxifen. Their littermates KspCre* or Piezo1^{lox/lox} genotype were used as controls. Since no significant difference was observed between both lines, data were merged (control). The genetic background was C57BL/6 J and the genotyping was performed as previously described [41]. Eight-week-old adult male mice were injected once per day for 4 days

intraperitoneally with TAM (50 mg/kg/day dissolved in peanut oil, EtOH 10 %). Mice were studied 6 weeks after TAM injection. To determine the deletion of Piezo1, the inner medulla and cortex were dissected from both KspCre* Piezo1^{lox/lox} and control mice and Piezo1 expression was quantified by real-time PCR. In addition, a KspCre* Piezo1^{del/lox} mouse model was used, in which one Piezo1 allele was constitutively deleted (del) and the other Piezo1 allele was floxed [41].

All experiments were performed according to policies on the care and use of laboratory animals of the European Community Legislation. The study was approved by the local committee for ethical and safety issues (CIEPAL-Azur). All efforts were made to minimize animal suffering and reduce the number of animals used. The animals were housed under controlled laboratory conditions with a 12-h dark–light cycle, a temperature of 21 ± 2 °C, and a humidity of 60–70 % and had free access to standard rodent diet and tap water (unless specified). For water and food deprivation challenges, mice were individually housed in metabolic cages (Tecniplast, Italy) on a 12-h light/12-h dark cycle. After 3 days of acclimatization, body weight, urinary volume, food intake, and water absorption were measured for the next 2 days, considered as baseline. Anesthesia was used for all invasive procedures, as indicated in specific sections.

Urine biochemistry

Urine samples were centrifuged immediately at 2000g for 10 min and frozen until assayed. Creatinine and urea nitrogen were measured using RANDOX kits following the manufacturers' instructions. Measure absorbances were performed using a Thermo Scientific™ Multiskan™ FC Microplate Photometer. Osmolalities were measured using the Vapro® vapor pressure osmometer (Wescor Inc.), an electronic adaptation of the hygrometric method of vapor pressure determination.

RNA isolation, real-time RT-PCR, and Western blots

Cortex and inner medulla tissues from adult control and KspCre* Piezo1^{lox/lox} were dissected and immediately snap-frozen in liquid nitrogen or immersed in RNAlater solution (Qiagen) for stabilization of RNA. Total tissues were stored at -80 °C until extraction. Each sample was simultaneously homogenized using a bead-based Precellys Evolution instrument (Bertin, France) in separate plastic tubes. Total RNA was extracted following the manufacturers' instructions of the miRNeasy kit (Qiagen) processed by the robotic workstation QIAcube (Qiagen). Complementary DNAs (cDNAs) were synthesized using the SuperScript® VILO™ Master Mix (Invitrogen). The cDNAs were amplified using specific primers for Piezo1, Piezo2, and the reference gene topoisomerase 1 (TOP1) [34] in a LightCycler 480 system (Roche). C_T

were estimated by the manufacture software release 1.5.0. For each sample, the relative expression of each gene *versus* TOP1 ($2^{-\Delta C_T}$) was calculated and then averaged.

Western blots were performed as previously described [40]. Antibodies against AQP2 and urea transporter A1 (UT-A1) were from Novus Biologicals (NB110-74682 1/2000 and UTA1 Abcam ab95365 1/1000, respectively).

Frozen sections and lacZ/aquaporin 2 stainings

The kidneys were dissected away, decapsulated, and fixed in 4 % paraformaldehyde in Dulbecco's Phosphate Buffered Saline (DPBS; Gibco BRL Life Technologies) for 48 h. After washing the fixative solution with PBS, the kidneys were incubated with 18 % sucrose for 4 h at 4 °C. Tissues were embedded in OCT compound (Tissue-Tek, USA) and frozen at -80 °C. Serial cryosections were cut at 5 μ m thickness and collected onto Superfrost™ Plus microscope slides. LacZ staining was performed following the manufacturers' instructions of the β -Galactosidase Reporter Gene Staining Kit (Sigma) at 37 °C overnight. Sections were counter stained in 0.25 % eosin solution. The sections were observed under a Zeiss Axio Observer fluorescence microscope. Aquaporin 2 protein expression was visualized using standard immunofluorescence protocols and a goat polyclonal AB (sc-9882) from Santa Cruz Biotechnology (Texas, USA).

Electrophysiology

Electrophysiological procedure has been previously described elsewhere [35, 43]. Briefly, single channel cell-attached patch clamp recordings were performed on isolated renal tubules (mean pipette resistance of 4.0 M Ω), isolated mouse CD cells, or immortalized IMCD-3 cell line (mean pipette resistance of 1.4 M Ω). For recordings on isolated renal tubules the pipette medium contained the following (in mM): NaCl 150, KCl 5, CaCl₂ 2, and HEPES 10 (pH 7.4 with NaOH). The pipette solution also contained 10 mM TEA, 5 mM 4AP, and 10 μ M glibenclamide to inhibit eventual contaminating K⁺ channels. The bath medium contained the following (in mM): KCl 155, EGTA 5, MgCl₂ 3, and HEPES 10 (pH 7.2 with KOH). The osmolarity of all solutions was adjusted to 310 mOsm. For recordings on isolated mouse CD cells or IMCD-3 cell line the pipette medium contained the following (in mM): 300 NaCl, 5 KCl, 2 CaCl₂, 10 HEPES, 600 Urea, and 1 MgCl₂ (pH 7.4 with NaOH). Osmolarity of the solution was adjusted to 1200 mOsm. The pipette solution also contained 10 mM TEA, 5 mM 4-AP, and 10 μ M glibenclamide to inhibit eventual contaminating K⁺ channels. The bath medium contained the following (in mM): 150 NaCl, 155 KCl, 2 CaCl₂, 10 HEPES, 600 Urea, and 1 MgCl₂ (pH 7.4 with NaOH). The osmolarity of all solutions was adjusted to 1200 mOsm.

Membrane patches were stimulated with 300 ms long negative pressure pulses of -10 mmHg increments with a period of 3 s, through the recording electrode using a fast pressure clamp device (ALA High Speed Pressure Clamp-1 system, ALA-scientific). The holding voltage for all experiments was -80 mV for SACs recordings, unless otherwise indicated.

The experiments were carried out at room temperature. Currents were filtered at 1 kHz, digitized at 10 kHz and analyzed with the pCLAMP9.2 (Axon Instruments) and ORIGIN8.5 (Microcal, Northampton, MA) softwares. Pressure-effect curves were fitted with a Boltzmann function. Single channel analysis was performed with the Single Channel Detection and Analysis mode of pCLAMP9.2 (Axon Instruments).

Isolation of medulla renal tubules

C57BL/6 J mice (8 weeks old, 15–20 g body weight; Charles River, l'Arbresle, France) were anesthetized using xylazine (Rompun®) 8 mg/kg and ketamine 1000 (Virbac) 80 mg/kg. The kidneys were perfused via the heart with 15 ml of L-15 Leibovitz medium (Sigma, Saint Quentin Fallavier, France) supplemented with collagenase (CLS II, Worthington; 300 U/ml) and removed, as previously described [34]. Small pieces of the medulla were incubated at 37 °C for 20–50 min in the same collagenase-containing medium, rinsed, and kept at 4 °C. Tubular fragments were dissected out under a stereomicroscope just before use and transferred to a petri dish placed on the stage of an inverted microscope (Axiovert 40, Zeiss) for patch recordings.

Isolation of inner medulla cells

Anesthetized mice (xylazine (Rompun®) 8 mg/kg and ketamine 1000 (Virbac) 80 mg/kg) were perfused with 15 ml Leibovitz L15 medium (Sigma) containing 300 U/ml collagenase II (Worthington) by intracardiac perfusion. The kidneys were removed, the inner medulla dissected and cut into small pieces. They were then incubated for 3 h at room temperature with agitation in freshly prepared IMCD media (DMEM/F12 (1:1); 10 % FBS; adjusted to 900 mOsmol/l with mannitol) containing in addition 300 U/ml collagenase type 1 (Worthington); 300 U/ml collagenase type 2 (Worthington). Digested inner medulla tubules were rinsed twice on ice with IMCD media and dissociated with the help of a P1000 micropipette. Tubules were transferred to 35-mm petri dishes coated with rat tail Collagen I (Invitrogen) and filled with IMCD media. Cultures were maintained at 37 °C in a 5 % CO₂-95 % air water-saturated atmosphere. The medium was removed 4 days after seeding and then every 2 days.

Statistical analysis

For quantitative PCR and metabolic cage experiments, control and KspCre* Piezo1^{lox/lox} mice were compared by non-parametric *t* test followed by the Mann–Whitney test. For patch clamp experiments, peak currents were measured and significance of the differences was tested with a permutation test (R Development Core Team: <http://www.r-project.org/>) ($n < 30$) or two sample *t* test ($n > 30$). One star indicates $p < 0.05$, two stars $p < 0.01$, and three stars $p < 0.001$. Data represent mean \pm standard error of the mean. The number of independent times each experiment has been repeated (n) is indicated throughout the manuscript and is shown in figures.

Results

Piezo1 is preferentially expressed in mouse CD principal cells of the inner medulla at the adult stage

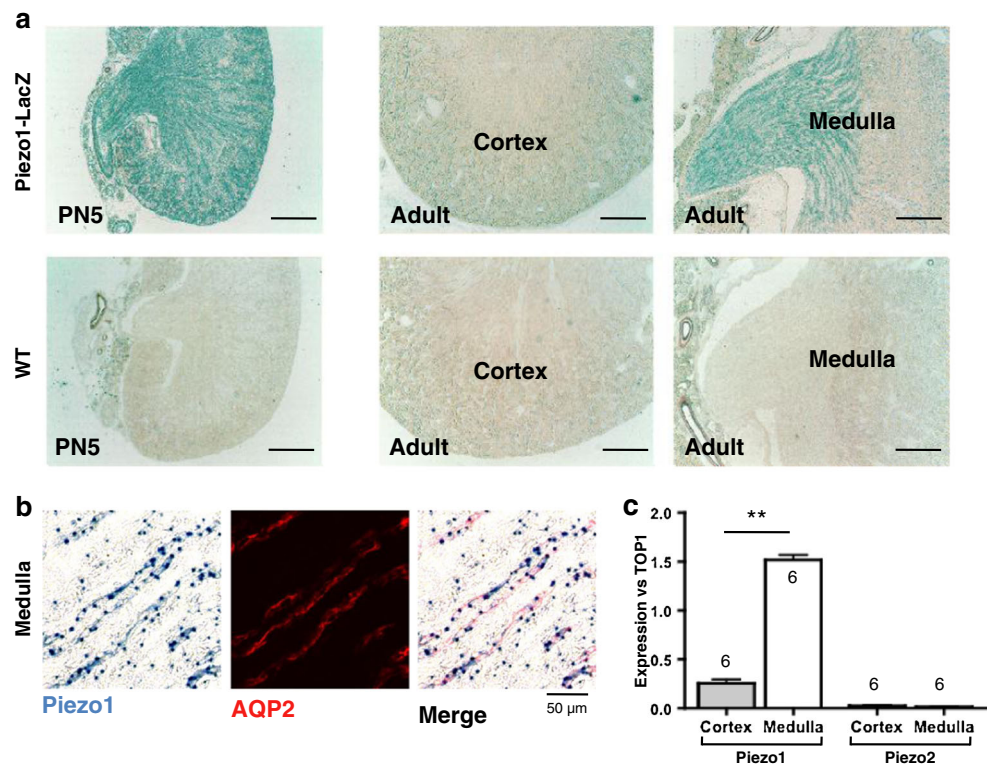
We took advantage of a LacZ reporter mouse line [38, 41] to visualize Piezo1 expression in the kidney from post-natal development to adulthood. Piezo1 expression (as visualized by LacZ staining in blue) was present both in the cortical and medullar region of the kidney at post-natal day 5 (Fig. 1a). At the adult stage, Piezo1 expression became more restricted to the inner medulla and co-localized with AQP2, a marker of CD principal cells (Fig. 1b). Of note, this pattern of expression from after birth to adulthood also corresponds to the capacity of the mice to concentrate urine.

Predominant mRNA expression of Piezo1 in the inner medulla (*vs* cortex) of adult mice was confirmed through quantitative PCR (qPCR) analysis (Fig. 1c). In contrast, Piezo2 expression was negligible in both the cortex and medulla (Fig. 1c). Thus, Piezo1 is preferentially expressed in mouse CD epithelial cells at the adult stage. Next, using transfection of siRNAs in inner medullary collecting duct cells, we explored the link between Piezo1 and SACs.

SAC activity in the mouse IMCD-3 cell line is critically dependent on Piezo1

Cell-attached patch clamp recordings were performed on cultured IMCD-3 cells at a holding potential of -80 mV. Pressure steps of increasing amplitude were applied at the back of the patch pipette using a high-speed pressure clamp system (Fig. 2a). SAC currents with a reversal potential of about 0 mV were observed upon pressure stimulation with a threshold of about -20 mmHg (Fig. 2a, b). Channel opening saturated at the higher pressure values and the pressure-effect curve could be described with a Boltzmann function ($P_{0.5} = -36.4 \pm 0.5$ mmHg, $k = 7.5 \pm 0.5$, $n = 35$) (Fig. 2b). Notably, SAC currents were non-inactivating and deactivation

Fig. 1 Expression of Piezo1 in mouse kidney during post-natal development and adulthood. **a** β -Galactosidase (shown in blue) reporter mouse (Piezo1-LacZ) was used to visualize Piezo1 expression in kidney sections of new born (post-natal day 5; *top left panels*) and adult (*top middle and right panels*) mice. The comparison is made with their wild-type littermates (WT, *bottom panels*). Scale bars: 500 μ m in post-natal day 5; 1 mm in adult. **b** Piezo1 expression (Piezo1-LacZ in blue) and aquaporin 2 (AQP2, in red) co-staining on a kidney section from an adult Piezo1 lacZ mouse. **c** mRNA expression of Piezo1 and Piezo2 normalized to topoisomerase1 (TOP1) in isolated cortex and inner medulla of adult WT mice ($n = 6$). Data are presented as mean \pm SEM. ****** $p < 0.01$



was remarkably slow (Fig. 2a). When IMCD-3 cells were transfected with siRNAs directed against Piezo1, SAC currents were dramatically reduced, as compared to control non-targeting (NT) siRNAs (Fig. 2a, b). Thus, SACs in IMCD-3 cells are critically dependent on Piezo1. Next, using a conditional renal specific knock-out mouse model, we further investigated the functional role of Piezo1 in CD epithelial cells.

Piezo1-dependent SACs in mouse CD epithelial cells

Cell-attached patch clamp recordings were performed at the basolateral side of isolated medullary collecting ducts (Fig. 3a). Stepwise pulses of increasing negative pressure were applied at a holding of -80 mV. Large SAC currents were observed in response to pressure stimulation, again lacking inactivation and showing a slow deactivation (Fig. 3a). These non-selective SAC currents reversed at a holding potential of 0 mV (Fig. S1).

We used an inducible system (KspCre* Piezo1^{lox/lox}) to conditionally and selectively delete Piezo1 in the kidney upon tamoxifen (TAM) injection at the adult stage. The KspCre* system allows excision of floxed alleles in the CD, but also in the connecting and distal tubules [26]. Six weeks after TAM induction, we observed a significant reduction of Piezo1 transcript expression in the inner medulla tissue, without a compensatory increase of Piezo2 expression (Fig. 3b). However, this decrease was only partial, even when increasing TAM

concentration, number of injections, or increasing delay after induction (not shown). These findings suggest that either deletion is incomplete and/or that some cells in the CD do not express the active Cre* recombinase (i.e., are Ksp negative) or fail to be activated by TAM due to limited drug diffusion. Another possibility might be the contribution of other cell type positives for Piezo1, such as vascular cells [28, 38, 41]. SAC currents in isolated CD cells from control animals were comparable to those recorded on whole tubules (Fig. 3c). Remarkably, when KspCre* was induced by TAM injections, SAC activity completely disappeared in CD cells within the whole pressure range studied, despite only a partial mRNA deletion (Fig. 3b–d). These findings indicate either that a partial deletion of Piezo1 is sufficient to ablate its function at the plasma membrane or that cells lacking KspCre expression were lost during the cell isolation/culture procedure. Altogether, these findings further indicate that Piezo1 is critically required for SAC activity in CD principal cells (Figs. 2 and 3). Next, we investigated whether Piezo1/SAC activity in CD epithelial cells might influence the regulation of water balance.

Piezo1 influences urinary dilution and urea concentration following dehydration

Piezo1 deletion in CD cells did not affect the effect of drinking restriction on both urine output and osmolarity (Figs. 4a and S2). However, when drinking water was

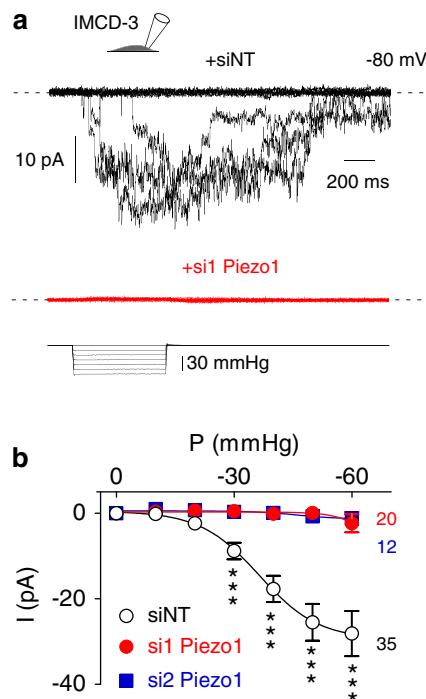


Fig. 2 SACs in IMCD-3 cells depend on Piezo1. **a** Cell-attached patch clamp recordings at a holding potential of -80 mV were performed on cultured IMCD-3 cells. Zero current is indicated by a dashed line, and inward SAC currents (top traces) are elicited in the control condition (siNT) at increased negative pressure. Pressure pulses of increasing magnitude were applied at the back of the patch pipette using a fast pressure clamp system (bottom trace). Cells were either transfected with non-targeting siRNAs (siNT) or siRNAs directed against Piezo1 (si1 Piezo1 shown in red). **b** Pressure-effect curves for SAC currents in siNT, si1 Piezo1, and si2 Piezo1. Data are presented as mean \pm SEM. Number of patches is indicated

added back, the rapid dilution of urine which occurred within 1 hour in control mice, was significantly delayed in the KspCre* Piezo1^{lox/lox} mice. Indeed, osmolarity and urea concentration were significantly higher in the KspCre* Piezo1^{lox/lox} mice, although water intake and urine production were not significantly different (Figs. 4 and S2). However, 24 h after resuming drinking, urine osmolarity was fully recovered in the KspCre* Piezo1^{lox/lox} mice (Figs. 4a, b, S2, and S3). Thus, Piezo1 influences the acute recovery of urine osmolarity (i.e., dilution) after water deprivation. Next, we investigated whether urine dilution associated with food deprivation might be similarly modulated by Piezo1.

Urinary dilution during fasting is impaired in the absence of Piezo1

We studied the influence of Piezo1 on urine dilution caused by fasting for 24 h (Figs. 5, S4, and S5) [2, 45]. In this model, urine dilution has been shown to be independent of AVP [45]. At baseline (i.e., when mice had free access to food and

water), no significant difference was seen in urine osmolarity or urea concentration (Fig. 5a, b). However, fasting (with water available) for 24 h induced a significant urinary dilution and lowered urea concentration in control mice, as previously reported [2, 45]. Remarkably, this fasting-induced urine dilution and decrease in urea concentration were lost in the KspCre* Piezo1^{lox/lox} mice (Figs. 5, S4, and S5).

In addition, we used a KspCre* Piezo1^{del/lox} mouse model in which one Piezo1 allele was constitutively deleted and the other one was floxed, in view of improving Piezo1 knock-down in the kidney [41]. At 6 weeks post-TAM induction, the level of Piezo1 mRNA expression in the inner medulla, as determined by qPCR, showed a more substantial decrease (although not yet total), as compared to the KspCre* Piezo1^{lox/lox} mouse line (Figs. 3a and 6a). These findings suggest a rather limited accessibility of the Cre recombinase to the floxed Piezo1 locus in renal cells. Of note, AQP2 or UT-A1/UT-A3 mRNA expressions were not significantly altered upon deletion of Piezo1 (Fig. 6b, c). In line with these findings, Western blot analysis revealed no difference in total AQP2 or UT-A1 protein expression in the inner medulla when Piezo1 was deleted in the TAM-injected KspCre* Piezo1^{del/lox} (Figs. S6 and S7).

Again, in the KspCre* Piezo1^{del/lox} mice, we observed a significant delay in urine dilution following rehydration, confirming our earlier findings obtained with the KspCre* Piezo1^{lox/lox} mouse line (Fig. 6d).

Thus, Piezo1/SACs in CD epithelial cells influences the mechanisms of urine dilution and urea transport, either following water deprivation or fasting.

Discussion

Our findings indicate that Piezo1 is critically required for SAC activity in CD tubular epithelial cells. Strikingly, native SACs in CD cells lack inactivation, although Piezo1 shows a prominent inactivation upon heterologous expression in a variety of cell types [10, 34]. Whether the lack of inactivation might be explained by a Piezo1 variant, an interaction with a partner or is due to the specific visco-elastic properties of the renal cells is unknown at this stage. This unconventional behavior indicates that Piezo1 is likely to be involved in the response to both acute and chronic mechanical stimuli (i.e., rhythmic pelvic contractions and steady shear stress).

We demonstrate that Piezo1 influences urine dilution and urea concentration following dehydration (in accordance with its prominent expression in inner medulla where maximal urinary concentrating ability takes place). Several potential mechanisms might explain our results:

1. Total AQP2 protein expression in the medulla is independent of Piezo1. However, the retrieval of phosphorylated

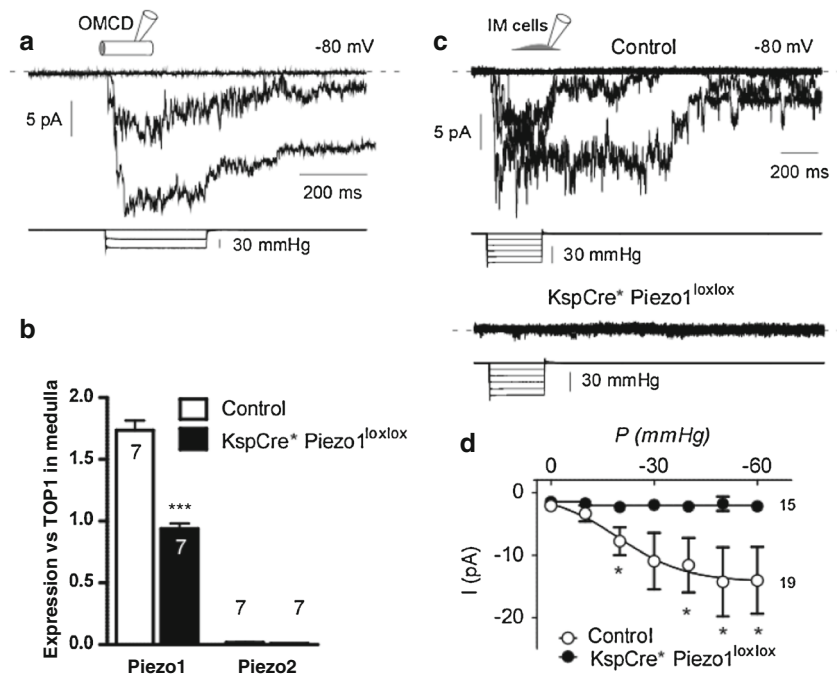


Fig. 3 Piezo1-dependent SACs in mouse CD epithelial cells. **a** Cell-attached patch clamp recordings at the basal side of an isolated outer medulla collecting duct with a holding potential of -80 mV. Zero current is indicated by a dashed line, and inward SAC currents are elicited in the control condition at increased negative pressure (top trace). Pressure pulses of increasing magnitude were applied at the back of the patch pipette using a fast pressure clamp system (bottom trace). **b** Piezo1 and Piezo2 ($n=7$) relative (to TOP1) mRNA expression in

inner medulla tissue of control (white bars) and knock-out (KspCre* Piezo1^{lox/lox}, black bars) mice. *** $p < 0.001$ vs. control kidney tissues. **c** SAC currents in freshly isolated cells from inner medulla of control and knock-out (KspCre* Piezo1^{lox/lox}) mice. **d** Pressure-effect curves for SAC currents in control, and Piezo1 knock-out inner medulla isolated cells (KspCre* Piezo1^{lox/lox}). Data are presented as mean \pm SEM. Number of cells is indicated. * $p < 0.05$

AQP2 from the apical side of CD principal cells upon rehydration might be delayed in the absence of Piezo1. AVP stimulation leads to the swelling of principal CD cells that might contribute to the opening of Piezo1 [9, 27]. Moreover, when urine becomes diluted during rehydration, CD cells are also known to swell [24].

The calcium entry through Piezo1 in swollen CD cells might decrease the production of cAMP via inhibition of adenylyl cyclase 6 and increase hydrolysis of cAMP by stimulation of the phosphodiesterase PDE1, thereby speeding up the retrieval of AQP2 from the plasma membrane. Conversely, in the absence of

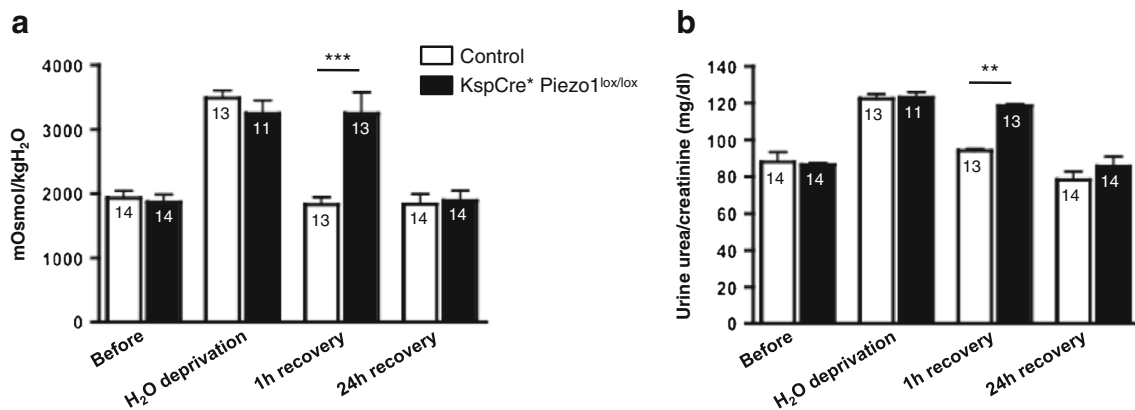
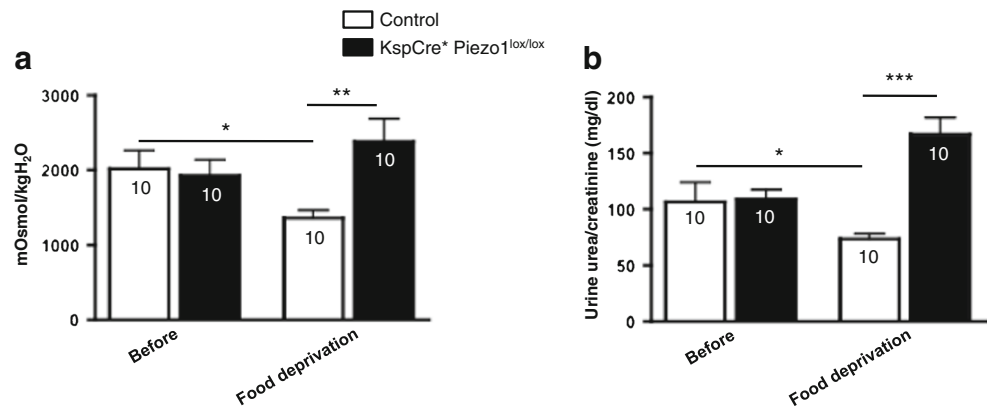


Fig. 4 Piezo1 in CD epithelial cells influences mouse urine osmolarity and urea concentration following water deprivation. **a** Quantitative analysis of metabolic cage experiments for urine osmolarity before, during, and after (1 and 24 h) drinking deprivation in control (white

bars) and knock-out (KspCre* Piezo1^{lox/lox}, black bars) mice. **b** Urine urea concentration. Data are presented as mean \pm SEM. Number of mice is indicated in the bar graphs. ** $p < 0.01$; *** $p < 0.001$

Fig. 5 Piezo1 in CD epithelial cells influences mouse urine osmolarity and urea concentration following fasting. **a** Quantitative analysis of metabolic cage experiments for urine osmolarity before and after fasting for 24 h in control (*white bars*) and knock-out (*KspCre* Piezo1^{lox/lox}*, *black bars*) mice. **b** Urine urea concentration. Data are presented as mean \pm SEM. Number is mice are indicated in the bar graphs. ****** $p < 0.01$



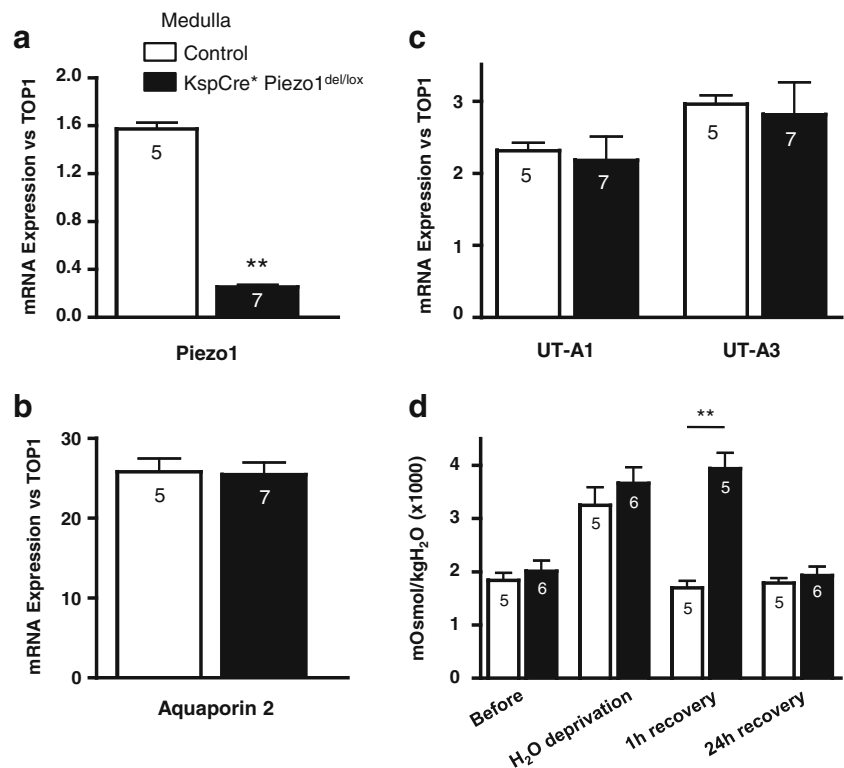
Piezo1, cAMP levels would be increased and consequently it might delay AQP2 retrieval during rehydration. Of note, stimulation of calcium inhibitable adenylyl cyclases and inhibition of calcium-dependent cAMP phosphodiesterases also likely account for the increased level of cAMP in the autosomal dominant polycystic kidney disease (PKD), which primarily affects the CD (for review [8]).

- Urea transporters trafficking might be similarly influenced by Piezo1 opening. Increased urea permeability, another key mechanism required for producing concentrated urine, is also AVP-driven and involves the PKA phosphorylation of urea transporters, including UT-A1 [6, 20]. Thus, as discussed above, calcium entry through Piezo1 might possibly influence this mechanism by

interfering with intracellular cAMP levels, although total UT-A1 protein expression in the medulla is independent of Piezo1. This hypothesis will need to be tested, when specific antibodies directed against the phosphorylated forms of these proteins will become commercially available.

- An additional mechanism of urine dilution, independent of both AVP (and hypoglycemia), has been reported upon food deprivation [2, 45]. These changes are correlated with a significant decrease in AQP2 in the outer medulla, both at the mRNA and protein levels [45]. Of note, prostaglandins have been shown to be increased during fasting [13]. Moreover, prostaglandins antagonize the effect of AVP on water transport in the CD, as for instance after the release of ureteral obstruction [16, 31, 32]. Adrenal

Fig. 6 Regulation of urine osmolarity in a *KspCre* Piezo1^{del/lox}* mouse model. **a** Piezo1 mRNA expression as determined by qPCR in the inner medulla of TAM-induced control or *KspCre* Piezo1^{del/lox}* mice. **b** AQP2 mRNA expression. **c** UT-A1 and UT-A3 mRNA expression. **d** Quantitative analysis of metabolic cage experiments for urine osmolarity before, during, and after (1 and 24 h) drinking deprivation in control (*white bars*) and knock-out (*KspCre* Piezo1^{del/lox}*, *black bars*) mice. Numbers of mice are indicated. ****** $p < 0.01$



gland steroids also play a key role in the regulation of urinary concentrating mechanisms [22, 42]. Whether Piezo1 opening in CD epithelial cells influences the synthesis and/or release of hormones regulating urine osmolarity is another possibility.

In conclusion, we have demonstrated that Piezo1 is preferentially expressed in renal CD principal cells of adult mice and is critically required for non-selective SAC activity. Its specific deletion in renal epithelial cells at the adult stage alters urinary concentration following dehydration or fasting. Altogether, these findings indicate that Piezo1 expression in CD principal cells plays a functional role in the regulation of urinary osmolarity.

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