MOLECULAR AND GENOMIC PHYSIOLOGY

The inositol Inpp5k 5-phosphatase affects osmoregulation through the vasopressin-aquaporin 2 pathway in the collecting system

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Abstract Inositol *Inpp5k* (or Pps, SKIP) is a member of the inositol polyphosphate 5-phosphatases family with a poorly characterized function in vivo. In this study, we explored the function of this inositol 5-phosphatase in mice and cells overexpressing the 42-kDa mouse *Inpp5k* protein. *Inpp5k* transgenic mice present defects in water metabolism characterized by a reduced plasma osmolality at baseline, a delayed urinary water excretion following a water load, and an increased acute response to vasopressin. These defects are associated with the expression of the *Inpp5k* transgene in renal collecting ducts and with alterations in the arginine vasopressin/aquaporin-2 signalling pathway in this tubular segment. Analysis in a mouse collecting duct mCCD cell line revealed that *Inpp5k* overexpression leads to increased

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expression of the arginine vasopressin receptor type 2 and increased cAMP response to arginine vasopressin, providing a basis for increased aquaporin-2 expression and plasma membrane localization with increased osmotically induced water transport. Altogether, our results indicate that *Inpp5k* 5-phosphatase is important for the control of the arginine vasopressin/aquaporin-2 signalling pathway and water transport in kidney collecting ducts.

Keywords Water transport · Aquaporin · Phosphoinositide metabolism · 5-Phosphatase · Collecting duct

Introduction

Phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2) and phosphatidylinositol (3,4,5)-trisphosphate (PtdIns (3,4,5)P3) demonstrate second messenger functions in multiple biological processes. PtdIns(4,5)P2 controls, among others, the dynamic turnover of actin cytoskeleton, assembly/disassembly of vesicular coats, secretion, gene expression and the fate of endocytic vesicles [13, 17, 29, 40]. It also regulates the activity of many ion channels and transporters [35, 36]. PtdIns(4,5)P2 is mainly localized in the inner leaflet of plasma membranes, but smaller pools have been detected in intracellular membranes, including Golgi, endoplasmic reticulum, endosomes and lamellipodia [13]. Mechanistically, a very large number of proteins are recruited to PtdIns(4,5)P2 via pleckstrin homology (PH), phagocyte oxidase (PX), Fab1p, YOTB, Vac1p and EEA1 (FYVE), epsin N-terminal homology (ENTH) domains or through small patches of basic amino acids [13, 18, 28]. PtdIns(4,5,)P2 also serves as precursor of five essential signalling molecules: diacylglycerol, inositol (1,4,5)-trisphosphate, PtdIns4P, PtdIns5P and PtdIns(3,4,5)P3 [13, 17]. In non-stimulated cells, PtdIns(3,4,5)P3 levels are very low in the plasma membrane, reaching ~0.1% of the level of PtdIns(4,5)P2 [13, 17]. Upon cell activation, PtdIns (3,4,5)P3 levels are transiently increased by a factor ranging from 2- to 100-fold. This phosphoinositide is implicated in the control of cell survival, growth and proliferation, resistance to apoptosis, regulation of cytoskeleton dynamics, membrane trafficking, cell migration, and many of the metabolic responses to insulin [12, 13]. PtdIns(3,4,5)P3 regulates a wide variety of effector proteins primarily by binding and recruiting specific proteins.

In mammalian cells, PtdIns(4,5)P2 and/or PtdIns(3,4,5) P3 are substrates for several members of the phosphoinositide 5-phosphatases family, including Inppl1 (or SHIP2), Inpp5e and Inpp5k (or SKIP, for skeletal muscle and kidney enriched inositol phosphatase, and Pps, for putative phosphatase) [2, 7, 30, 32, 33]. These enzymes tightly control the level of the above-mentioned phosphoinositides

in a tissue-, cellular-, subcellular- and signalling cascade-specific way. Importantly, inactivation or overexpression of most members of this family has been associated with diseases in mouse and man [3, 30, 32, 33]. For example, Inpp5e is localized in the primary cilium of differentiated/ quiescent cells; mice and man with genetic alterations in the Inpp5e gene develop ciliopathies [6, 22]. SHIP2 knock-out mice are resistant to high fat diet-induced obesity and have glucose metabolism alterations [10, 34]; polymorphisms in the *SHIP2* gene have been identified in cohorts of type 2 diabetic patients, and overexpression of SHIP2 in transgenic mice is associated with glucose metabolism and insulin sensitivity defects [25, 26, 31].

Inpp5k is a PtdIns(4,5)P2 and PtdIns(3,4,5)P3 5-phosphatase. It is highly expressed in human skeletal muscles and kidney, hence its previous name SKIP [19]. In N1E-115 neuroblastoma cells, human Inpp5k regulates the actin cytoskeleton [19]. Human Inpp5k is translocated from the endoplasmic reticulum to plasma membrane ruffles following activation by EGF and insulin in Cos-7 and skeletal muscle C2C12 cell lines, respectively [16, 39]. Activation of PKB and p70 S6 kinases as well as GLUT4 glucose transporter translocation to the plasma membrane and membrane ruffles formation were significantly decreased in human Inpp5k-transfected and insulin-stimulated CHO cells [20]. Mice totally deficient for Inpp5k die during embryonic life for still unknown reason; Inpp5k$^{+/-}$ mice have increased insulin sensitivity in skeletal muscles, confirming the results obtained in different cell lines [21]. However, the role of Inpp5k in the kidney, another tissue where the phosphatase has been reported to be highly expressed, and its potential implication in a metabolic or a pathologic process in this tissue remain undefined.

In order to explore the function of Inpp5k in vivo, we generated and characterized a transgenic mouse line expressing the mouse Inpp5k 5-phosphatase in many tissues. Our in vivo and in vitro results reveal that overexpression of Inpp5k in kidney collecting duct cells is associated with defects in water handling paralleled by alterations in the arginine vasopressin (AVP)/ aquaporin-2 (AQP2) signalling pathway resulting in impaired osmoregulation.

Materials and methods

RNA analysis in mouse tissues and cells

For Northern blot analysis, total RNA was extracted from mouse tissues using the TriPure Isolation reagent (Roche Applied Sciences) and loaded (10 µg/lane) on agarose gel. A 944-bp DNA fragment of the mouse Inpp5k cDNA served as a radiolabelled probe. A radiolabelled probe

consisting of a 600-bp DNA fragment of the mouse hypoxanthine–guanine phosphoribosyltransferase (HPRT) cDNA served as loading control.

For reverse transcription-polymerase chain reaction (RT-PCR) analysis on FACS-purified populations of dendritic cells, mast cells, macrophages, neutrophils, eosinophils, T and B lymphocytes, RNA was extracted and reverse-transcribed using random hexamers. cDNA was amplified by PCR using forward and reverse primers located in exon 8 and exon 12 of the mouse Inpp5k gene, respectively (see Table S1 for the primers sequence; annealing temperature, 55.5°C; 30 cycles). For RT-PCR and real-time RT-PCR analysis on 6-month-old mouse kidney and brain and on mCCD cells, total RNA and cDNA were obtained as described [24]. The primers (Table S1) were designed using Primer3. Changes in target gene mRNA levels were determined in duplicate by relative RT-qPCR with a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using iQ™ SYBR Green Supermix (Bio-Rad) detection of single PCR product accumulation. The PCR conditions, the negative controls, the preparation of the standard curves and the calculation of the reaction efficiencies were as previously described [1].

Antibodies

An affinity-purified rabbit antibody against the RSFLREDTLYEPEPQI carboxy-terminal mouse Inpp5k peptide was obtained from Eurogentec, Liège, Belgium. Pre-incubation of the anti-Inpp5k antibody with the immunogenic peptide resulted in the disappearance of the signal in Western blot, immunoprecipitation and immuno-histochemistry analyses (data not shown). Rabbit polyclon-al antibodies raised against the AQP2 (Sigma-Aldrich), phosphorylated Ser256 AQP2 (p-AQP2) [8] and NKCC2 (Millipore), sheep anti-megalin (a gift from P. Verroust, Paris, France) [9], mouse monoclonal antibodies against the M2 clone of Flag® tag (FITC-conjugated, Sigma-Aldrich) and β-actin (Sigma-Aldrich), and Alexa633-labelled sec-ondary anti-IgG antibodies (Invitrogen, Belgium) were also used.

Protein analysis

Tissues were homogenized in a buffer containing NP-40 (1%), Tris–HCl pH 8 (50 mM), NaCl (150 mM), and protease inhibitors (Complete™, Roche Diagnostics GmbH). Cell lysates were obtained by solubilizing frozen pellets in lysis buffer containing Complete Mini (Roche), followed by sonication and centrifugation at 16,000×g for 1 min at 4°C. When needed, total membrane and cytosolic fractions were separated by centrifugation (100,000×g for 2 h). Protein concentrations were determined using BCA

protein assay (Pierce, Aalst, Belgium). Cell and tissue samples were normalized for protein levels, diluted in Laemmli sample buffer, separated by SDS-PAGE in reducing conditions, and blotted onto nitrocellulose or PVDF membranes. After blocking with non-fat milk, membranes were incubated overnight at 4°C with the primary antibody, washed, incubated with peroxidase-labelled secondary antibody, and visualized with enhanced chemiluminescence (Perkin Elmer).

When indicated, protein analysis was performed after immunoprecipitation of the Inpp5k protein either with the anti-Flag or the anti-Inpp5k rabbit polyclonal antibodies and protein A Sepharose CL-4B (Amersham). In these conditions, an HRP-coupled anti-Flag monoclonal antibody and a biotin-coupled anti-Inpp5k polyclonal antibody were used as primary antibodies in the Western blotting. For surface biotinylation assay, 10-day-old confluent mono-layers of mCCD cells were incubated with Sulfo-NHS-biotine (1.5 mg/ml; Pierce) at 4°C. Excess biotine was quenched by incubation with 100 mM glycine and cells were lysed in Ripa buffer containing protease inhibitors. Lysate was centrifuged, and its supernatant was incubated with streptavidine-conjugated agarose droplets (Pierce) to purify apical membranes from the cytosolic fraction. Proteins were extracted from renal cortex and medulla samples as described [24].

Malachite phosphatase assay

Inpp5k protein was immunoprecipitated from kidney extracts as described above. Inpp5k phosphoinositide phosphatase activity was measured with a phosphate release assay using an acidic malachite green. Briefly, Inpp5k protein immunoprecipitates were incubated with 100 μM of either PtdIns(3,4,5)P3 di-C16 or PtdIns(4,5)P2 di-C16 in mixed vesicles of Ptdserine for 10 min at 37°C. Reactions were stopped by addition of EDTA. After centrifugation, supernatants were incubated with malachite green reagent for 10 min. Absorbance was measured at 650 nm. As negative controls, we used the anti-Inpp5k antibody pre-incubated with the immunogenic peptide before adding the protein extract, and the anti-Flag antibody without addition of any kidney extract. Inorganic phosphate release was quantified by comparison to a standard curve of KH_2PO_4 in dH_2O.

Lentivirus construction and production

pWPXLd, psPAX2 and pMD2.G plasmids were obtained from D. Trono (Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland). The chicken β actin promoter/ enhancer, the cytomegalovirus immediate-early enhancer (altogether referred as the CAGG promoter), a β globin

intron, the eGFP cDNA flanked by loxP sites and the octapeptide Flag amino-terminal-tagged mouse Inpp5k cDNA corresponding to the 42-kDa isoform of the Inpp5k protein (NM-008916) were introduced between the two LTR sequences in the pWPXLd plasmid. This plasmid was named pWPXLd/GFP/Inpp5k. A pWPXLd/Inpp5k plasmid was obtained after transformation of 294 Cre-expressing bacteria with the pWPXLd/GFP/Inpp5k plasmid. Recombinant lentiviruses were obtained after transfection of 293 T cells with the pWPXLd/GFP/Inpp5k, psPAX2 and pMD2.G plasmids. After 2 days, the lentivirus-containing supernatant was harvested, purified and concentrated by ultracentrifugation, and resuspended in KSOM embryo culture medium (Specialty Media). The concentrated lentiviral suspension was titrated by infection of 293T cells for 2 days followed by GFP expression analysis using a flow cytometer.

Production of lentigenic mice

Two to four cell embryos were recovered from superovulated (C57BL/6 × Balb/c)F1 female mice crossed with F1 males. Embryos were depellucided with tyrode acid (Sigma) and incubated with $5–10×10^3$ lentiviral particles in a drop of KSOM medium under mineral oil (Sigma). After 2 days at 37°C and 5% CO_2, viable embryos were rinsed several times with M2 embryo culture medium (Sigma) and reimplanted in the uterine horns of pseudogestant CD1 females (Harlan). Newborns were screened for GFP expression in the tail by fluorescent microscopy (Nikon Eclipse TE300). GFP/Inpp5k transgenic founders were crossed with PGK-Cre mice in order to remove the GFP cDNA from the inserted transgene and to obtain Inpp5k transgenic mice. GFP/Inpp5k and Inpp5k transgenic mice were backcrossed with C57BL/6 mice for five generations before analysis. No Cre transgene was left in the experimental mice. Non-transgenic littermates resulting from the crossing between Inpp5k transgenic mice and C57BL/6 mice, as well as age-matched GFP/Inpp5k transgenic mice served as controls. All animal studies were authorized by the Animal Care Use and Review Committee of the Université Libre de Bruxelles.

DNA analysis

Genomic DNA was extracted from tail tips and analyzed by Southern blotting and PCR. DNA was digested with *Eco*RI or *Spe*I, loaded on an agarose gel and transferred onto a nitrocellulose membrane. A 950-bp DNA fragment of the mouse Inpp5k cDNA was used as a radiolabelled probe. For genotyping, PCR was used to amplify a DNA fragment of eGFP or Inpp5k cDNAs present in the transgene (see Table S1 for primer sequences).

Renal salt and water handling protocols

Experiments were conducted on age- and gender-matched mice. Water handling at baseline and during various protocols was assessed in individual metabolic cages, after appropriate training, as described [1, 5]. Plasma samples and 24-h urine collections were obtained at baseline, and urinary concentrating ability was tested after 24-h water deprivation. The capacity to excrete a water load was tested after i.p. injection of 2 ml of sterile water; urine was collected under a plastic-wrapped container on an hourly basis for the next 6 h as described [1]. The acute response to vasopressin was investigated by treating mice with an i.p. injection of 1 ng/g body weight of dDAVP (desmopressin) (Minirin®, Ferring, Switzeland) in 100 µl of saline i.p. and collection of urine 5 h later to measure osmolality.

Plasma and urine analyses

Plasma urea, glucose and creatinine were measured after appropriate sample dilution with a Synchron CX5 Analyzer (Beckman Coulter), and osmolality was measured using a Fiske Osmometer (Needham Heights, MA), as described [1, 5].

Histology and immunohistochemistry

Kidney samples were fixed in 4% paraformaldehyde (Boehringer Ingelheim, Heidelberg, Germany) in 0.1% mol/l phosphate buffer (pH 7.4) before embedding in paraffin, and processed for staining and antibodies incubation as described [1]. Sections were viewed under a Zeiss LSM510Meta Confocal microscope (Zeiss, Belgium). Fluorescence measurements were performed with a x63/1.4 Plan-Apochromat oil-immersion objective (Zeiss).

mCCD cell culture, transfection, immunostaining, response to vasopressin and fluid transport measurement

mCCD cells were cultured on collagen-coated PTFE filter membranes as described [14]. At 60–80% confluency, mCCD cells were transfected or not with a pWPXLd plasmid expressing either the wild-type or the catalytically inactive Inpp5k protein by the use of Fugene HD (Roche). The catalytically inactive 42-kDa mouse Inpp5k protein was obtained after mutation of the wild type Inpp5k cDNA, according to Communi et al. [11] and Wada et al. [38]: the P^{283}, D^{287} and R^{288} of the catalytic domain of the Inpp5k protein were respectively mutated in A, A and G. Six- to ten-day-old confluent monolayer cells were used in the experiments. For immunostaining, cells were fixed, permeabilized and incubated with a goat anti-rabbit AQP2 and a monoclonal anti-Flag® antibodies coupled with Alexa633 and FITC, respectively. Nuclei were stained with DAPI. Cells were

analyzed with a Zeiss LSM510Meta Confocal microscope (Zeiss, Belgium). The response to vasopressin was tested by treating the filter-seeded cells (transfected or not, as described above) with 10 nM dDAVP (Sigma) for 5 min in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma). After treatment, cells were washed, incubated with sample diluent for 10 min at room temperature and centrifuged at $600 \times g$ at 4°C for 15 min. Fifty µl of the supernatant were used to assay cAMP, using a direct cAMP enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, USA) according to the Manufacturer's instructions. Data are based on three independent experiments performed in duplicate.

Cellular fluid transport

Fluid transport was determined in confluent monolayers of mCCD cells at day 10 as described previously [15]. For baseline volume measurement, cell culture medium was changed and replaced with fixed volume of media introduced in apical and basolateral sides. After an incubation time of 2 or 15 h, the apical and basolateral medium were collected and measured on a precision balance. For the effect of an osmotic gradient on fluid transport, a fixed volume of diluted culture medium (2/5) was added to the apical side, while undiluted (5/5) culture medium was added to the basolateral side. After 2 h, both apical and basolateral volumes were collected and measured. $HgCl_2$ (1 mM), the aquaporins inhibitor, was added to the apical side in the presence of a concentration gradient (2/5 apical and 5/5 basolateral). After 2 h, apical and basolateral volumes were collected and measured. Protein levels from each cell culture were determined with a BCA kit (Pierce), and were found to be similar in all conditions.

Nephron segments microdissection

Eight-week-old C57Bl/6 mice were used for microdissection of individual nephron segments. Briefly, thin coronal kidney slices were prepared, after which cortex and medulla were separated and digested with collagenase type II (1 mg/ml) for 30 min (for proximal tubules) or 1 h (for other tubules) at 37°C. Then, tubule suspension was washed and placed on a stage of an inverted microscope. A total of 50 proximal tubules (S1S2 and S3), thick ascending limbs of Henle's loop (TAL), and collecting ducts (CD) were collected and placed in 300 µl RLT buffer (Qiagen). Total RNA was extracted immediately after microdissection using the RNAeasy Micro kit (Qiagen).

Statistics

All values are expressed as means ± standard error of the mean (SEM). Significance of differences between two means was calculated using the GraphPad InStat software (GraphPad Software, San Diego, CA, USA). The significance level of the differences of the means (P value) is indicated in the legend of each figure.

Results

Production and characterization of Inpp5k transgenic mice

In order to study the role of mouse Inpp5k phosphatase in vivo, a transgenic mouse expressing a Flag-tagged 42-kDa Inpp5k protein was generated by infection of embryos with a recombinant lentivirus. Since our preliminary results indicated that Inpp5k expression is ubiquitous in mouse (Suppl. information and Fig. S1), a transgene was constructed containing the chicken β actin promoter/enhancer and the cytomegalovirus immediate-early enhancer (altogether referred as the CAGG promoter) which are known to direct expression of the transgene in many tissues in genetically modified mouse (Fig. 1). Downstream of the CAGG promoter, the transgene contained a β globin intron, the GFP cDNA flanked by loxP sites, and the octapeptide Flag-tagged mouse Inpp5k cDNA. The transgene was inserted into the pWPXLd lentiviral plasmid and successfully tested for mutually exclusive expression of GFP or Inpp5k proteins in 293 cells (Suppl. information and Fig. S2). Mouse embryos were infected with lentiviral particles and reimplanted in pseudogestant females. The presence of founders in the progeny was checked at birth by tail fluorescence analysis and confirmed by PCR analysis. Out of 21 newborns, two were fluorescent and both transmitted the transgene to their progeny (data not shown). Experiments were done to demonstrate that both lentiviral transgenic lines A and B had sole transgene integration in the genome, produced the Inpp5k transgene only after Cre recombination, and that the transgenic Inpp5k protein was ubiquitously expressed and catalytically active in both lines (Suppl. information, Fig. S3 and data not shown). We also confirmed that both PtdIns(4,5)P2 and PtdIns(3,4,5)P3 are substrates for the murine Inpp5k protein (Suppl. information and Fig. S3). Given the higher amount of Inpp5k protein and 5-phosphatase activity detected in tissues of transgenic line B, this line was used in the following experiments. Age-matched non-transgenic littermates and transgenic line B mice expressing the GFP protein served as controls. As no significant difference was observed between both groups of control mice, a potential effect of the transgene integration site on the observed phenotype was discarded, and these two groups were finally pooled.
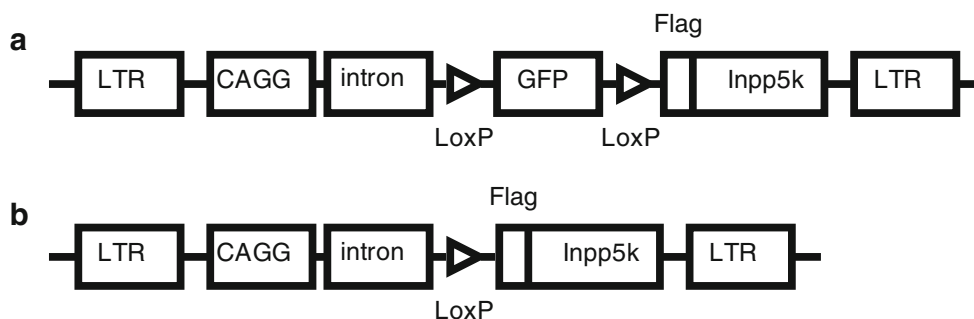
Fig. 1 Inpp5k transgene structure. Transgene structure before (**a**) and after (**b**) Cre recombination showing the CAGG promoter (including the CMV enhancer), the β globin intron, a first loxP site, the enhanced green fluorescent protein (*GFP*) cDNA, a second loxP site, and the Flag-tagged mouse Inpp5k cDNA. A viral LTR is present at both transgene extremities

Altered osmoregulation in Inpp5k transgenic mice

Inpp5k transgenic mice were fertile and had a normal life span and general behaviour. No evident macroscopic nor microscopic alterations were detected in all tissues examined (data not shown). As Inpp5k mRNA and protein have been reported to be highly expressed in the kidney [19], renal function and osmoregulation parameters at baseline were investigated. Control and Inpp5k transgenic mice were similar in terms of body weight, water intake, urinary flow rate and renal function (plasma urea and creatinine) (Table S2). However, transgenic Inpp5k mice had a significantly lower plasma osmolality than control mice (350 ± 3.7 and 335 ± 4.3 mOsm/kg H_2O in control and transgenic mice, respectively; $P<0.03$, 5–6 mice per group). These data suggested an abnormal osmoregulation in Inpp5k transgenic mice and led us to analyze the renal expression of endogenous and transgenic Inpp5k as well as water handling by the kidney in these mice.

Inpp5k expression in control and Inpp5k transgenic mice

Endogenous Inpp5k mRNA expression was investigated by RT-PCR in the different nephron segments of wild-type kidneys: endogenous Inpp5k mRNA was found in all segments, with the highest expression in the proximal tubule segments (Fig. 2a and b). The presence of the 44-kDa Flag-tagged Inpp5k transgenic protein was identified in both cortical and medullary fractions of Inpp5k transgenic kidneys (Fig. 2c). As expected, total Inpp5k protein expression was increased in transgenic kidneys, as compared to control kidneys. Kidney sections from control and Inpp5k transgenic mice were stained for total and transgenic Inpp5k proteins with an anti-Inpp5k and an anti-Flag antibody, respectively, and analyzed at the confocal microscope (Fig. 3a). The Flag-tagged transgenic Inpp5k protein was detected in the proximal tubules and the medullary collecting ducts of transgenic kidneys, but not in control kidneys, as expected. As compared with endogenous Inpp5k expression in control kidneys, total Inpp5k protein expression was increased in transgenic kidneys. Cellular co-localization of the Flag-tagged Inpp5k transgenic protein with megalin (a proximal tubule marker) and AQP2 (a collecting duct marker) confirmed the expression of the transgenic Inpp5k protein in these specific nephron segments (Fig. 3b). Interestingly, the expression of AQP2 was distinctly upregulated in the medullary collecting ducts of Inpp5k transgenic kidneys, whereas megalin and NKCC2 (a thick ascending limb of Henle's loop marker) were found unchanged, as compared with control kidneys (Fig. 3b and data not shown).

Abnormal water handling in Inpp5k transgenic mice

The altered plasma osmolality associated with the expression of the transgenic Inpp5k protein in specific nephron segments known to control water and/or ions transport suggested abnormal osmoregulation in Inpp5k transgenic mice. In order to substantiate this defect, we first tested the urinary concentrating ability of Inpp5k transgenic mice following a water deprivation test. The water deprivation resulted in a similar weight loss in control and Inpp5k transgenic mice (averaging $14\pm0.4\%$ in control mice and $13\pm0.8\%$ in Inpp5k transgenic mice, $n=5$ pairs) and, as expected, a significant reduction in the urine volume (Fig. S5). These changes were similar in both groups of mice, indicating that the mechanisms of solute accumulation in the medulla (i.e., transport of Na^+ and urea) allowing a maximal urinary concentration were not affected by Inpp5k overexpression. The latter hypothesis was also supported by the similar response of the control and Inpp5k transgenic mice to the loop diuretic furosemide (data not shown).

We next evaluated the capacity of the transgenic kidney to eliminate a water load by diluting urine (Fig. 4a). As compared with control mice, Inpp5k transgenic mice showed a significant delay in their ability to excrete the water load, pointing to a defective handling of water in the collecting duct. The acute response to vasopressin was also
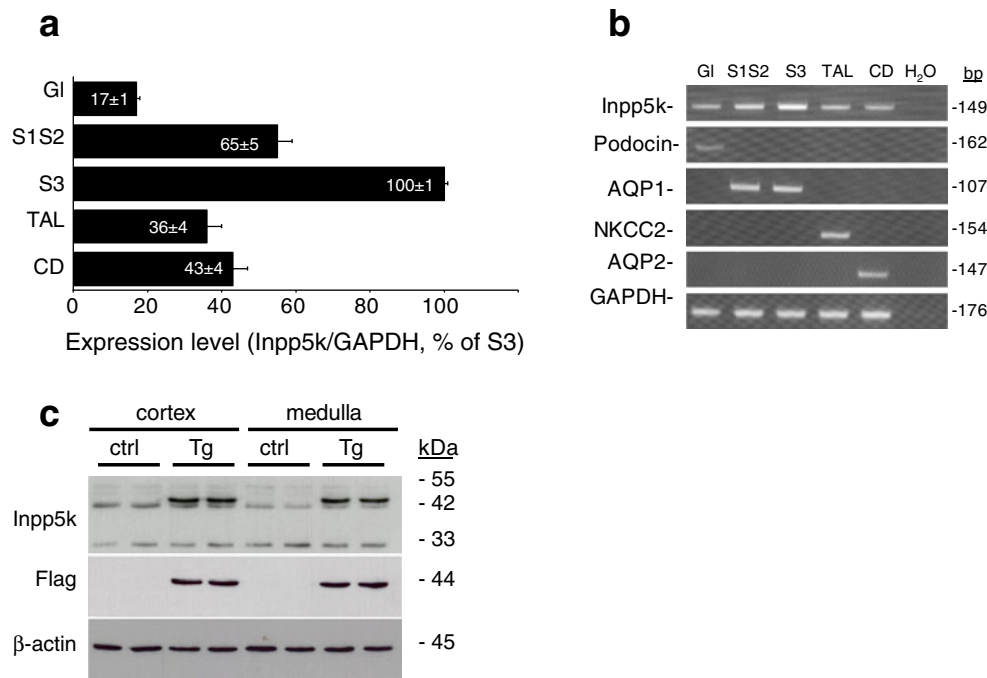
Fig. 2 Inpp5k expression in the kidney of control and Inpp5k transgenic mice. **a**, **b** Comparative expression of endogenous Inpp5k mRNA in different nephron segments from wild-type kidney. The expression level of Inpp5k was analyzed by quantitative RT-PCR. Nephron segments are characterized by the enrichment for respective markers (**b**): podocin for glomeruli (*Gl*), AQP-1 for the S1S2 and S3 segments of the proximal tubule, NKCC2 for the thick ascending limb (*TAL*) and AQP-2 for the collecting duct (*CD*). **c** Representative immunoblot on cortical and medullary fractions of wild-type (*ctrl*) and Inpp5k transgenic (*Tg*) mice. Cytosolic proteins (20 μg) were separated by SDS-PAGE and blotted onto nitrocellulose membrane, before incubation with anti-Inpp5k (1/1,000) or anti-Flag (1/1,000) affinity-purified antibodies. Loading control was performed after membrane stripping and incubation with monoclonal anti-β-actin antibody (1/10,000)

investigated in these mice, revealing that the maximal urine osmolality was significantly higher in Inpp5k transgenic mice as compared with controls ($3{,}815\pm100$ vs. $2{,}561\pm254$ mOsm/kg H_2O, $P<0.005$; Fig. 4b). Together, the lower plasma osmolality, impaired ability to excrete a water load and increased response to dDAVP indicated that Inpp5k transgenic mice had a significant defect in osmoregulation, which could be related to a specific effect of Inpp5k on the vasopressin/AQP2 axis in the kidneys.

Abnormal arginine vasopressin signalling pathway in Inpp5k transgenic mice

In order to investigate the mechanism of altered water handling in Inpp5k transgenic mice, real time RT-PCR was first used to test for the differential expression of transcripts that are primarily involved in the AVP signalling pathway, including AVPR2 receptor and AQP2 in the kidney, and AVP in the brain (Fig. 4c). The expression of AVPR2 and AQP2 mRNA were significantly increased in the kidneys of the Inpp5k transgenic mice as compared with controls. By contrast, the kidney expression of transcripts corresponding to pathways involved in Na^+ or K^+ handling (e.g., Na^+/K^+-ATPase and the different ENaC subunits) was unchanged

(Fig. 4b). In the brain, AVP mRNA expression was found significantly decreased in Inpp5k transgenic mice, as expected from a feedback response of the lower plasma osmolality values (Fig. 4c). We next investigated AQP2 protein expression and phosphorylation in transgenic kidneys by Western blotting (Fig. 4d and e). Immunoblot analyses essentially confirmed the immunostaining results presented in Fig. 3b: a significant overexpression of AQP2 protein was detected in the transgenic kidney as compared with control kidney. The proportion of phosphorylated AQP2 protein was also significantly increased in Inpp5k transgenic kidney (Fig. 4e).

Together, these results indicate that the osmoregulation defect detected in Inpp5k transgenic mice is caused by alteration in the AVPR2/AQP2 signalling pathway in kidney collecting ducts.

AQP2 and AVPR2 expression, cAMP production and water transport in mouse collecting duct cells overexpressing Inpp5k

The mouse CCD (mCCD) cells represent a well established model to investigate the mechanisms of water reabsorption in the collecting duct. In order to confirm and substantiate

Fig. 3 Inpp5k immunostaining on kidney sections from control and Inpp5k transgenic mice. Control and Inpp5k transgenic (*Tg*) kidneys were stained with **a** anti-Inpp5k (1/200) and anti-Flag (1/100) antibodies, and with **b** anti-megalin (1/10,000), anti-AQP2 (1/200) and anti-Flag (1/100) antibodies. The transgenic Inpp5k protein is located in the proximal tubules (cytoplasmic pattern, with expression of megalin in the apical membrane) and in the medullary collecting ducts (co-expression with AQP2, which is distinctly upregulated in these profiles). Total Inpp5k expression and AQP2 expression are increased in Inpp5k transgenic kidneys. Bar: 10 μm
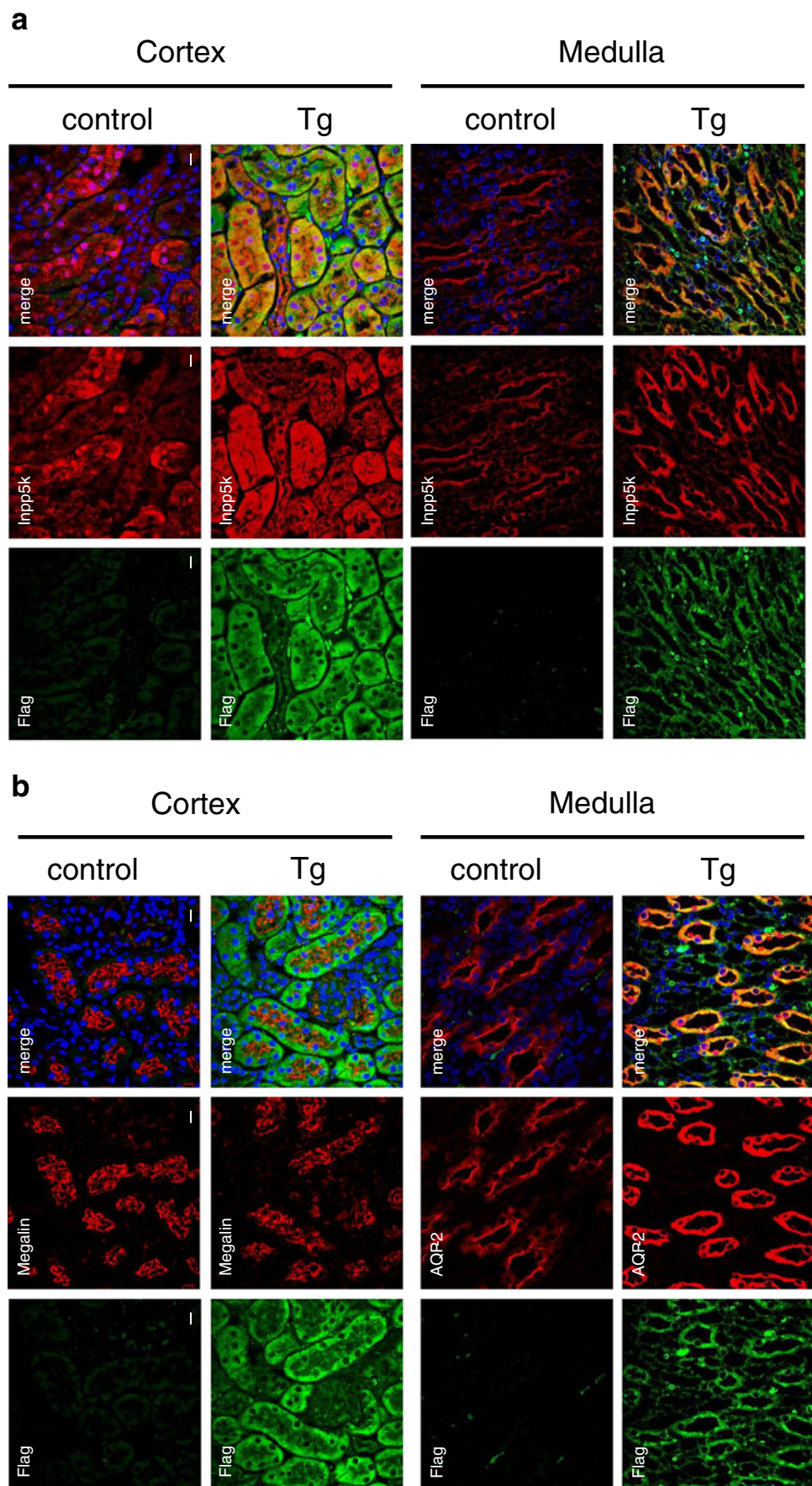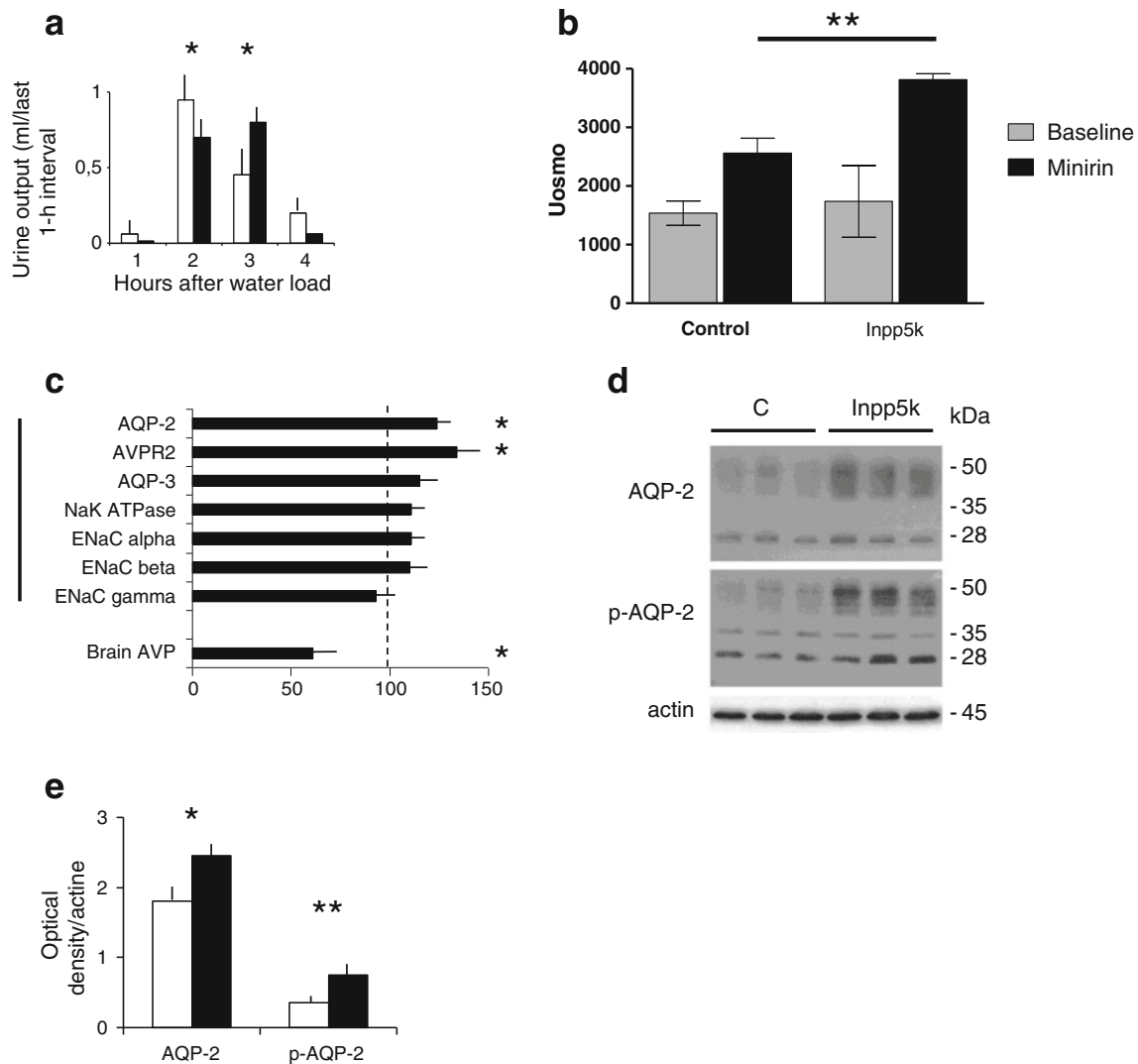
Fig. 4 Abnormal water handling, acute response to vasopressin and renal AQP2 expression in Inpp5k transgenic mice: **a** The urine output was analyzed in control (*white bars*) and Inpp5k transgenic (*black bars*) mice during 4 h in a test of acute water loading (2 ml intraperitoneally). Results are means±SEM of six pairs of 5-month-old male mice and are expressed as milliliter of urine excreted over the last 1-h interval. *P<0.05. **b** Effect of dDAVP treatment on urine osmolality in control and Inpp5k transgenic mice. After adaptation and baseline collection, mice were treated with 1 ng/g body weight of dDAVP (Minirin®) and urine was collected 5 h later to measure osmolality (Uosmo). The two groups of mice show similar urine osmolality values at baseline, whereas treatment with dDAVP induces a significantly higher response in the Inpp5k transgenic mice vs. controls. Data were obtained on four pairs of control vs. transgenic Inpp5k mice. **P<0.005. **c** Real-time RT-PCR quantification of mRNA expression in the kidney and the brain. Messenger RNA levels were first adjusted to glyceraldehydes-3-phosphate deshydrogenase (*GAPDH*), then normalized to the control mice level set at 100% using the following formula: Ratio = $2^{\Delta Ct(GAPDH-targetInpp5ktransgenicmice)-\Delta Ct(GAPDH-targetcontrolmice)}$. The normalized values (mean±SEM) are shown on the *right-hand side* of the panel. Eight pairs of kidney and brain from 5-month-old male mice were analyzed. *P<0.05. **d** Western blot analysis of AQP2, phosphorylated AQP2 (p-AQP2) and actin (as a loading control) in kidney extracts from control (*C*) and Inpp5k transgenic mice (*Inpp5k*). Five pairs of kidney from 5-month-old male mice were analyzed. **e** Densitometry analyses (core and glycosylated bands) confirm that there is a significant increase of both AQP2 and p-AQP2 proteins in the kidney extracts from Inpp5k transgenic (*black bars*) mice, as compared with control (*white bars*) mice. Results are means±SEM and are expressed as a ratio between AQP2 or p-AQP2 signals and actin signal. *P<0.05, **P<0.005

the link between Inpp5k overexpression and the water handling defect observed in mice, mCCDcells were plated on filters and transfected or not with either a catalytically active or inactive Inpp5k expression vector. The cells were then cultured to confluency to develop a polarized monolayer. Transfection of mCCD cells with wild type Inpp5k resulted in higher AQP2 protein expression and plasma membrane localization than in non-transfected cells (Fig. 5a–c). An intact Inpp5k enzymatic activity was required to observe the AQP2 expression and localization
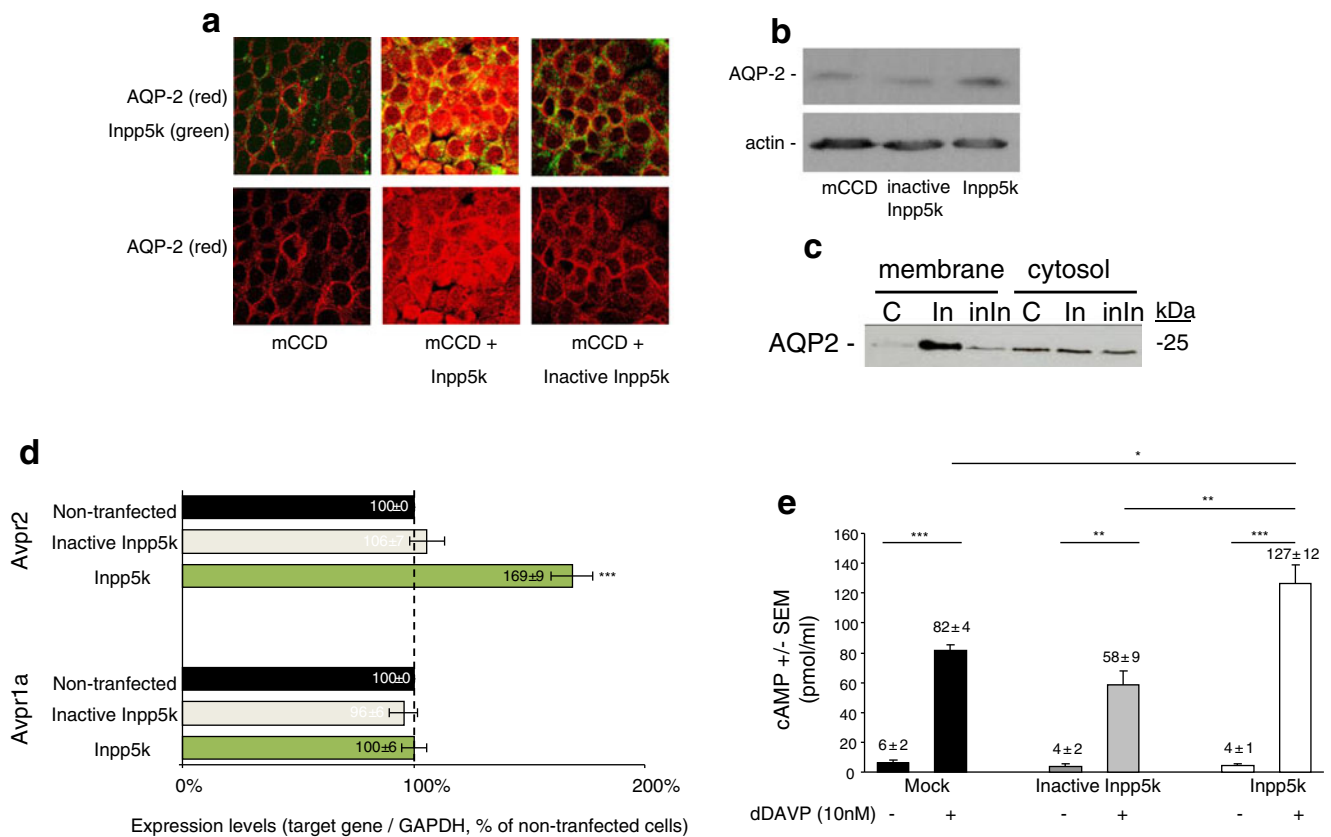
Fig. 5 Increased AQP2 and Avpr2 expression and dDAVP-induced cAMP production in Inpp5k-transfected mCCD cells: **a** Non-transfected, wild-type or catalytically inactive Inpp5k-transfected mCCD cells were cultured to confluency on filters to obtain a polarized cell monolayer. Anti-AQP2 (*red*) and anti-Inpp5k (*green*) antibodies were incubated with permeabilized cells and analyzed by confocal microscopy. **b** Western blot analysis of non-transfected and wild type or catalytically inactive Inpp5k-transfected mCCD cell protein extracts with an anti-AQP2 antibody. Actin served as loading control. **c** Plasma membrane of 10-day-old confluent mCCD cells under control conditions (**c**) or after transfection with either wild type Inpp5k (*In*) or catalytically inactive Inpp5k (*inIn*) were obtained after biotinylation assay. Plasma membrane and cytosol fraction were analysed by Western blotting with an anti-AQP2 antibody. Equal amounts of protein (20 μg/lane) were charged. **d** Increased expression

of Avpr2 in Inpp5k-transfected mCCD cells. Quantification (real-time PCR) of target mRNA in mCCD, expressed as relative expression over non-transfected cells ($n=4$). Significant differences (***$P<0.001$) between different conditions are indicated. **e** Effects of dDAVP (10 nM) on cAMP production in mCCD cells transfected with Mock or inactive Inpp5k or Inpp5k plasmid. mCCD grown on filter membranes were pretreated with 1 mM 3-isobutyl-1-methylxanthine (*IBMX*) for 10 min. Where indicated (+), cells were stimulated with 10 nM of dDAVP for 5 min in the continuous presence of IBMX. Cells were lysed and subjected to cAMP measurements. The mean cAMP concentration (cAMP ± SEM (in pmol/ml)) was determined from three independent experiments performed in duplicate (six filters per condition). Significant differences (*$P<0.05$, **$P<0.01$, ***$P<0.001$) between different conditions are indicated. Data are mean±SEM

defects, since transfection of mCCD cells with a catalytically inactive Inpp5k protein had no effect (Fig. 5). Further studies demonstrated that cells transfected with wild-type Inpp5k had a significantly higher mRNA expression of AVPR2 (Fig. 5d), which was reflected by a significantly (>2-fold) accentuated cAMP response to treatment with dDAVP (Fig. 5e). Again, these changes were not observed in cells transfected with the catalytically inactive Inpp5k.

These AQP2 and AVPR2 studies were correlated with measurement of net water transport across polarized mCCD cell monolayers (Fig. 6). Indeed, 2 and 15 h after placing iso-osmotic media on both sides of the mCCD cell monolayer, an increased water transport was detected in

wild-type Inpp5k-transfected cells, as compared with non-transfected and catalytically inactive Inpp5k transfected cells. When a hypo-osmotic medium was placed at the apical side of the mCCD cell monolayer, water transport was increased in all three conditions tested. However, water transport was still significantly increased in wild type Inpp5k transfected cells (Fig. 6). Addition of HgCl$_2$, which blocks AQP2 by interacting with a critical cysteine residue in the water pore, nearly completely blocked the osmotically induced water transport in all cells tested (Fig. 6). Together, these results indicate that the active form of Inpp5k tightly controls AQP2 expression and plasma membrane localization, as well as the osmotically induced water transport in mouse collecting duct cells.
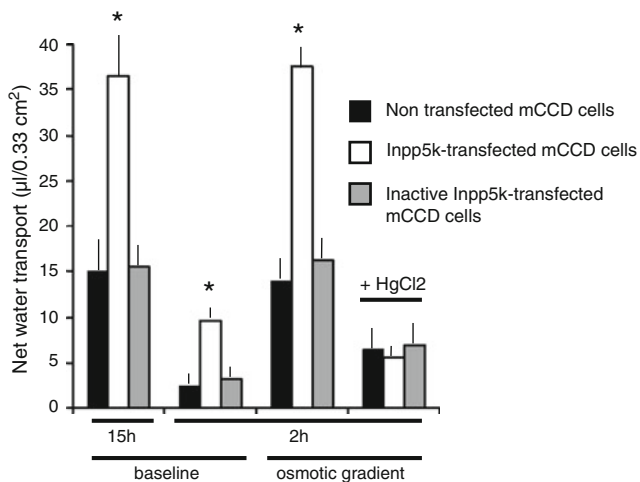
Fig. 6 Increased water transport in Inpp5k transfected mCCD cells: Non-transfected and wild type or catalytically inactive Inpp5k transfected mCCD cell monolayer were incubated with iso- (baseline) or hypo- (osmotic gradient) osmotic medium at the apical side. After 2 and 15 h, medium volumes at the apical and basolateral sides of the cell monolayer were measured and the net water transport calculated. The aquaporins inhibitor $HgCl_2$ (1 mM) was added to the medium at the apical side of the cell monolayer. For each condition, results are representative of six independent experiments performed in triplicate. Mean±SEM are represented. *$P<0.001$ when comparing wild-type Inpp5k-transfected cells with either non-transfected or catalytically inactive Inpp5k-transfected cells

Discussion

We report here that transgenic mice overexpressing the phosphoinositide 5-phosphatase Inpp5k present an altered water metabolism characterized by a decreased plasma osmolality in basal conditions, a decreased ability to excrete a water load, and an increased urinary concentrating response to arginin vasopressin. These physiological alterations are associated with defects in the AVPR2/AQP2 signalling pathway in the collecting ducts of the kidney. Indeed, overexpression of wild type, but not that of catalytically inactive Inpp5k in mouse collecting duct cells results in an increased water transport associated with increased AVPR2 expression and cAMP response to vasopressin, and with increased AQP2 expression and plasma membrane localization, providing a mechanism to explain the defects observed in Inpp5k transgenic mice. Together, we conclude that Inpp5k as an important regulator of the AVPR2/AQP2 signalling pathway and water metabolism.

Inpp5k is a member of the phosphoinositide 5-phosphatases family with catalytic activity directed towards PtdIns(4,5)P2 and PtdIns(3,4,5)P3 [19–21, 37]. Our Northern blot, RT-PCR and Western blot analyses have identified mouse Inpp5k as a ubiquitously expressed phosphatase. Several Inpp5k mRNA and protein molecular weight forms have been detected in mouse tissues, as in man [19]. In the

kidney, Inpp5k mRNA is also differentially expressed along the nephron and the collecting duct, and three molecular forms were identified in the cortex and the medulla. This very complex pattern of expression is probably of significant relevance and must serve specific physiological function for the Inpp5k enzyme in the different regions of the kidney and other tissues.

Recently, Pps^{Brdm1} mice with a frameshift mutation in the Inpp5k/Pps coding sequence downstream of exon 7 have been generated [21]. The homozygous Pps^{Brdm1} mutation caused embryonic lethality of unknown origin. In adult heterozygous $Pps^{Brdm1/+}$ mice, a significant increase in insulin sensitivity was observed in skeletal muscles, and these mice were also resistant to a high fat diet-induced obesity. By contrast, no difference was detected between control and $Pps^{Brdm1/+}$ mice for insulin signalling in the adipose tissue and for hepatic glucose production following insulin administration. Although our transgenic mice expressed a high amount of catalytically active Inpp5k in skeletal muscles, no significant difference was found in insulin and glucose tolerance tests as compared to control mice (data not shown). This negative result is rather surprising, because overexpression of Inpp5k in insulin-stimulated CHO cells was reported to inhibit phosphoinositide 3-kinase signalling by reducing PtdIns(3,4,5)P3 concentrations and downstream signalling [20]. In addition, we expected to observe a frank insulin resistance in our transgenic mice which mirrored the increase in insulin sensitivity observed in $Pps^{Brdm1/+}$ mice. The possible reason(s) of this discrepancy between our results and those of Takenawa are currently unknown. It could result from the use of different Inpp5k molecular weight forms with potentially different function and/or 5-phosphatase activities to express Inpp5k in our transgenic mice (42 kDa) and in CHO cells (approximately 50 kDa). Alternatively, the expression of the catalytically active Inpp5k protein in muscles of our transgenic mice is may be not sufficient to reproduce the in vitro effects or to mirror the phenotype detected in the $Pps^{Brdm1/+}$ mice. Indeed, the presence of compensatory mechanisms involving other phosphoinositide phosphatases expressed in the muscles of our transgenic mice cannot be ruled out, similarly to the compensation of the absence of the OCRL protein by the 75-kDa protein [23].

AVP, AVPR2 and AQP2 are key components in the signalling pathway involved in the control of the final urine concentration by the principal cells lining the collecting ducts. Under basal conditions, AQP2-containing vesicles are maintained in the subapical region of the cells. When the organism is exposed to a loss of free water, plasma osmolality increases, stimulating the release of AVP by the posterior pituitary into the circulation. In turn, AVP binds to the AVPR2 on principal cells, leading to increased cAMP

levels stimulating the expression of AQP2 as well as the phosphorylation and shuttle of AQP2 from subapical storage vesicles to the apical membrane, thereby increasing the osmotically induced water transport. Our data demonstrate that AVPR2, AQP2 and p-AQP2 expression is upregulated in Inpp5k transgenic kidneys. The overexperssion of these three factors in collecting duct cells would substantiate an increase in water reabsorption and the subsequent reduced basal osmolality and delayed ability to excrete a water load. The fact that this phenotype results from a primary renal deficit is confirmed by the physiological response of the brain, i.e., a decreased AVP mRNA expression secondary to lower plasma osmolality. It is noteworthy that the AQP2 mRNA and protein expressions are distinctly upregulated in the medullary collecting ducts of Inpp5k transgenic mice, whereas markers of other segments (i.e., megalin and NKCC2) are found unchanged. Furthermore, our studies in the mCCD cells demonstrate that Inpp5k overexpression alone, and not its catalytic mutant, is sufficient to upregulate AVPR2, increasing the cAMP response to dDAVP, which could then explain the observed increase in AQP2 expression and osmotic water transport. Unfortunately, we were unable to investigate the effects of a decreased Inpp5k protein expression on the AVP/AQP2 signalling pathway in this cellular system, because of the different kinetics of AQP2 expression and siRNA-induced Inpp5k downregulation in mCCD cells (data not shown).

Interestingly, no proximal tubule phenotype was detected in Inpp5k transgenic mice, despite expression of the transgene in this nephron segment. This may be related to the fact that the AQP1 water channels expressed in these tubules are constitutively inserted in the plasma membrane, without trafficking regulation nor response to AVP.

The mechanism leading to increased expression of AVPR2 and AQP2 mRNA as well as to increased AQP2 plasma membrane localization in collecting duct cells overexpressing Inpp5k certainly requires an intact phosphoinositide 5-phosphatase activity. Phosphoinositides signalling occurs in many different intracellular membranes, including the inner surface of the plasma membrane and the membrane vesicles that move to the plasma membrane, providing a potential explanation for the increased AQP2 expression at the cell surface of collecting duct cells when catalytically active Inpp5k is overexpressed. It is noteworthy that a still poorly defined nuclear phosphoinositide signalling pathway, including PtdIns(4,5)P2 signalling, also occurs and has been shown to control gene expression, mRNA export as well as chromatin remodelling [4, 27]. The overexpression of the inositol Inpp5k 5-phosphatase in mCCD cells represents thus a new model to understand how phosphoinositides enter the nucleus and impact on nuclear processes.

In conclusion, we have here for the first time identified a role for the inositol 5-phosphatase Inpp5k in the mouse kidney: Inpp5k controls plasma osmolality via the regulation of the AVPR2/AQP-2 signalling pathway and of water transport in kidney collecting duct cells. Although the role of Inpp5k in the human kidney has not been investigated, our data suggest that Inpp5k could be important in patients with inappropriate water handling by the collecting duct.

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