Cellular Localization of THIK-1 (K_{2P} 13.1) and THIK-2 (K_{2P} 12.1) K^+ Channels in the Mammalian Kidney

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Key Words

K_{2P} • THIK channel • K^+ -transport • Nephron • Renal epithelium

Abstract

K^+ -channels fulfill several important functions in the mammalian kidney such as volume regulation, recirculation and secretion of K^+ ions, and maintaining the resting potential. In this study we used immunocytochemical methods, *in situ* hybridization, and nephron segment-specific RT-PCR to obtain a detailed picture of the cellular localization of two tandem pore domain potassium (K_{2P}) channels, THIK-1 (K_{2P} 13.1, KCNK13) and THIK-2 (K_{2P} 12.1, KCNK12). Monospecific antibodies against C-terminal domains of rat THIK-1 and THIK-2 proteins (GST-fusion proteins) were raised in rabbits, freed from cross-reactivity, and affinity purified. All antibodies were validated by Western blot analysis, competitive ELISA, and preabsorption experiments. The expression of THIK channels in specific nephron segments was confirmed by double staining with marker proteins. Results indicate that in rat and mouse THIK-1 and THIK-2 were expressed in the proximal tubule (PT), thick ascending limb (TAL), connecting tubule (CNT), and cortical

collecting duct (CCD). In human kidney THIK-1 and THIK-2 were localized in PT, TAL and CCD. Immunostaining of rat tissue revealed an intracellular expression of THIK-1 and THIK-2 throughout the identified nephron segments. However in mouse kidney THIK-2 was identified in basolateral membranes. Overall, the glomerulus, thin limbs and medullary collecting ducts were devoid of THIK-1 and THIK-2 signal. In summary, THIK-1 and THIK-2 are abundantly expressed in the proximal and distal nephron of the mammalian kidney.

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Introduction

Potassium channels play an important role in the cell homeostasis to maintain the unequal distribution between intra- and extracellular compartments. The constantly low extracellular K^+ -concentration depends on effective renal K^+ -excretion. K^+ ions are freely filtered in the glomerulum and reabsorbed by the proximal tubule (PT). Potassium concentrations are tightly controlled by excretion or reabsorption in the distal nephron. In addition, K^+ -channels in the kidney maintain the negative cell membrane potential. Furthermore, they regulate cell volume and move

K⁺ ions across the luminal and basolateral membranes in order to facilitate other transport processes [1].

There is a variety of K⁺-channels expressed in the kidney which can be classified into inwardly rectifying K⁺ channels (Kir) [2-5], voltage-gated K⁺ channels (Kv) [1], Ca²⁺-activated K⁺ channels (K_{Ca}) [6-9] and tandem pore domain K⁺-channels (K_{2p}, see below). Among the specific renal functions of these channels (summarized in an excellent review by Hebert et al. 2005 [1]), Kir1.1 (ROMK, KCNJ1) is probably the most important and best characterized channel. It is implicated in K⁺ secretion and potassium recycling, thereby providing K⁺ ions as a necessary prerequisite for the Na⁺-2Cl⁻-K⁺-cotransporter (NKCC-2) in the luminal membrane of the thick ascending limb [10]. Kir1.1 channels also finetune K⁺-secretion in the cortical collecting duct [11]. This is emphasized by the fact that several mutations in the ROMK gene (*KCNJ1*) result in an inherited tubulopathy, the antenatal Bartter syndrome [12-15]. However, ROMK-independent pathways for potassium secretion have been recently described and may involve Ca²⁺-activated K⁺ channels [6-9].

In recent years there has been an additional focus on K_{2p} channel function in the kidney. They possess four transmembrane regions, two pore-forming domains, and a less conserved cytosolic C-terminal domain [16, 17]. It has been proposed that they function as dimers. Their function is tightly controlled by factors such as extracellular pH, mechanical stress, temperature differences, oxygen tension, or modification by lipids or Gq-type G-proteins [18, 19]. So far, 15 different K_{2p} channels have been cloned, eight of these show significant expression in the kidney. TWIK-1 (K_{2p}1.1, KCNK1) and the pH-sensitive TASK-2 (K_{2p}5.1, KCNK5) have been observed in the proximal and distal tubules and in the collecting duct [20-24], although recently a more widespread expression of TWIK-1 in kidney has been shown [25]. In the proximal tubule TWIK-1 and TASK-2 may balance cell swelling associated with solute transport. Disruption of the TASK-2 gene (*KCNK5*) leads to renal proximal tubular acidosis [24]. In the distal tubule TWIK-1 and TASK-2 may serve to stabilize the membrane potential during electrogenic Na⁺ reabsorption [1]. TREK-2 (K_{2p}10.1, KCNK10) expresses a kidney-specific splice variant in the proximal tubule [26], and TASK-1 (K_{2p}3.1, KCNK3) and TWIK-2 (K_{2p}6.1, KCNK6) were shown to be expressed in glomeruli by quantitative RT-PCR [27]. The recently described "Tandem Pore Domain Halothane Inhibited K⁺ channels" THIK-1 (K_{2p}13.1, KCNK13) and THIK-2 (K_{2p}12.1, KCNK12) showed a widespread distribution

in rat tissues, including kidney [28]. Functionally, THIK-1 may be involved in linking hypoxia to cell function as observed in central nervous tissue [29] whereas no clear function has been assigned to THIK-2. The cellular and segmental renal distribution of THIK-1 and THIK-2 are currently unknown [28, 29].

Here we give a detailed analysis of the localization of THIK-1 and THIK-2 channels in rat, mouse and human kidney using immunocytochemistry, *in situ* hybridization, and RT-PCR. Both channels are shown to be widely expressed in the kidney with minor species differences. The distribution of THIK-1 and THIK-2 in the kidney suggests an important functional role for these two channels in renal K⁺ handling.

Materials and Methods

Animals and fixation

Male Sprague Dawley rats weighing between 180-200 g and male C57 Black/6 mice weighing between 25-30g were obtained from the local animal facility. Animals were kept under standard conditions and had free access to standard chow and tap water. The experiments were conducted in accordance to the German Law for the protection of animals (Reg G 0357/97). Animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (0.06 mg/g body weight). Then kidneys were perfused retrogradely through the abdominal aorta using 3% paraformaldehyde (PFA) in phosphate buffered saline (PBS) as described previously [30].

Cell culture

Opossum kidney cells (OK-cells) were cultured on 35 mm plastic culture dishes with glass bottom using DMEM-media supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO₂, 95% air atmosphere.

Tissue processing for immunohistochemical evaluation

For cryostat sectioning, tissues were protected from freezing artefacts by subsequent overnight immersion in PBS adjusted to 800 mosmol/kg H₂O sucrose, shock-frozen and stored at -80°C. For paraffin embedding, tissues were postfixed in 3% PFA/PBS, dehydrated and embedded in standard paraffin. For pre-embedding tissue specimens were postfixed in 3% PFA/PBS containing 0.05% glutaraldehyde and stored in sucrose-PBS solution at 4°C.

Tissue processing for immunoblotting

For the isolation of membrane-enriched fractions, renal cortices and OK-cells were homogenized for 3 min using a tissue homogenizer (Diaz 600, Heidolph Instruments) in an ice-cold solution containing 250 mM sucrose, 10 mM triethanolamine with 1 tablet/50 ml of a protease inhibitor cocktail. The homogenate was centrifuged at 1,000 g for 10 min (Sigma

3K15 refrigerated centrifuge) to remove nuclei and incompletely homogenized membrane fragments. The supernatant was then centrifuged at 200,000 g for 1 h (Beckman LE 80 ultracentrifuge) to obtain a pellet containing plasma membranes and intracellular vesicles. The resulting pellets were resuspended in homogenization solution. Total protein concentration was measured using the Pierce BCA Protein Assay reagent kit (Pierce, Rockford, IL) and controlled by Coomassie staining.

Antibody production and purification

Three different fusion proteins were constructed for each THIK channel, using the bacterial expression vectors pGEX4T-1 (Glutathion-S-Transferase, GST), pQE40 (Dihydrofolatreductase-HisTag, DHFR) and pET32b(+) (Thioredoxin-HisTag, TR). For THIK-1 amino acids 295-334 (Acc.No. AAG32312) and for THIK-2 amino acids 314-356 (Acc.No. AAG32311), both located in the cytosolic carboxyterminal portion of the proteins, were used as a fusion part. Proteins were expressed in *E. coli* BL21DE3 cells and purified using Ni²⁺-NTA-agarose (Qiagen, Hilden, Germany) for HisTag fusion proteins and Glutathion-Sepharose 4B (Amersham Pharmacia, Freiburg, Germany) for GST fusion proteins as described by the manufacturer.

White 4 to 5 month old New Zealand rabbits were immunized with the THIK-GST fusion protein derived from the pGEX expression system following standard protocols [31] (Pineda Antikörper Service, Berlin). Two animals were used for each THIK protein. After bleeding and deplementation, 2 ml aliquots of individual immune sera were passed through a Superdex 200 column (Amersham Pharmacia) to remove IgMs, and fractions containing IgGs were pooled. Crude IgG-fractions were tested for cross reactivity [31] by enzyme-linked immunosorbent assays (ELISA). Cross reactivities against the GST-fusion part and the paralogous THIK channel were removed following a recent protocol [32], resulting in monospecific antisera. These antisera were finally purified by affinity chromatography using nitrocellulose membranes loaded with the cognate recombinant THIK-TR or THIK-DHFR protein as a specific antigen. After elution, purified antibodies were concentrated by ion exchange chromatography on SP-Sepharose (Pharmacia) and stored in aliquots at -80° C until further use.

Antibodies. In double staining experiments, segments were identified using phalloidin for actin filaments marking the brush border of the proximal tubule (PT), anti-Tamm Horsfall protein (THP) antibody (gift from J. Hoyer, Philadelphia, PA), anti-type 2 NKCC-2 antibody (gift from D. H. Ellison, Portland, OR) for thick ascending limb (TAL), anti-Na⁺-Cl⁻-cotransporter (NCC) antibody (gift from D. H. Ellison, Portland, OR) for the distal convoluted tubule (DCT), and anti-calbindin D28k antibody (SigmaAldrich, Taufkirchen, Germany) for the connecting tubule (CNT) and cortical collecting duct (CCD).

Immunohistochemistry

Immunofluorescence. Immunohistochemical staining was performed on cryostat sections of the kidney or on OK cells fixed with 3% PFA/PBS. Sections were blocked with 5% skim milk/PBS, incubated with the respective primary antibody (see

antibodies listed above) at 4°C overnight, followed by an incubation with suitable cy-2- or cy-3-coupled secondary antibodies (Dianova, Hamburg, Germany). Sensitivity was satisfactory using anti-THIK-2 antibody, but less so using anti-THIK-1. To improve conditions for THIK-1 staining, preembedding technique was applied as detailed below. For double-labeling, suitable secondary antibodies coupled to different fluorochromes were applied (Dianova), or serial sections were used for direct comparison. Nuclei were additionally stained with 4',6-diamino-2-phenylindole (DAPI blue). Specificity of the double-staining procedures was controlled by parallel incubation of consecutive sections, each incubated only with one single probe. Sections were analyzed with a digital Spot camera from Diagnostic Instruments, processed with Meta View software from Universal imaging, as supported by Visitron System (Puchheim, Germany), and viewed with a Leica DMRB light microscope equipped with an HBO fluorescence lamp.

Preembedding technique. For fine structural immunolabeling, an established protocol was used [33]. Vibratome slices of 30 µm thickness were generated, incubated overnight with anti-THIK-1 in a 1:10 or 1:20 dilution, followed by incubation with HRP-coupled goat anti-rabbit antibody. Specific signal was generated using DAB and H₂O₂. Tissue specimens were postfixed with 1% osmium tetroxide, rinsed in maleate buffer, stained en bloc with uranyl acetate, and flat-embedded in Epon 812. Semithin sections were produced and photographed using a light microscope (Leica). Control experiments were performed by replacing the primary antibody with PBS.

Electrophoresis and SDS-Page

After Laemmli's sample buffer was added, the proteins were solubilized at 95°C for 3 min. SDS gel electrophoresis was performed on 10% polyacrylamide gels. After electrophoretic transfer of the proteins to nitrocellulose membranes, equity in protein loading and blotting was verified by membrane staining using 0.1% Ponceau red. Membranes were probed overnight at 4°C and then exposed to HRP-conjugated secondary antibodies (DAKO, Glostrup, Denmark) for 1h at room temperature.

Immunoreactive bands were detected on the basis of chemiluminescence, using an enhanced chemiluminescence kit (Amersham Pharmacia) before exposure to X-ray films (Hyperfilm, Amersham).

RT-PCR analysis

Human nephron segments were isolated from healthy cortical kidney pieces of patients (with their written consent) undergoing tumor nephrectomy. Selected tubules of a total length of 200 mm or ~400 glomeruli were lysed in 4 M guanidinium hydrochloride buffer and total RNA was prepared using the RNeasy Mini Kit (Qiagen). Isolated total RNA was incubated with 10 U DNaseI (Promega, Heidelberg, Germany) at 37°C for one hour to digest trace amounts of genomic DNA. cDNA synthesis was performed using MULV reverse transcriptase (Promega) and random hexanucleotides (Roche Diagnostics, Mannheim, Germany). A small aliquot (1/30th) of the cDNA was then subjected to a 50 µl PCR reaction using 20

Fig. 1. Characterization of anti-THIK antibodies. (A, B) Competitive ELISA of anti-THIK antibodies; coating with recombinant THIK-1-GST (A) or THIK-2-GST fusion proteins (B). Preincubation of antibodies with the cognate antigen (as GST or as DHFR fusion protein) but not with other, unrelated antigens decreased immunoreactivity in a concentration-dependent manner. (C, D) Western blot analysis of anti-THIK-1 (C) and anti-THIK-2 (D) antibodies with different fusion proteins (THIK-1-GST [30.7 kDa], THIK-1-DHFR [31.7 kDa], THIK-1-TR [24.7 kDa], THIK-2-GST [30.6 kDa], THIK-2-DHFR [31.6 kDa], THIK-2-TR [24.6 kDa]). Only the cognate antigen shows a specific signal. M: Biorad protein marker, molecular weight indicated in kDa. Lower bands in lane for THIK-1-CT-GST and THIK-1-CT-DHFR represent proteolytic fragments.

pmol of THIK specific primers, and 1 U of Taq DNA polymerase (Qiagen).

Primers for human THIK-1 (5'-TCA TCG TGC TCT ACC TGC TG-3'; 5'-TCC TAC TGT CGC CGG AGT-3') amplified a 290 bp fragment, primers for human THIK-2 (5'-GCT CAT CGG CCT CTA CCT G-3'; 5'-GAG GTT GAA GAA CAG GAT GGT C-3') amplified a 358 bp fragment. To prevent amplification from contaminating genomic DNA both primer pairs were intron-spanning. Thermal cycling was as follows: an initial cycle at 3 min. 94°C, 30 sec. 53°C and 1 min. 72°C; 30 cycles at 30 sec. 94°C, 30 sec. 53°C and 1 min. 72°C and a final 10 min. elongation at 72°C.

THIK expression in OK cells was also verified by RT-PCR analysis. Therefore, RNA from OK cells was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using Sensiscript reverse transcriptase (Qiagen). PCR-fragments for THIK-1 (388 bp) and THIK-2 (400 bp) were amplified using Advantage Taq Polymerase mixture (Clontech) and opossum-specific intron-spanning primers according to Mikkelsen et al. [34]: opoTHIK-1: 5'-GAG CCT GGA TGA GAT TCG AG-3' and 5'-TAC ACA GAG GGC TTC CAT CC-3'; opoTHIK-2: 5'-GCT GCT CTC TTT CCT TCG AG-3' and 5'-ATG CCC AGG ATA AGC ATC AC-3'.

Human and opossum PCR products were visualized on a 2% agarose gel and verified by direct DNA sequencing.

In situ hybridisation

The mRNA expression of THIK-1 and THIK-2 was studied by *in situ* hybridization using digoxigenin-labeled riboprobes (Roche). Sense and antisense probes were generated by *in vitro* transcription of a ~700 bp THIK-1 (nucleotides 994-1701 of Acc.No. AF287301) and ~650 bp THIK-2 (nucleotides 12121866 of Acc.No. AF287300) cDNA from T7 and SP6 promoter sites of pGEM-T, respectively. *In situ* hybridization was performed on 5 µm thick paraffin sections ac-

cording to an established protocol [30]. Signal was generated with 4-nitroblue tetrazolium chloride. For control, sense and antisense probes were applied to alternating sections.

Results

Characterization of anti-THIK antibodies

For the generation of monospecific antisera directed against THIK channels (K_{2p} 12.1 and K_{2p} 13.1) sequences of ~40 amino acid of the less conserved cytosolic C-terminus were selected as a fusion part for GST. Polyclonal antisera directed against the THIK-GST fusion proteins were raised in rabbits, freed from cross-reactivities and affinity purified.

Quality of the antibodies was evaluated by competitive ELISA indicating that the affinity-purified antibodies interacted only with the cognate antigen and showed no cross-reactivity against the GST-fusion part, the paralogous THIK-fusion proteins or the C-terminal domains of TASK-1, TASK-3, and TASK-5 proteins (Fig. 1A and B). In addition, Western blot analysis using purified fusion proteins showed specific labeling for the cognate THIK-fusion proteins only (Fig. 1C and D). Western blot analysis of isolated rat and mouse kidney membrane fractions showed a specific band at ~52 kDa for THIK-1 and at ~49 kDa for THIK-2. Both THIK-proteins are slightly larger than the predicted molecular weights of 45.1 kDa (THIK-1) and 47.0 kDa (THIK-2) (Fig. 2A and C). This small difference may be the conse-

quence of aberrant electrophoretic mobility of the THIK membrane protein or an as yet unidentified posttranslational modification, such as glycosylation or phosphorylation. Preabsorption of the antibodies with the THIK-GST fusion protein resulted in strongly reduced or abolished signals, confirming specificity of our antibodies (Fig. 2B and D).

Identification of kidney structures

For the precise assignment of THIK-1 and THIK-2 expression to the distinct portions of the renal tubule, we used morphological criteria as well as the segmental coexpression of defined markers [35]. Glomerular and juxtaglomerular cells were identified by micromorphological criteria. The PT was recognized by the presence of its brush border which was identified morphologically or by phalloidin staining of the actin filament bundles. Thin limbs were identified from their respective transition points. Medullary and cortical thick ascending limb (mTAL and cTAL, respectively) were identified by the presence of THP and NKCC2, the macula densa (MD) by its juxtaglomerular localization and absence of THP, DCT by the presence of NCC, and parts of DCT and CNT by the expression of calbindin D28k. Collecting ducts were identified by the transition from calbindin-positive CNT towards CCD, their decreased epithelial height, and the presence of intercalated cells. Interstitial cells were identified based on their shape and location.

Localization of THIK-1 in rat and mouse kidney

Using preembedding technique it was demonstrated that THIK-1 was expressed in single scattered cells of the rat PT, DCT and CCD (Fig. 3A and B; for an overview, see Fig. 9). In cells of PT and principal cells of CCD and CNT signal was generally observed in intracellular compartments (Fig. 3A and B). Intercalated cells of CNT and CCD were generally devoid of THIK-1 protein. Preabsorption with the cognate recombinant GST fusion protein blocked specific staining of anti-THIK-1 (Fig. 3C and D).

Due to weak immunostaining in mice, *in situ* hybridization was considered more representative for the localization of THIK-1. Mouse kidneys showed a strong mRNA signal in the *pars recta* of PT of medullary rays (Fig. 4A). In the *pars convoluta* only a weak THIK-1 mRNA signal was present. TAL (Fig. 4A), CNT and CCD (Fig. 4B) revealed prominent staining of THIK-1 mRNA. The MD as a specified TAL segment has been positive for THIK-1 mRNA. Labeled sense THIK-1 mRNA did not produce a signal (Fig. 4C). These results are in good

Fig. 2. Expression analysis of THIK channels in rat and mouse kidneys. (A, C) Western blot analysis with anti-THIK-1 (A) and anti-THIK-2 (C) of membrane fractions from rat (left lane) and mouse kidney (right lane). Pre-incubation of the antibody with the respective GST-fusion protein results in decreased or absent THIK-1 (B) and THIK-2 signals (D). Molecular weight in kDa as indicated on the left.

agreement with our immunohistochemical data.

Furthermore, opossum kidney (OK) cells were used as a common cell culture model of PT epithelial cells to verify anti-THIK-1 PT labeling in rat and mouse kidney. The rat THIK-1 epitope used for immunization is 64% identical to the THIK-1 sequence of the recently sequenced opossum *Monodelphis domestica* genome [34]. THIK-1 labeling in OK cells showed strong cytoplasmic staining with a vesicular distribution (Fig. 5A). Cells were stained with fluorescent phalloidin which labels actin filaments for visualization in confluent monolayers (Fig. 5B and D).

No staining was observed when the anti-THIK-1 antibody was replaced by PBS (Fig. 5C). Western blot showed a specific band at ~52 kDa for THIK-1 protein (Fig. 5E). Preabsorption of western blot reduced this signal nearly completely (Fig. 5E). These results demonstrate strong expression of THIK-1 in OK cells with a dominant intracellular localization supporting proximal

Fig. 3. Localization of THIK-1 in rat kidney. (A and B) Immunostaining of THIK-1 protein on rat kidney cortex, semithin section. (A) Anti-THIK-1 staining is present in the proximal tubule (cross) and cortical collecting duct (CCD, circle). Positive individual proximal tubule cells (arrowheads) and principal cells of CCD (arrows) show strong intracellular labeling. (B) Anti-THIK-1 staining is present in the connecting tubule (CNT, asterisk) just underneath a cortical vein. CNT cells (arrowheads) reveal strong intracellular immunolabeling. (C and D) Anti-THIK-1 staining after pre-incubation of the antibody with specific GST-fusion protein on rat kidney cortex, semithin section. (C) No staining is present in the proximal tubule (cross) and CCD (circle, D) as well as in CNT (asterisk, D). Bar = 50 μ m throughout.

Fig. 4. Localization of THIK-1 mRNA in mouse kidney. (A and B) Localization of THIK-1 mRNA by in situ hybridization in mouse kidney. (A) THIK-1 mRNA in thick ascending limb (circle). Transition of the convoluted to the straight part of proximal tubule coincides with an increase in signal intensity (asterisk). (B) THIK-1 mRNA most prominently expressed in the connecting tubule (CNT, cross) and cortical collecting duct (CCD, double cross). Note, the transition of CNT to CCD. Bar = 50 μ m throughout. (C) No signal is apparent when sense THIK-1 mRNA was applied.

tubular expression. To verify expression of THIK channels in OK cells we amplified opossum specific DNA fragments from OK cell RNA by RT-PCR. The resulting OK cell (species *Didelphis virginiana*) THIK-PCR products of 388 bp (THIK-1) and 400 bp (THIK-2) were 97% and 98% identical to the sequences of *Monodelphis domestica* [34], unequivocally demonstrating that both THIK channels are expressed in OK cells.

Localization of THIK-2 in rat and mouse kidney

In rat kidney, antibodies against THIK-2 showed a weak intracellular labeling within single, scattered cells of the PT revealing stronger expression in the *pars recta* of outer stripe, than in the cortical parts (as indicated by an asterisk in Fig. 6B and D; for an overview, see Fig. 9). Double-labeling with anti-THP revealed an intracellular

expression of THIK-2 in mTAL (Fig. 6A and B) and cTAL (Fig. 6C and D). The MD, as identified by the absence of THP, also showed THIK-2 expression. Double-labeling with anti-calbindin revealed likewise an intracellular expression of THIK-2 protein in the CNT (Fig. 6E and F). Intercalated cells, as defined by the absence of calbindin D28k labeling within the distal convolutions and collecting duct system, showed no THIK-2 staining (arrowheads, Fig. 6E and F).

Immunoreactive THIK-2 protein in mouse kidney showed quite strong expression, particularly along the proximal nephron, and an even more pronounced signal in the distal nephron and collecting duct system (for an overview, see Fig. 9). In the PT anti-THIK-2 staining was observed in the basolateral membrane as shown by doublestaining of the actin skeleton in the brush border

Fig. 5. Localization of THIK-1 in opossum kidney cells. (A-D) THIK-1 expression in opossum kidney (OK) cells. (A and B) Confluent monolayers double-stained with fluorescence-labeled anti-THIK-1 (intracellular red staining, A) and fluorescent phalloidin staining of actin filaments (green, B). (C) For negative control, cells were incubated with omission of specific antibody and double-stained with phalloidin (green, D) nuclei were stained blue with DAPI. Bar = 25 μ m throughout. (E) Western blot analysis of THIK-1 expression in OK cells shows a prominent band at a molecular weight of 50-52 kDa (left lane); pre-incubation of the antibody with THIK-1 GST-fusion protein strongly reduced the signal. Molecular weight in kDa as indicated on the left. (F) RT-PCR analysis of THIK-1 and THIK-2 expression in OK cells. The arrow indicates the PCR products of the expected size (400 bp). DNA marker (in bp) are indicated.

and basement membrane (Fig. 7A-C). Both, mTAL and cTAL including the post-macula segment showed strong, basolaterally enhanced signals (Fig. 7D-M). The MD as well as positive for THIK-2 (data not shown). A strong signal was further detected in CNT (Fig. 7M-P). In transition to the CCD the intensity of the basolateral THIK-2 expression continuously tapered out towards the medulla (Fig. 7R-T). Intercalated cells of the distal

Fig. 6. Localization of THIK-2 in rat kidney. (A-D) Double labeling of Tamm-Horsfall protein (THP, A and C) for the identification of thick ascending limb (TAL) and THIK-2 (B and D). (A) Identification of medullary TAL by anti-THP staining. (B) Anti-THIK-2 staining is present in medullary TAL and is co-localized with THP. Occasional proximal tubular (asterisk) and collecting duct (double cross) staining is encountered. (C) Identification of cortical TAL by anti-THP labeling. (D) Anti-THIK-2 staining is present in cortical TAL. Proximal tubular staining is marked (asterisk). (E and F) Double labeling of calbindin D_{28k} (E) for identification of connecting tubule (CNT) and THIK-2 (F). Strong CNT labeling for THIK-2 was found. Intercalated cells (arrowheads in E and F) as identified by absent calbindin D_{28k} staining, show no THIK-2 expression. Bar = 50 μ m throughout.

convolute and collecting duct showed no specific staining.

Blockade with THIK-2 GST-fusion protein strongly reduced the specific labeling in mouse kidney (Fig. 7U).

For the verification of the immunohistochemical results, *in situ* hybridization (ISH) of THIK-2 mRNA was performed on mouse paraffin sections. A weak THIK-2 ISH signal was observed in the cortical PT, with an increase in the outer stripe (Fig. 7V). Most prominent ex-

Fig. 7. Localization of THIK-2 in mouse kidney. Nephron segments were identified by fluorescent phalloidin (A), anti-NKCC-2 (D, G), anti-NCC (K, N), and anti-calbindin D_{28k} staining (R) combined with double-staining for fluorescence-labeled anti-THIK-2 (B, E, H, L, O and S). Merge images (C, F, J, M, P, T) show nephron segment marker (green), anti-THIK-2 (red) and nuclear DAPI staining (blue). (A-C) Proximal tubule S3 segments of a medullary ray. (A) Fluorescent phalloidin stains actin filaments within brush border and basement membrane. (B) Anti-THIK-2 stains the basolateral membrane. (C) Merge. (D-F) Medullary thick ascending limb profiles. (D) Anti-NKCC2 labels the luminal aspects. (E) Anti-THIK-2 stains the basolateral membrane of medullary thick ascending limb. (F) Merge. Dashed line indicates border between outer and inner medulla. (G-J) Transition (bar) between cortical thick ascending limb (small circle; cTAL) and distal convoluted tubule (asterisk; DCT). (G) Anti-NKCC-2 staining ends with the begin of the DCT. (H) Anti-THIK-2 shows a strong continuous basolateral signal. (J) Merge, G marks the glomerulus. (K-M) Transition (bar) between cTAL (small circle) and DCT (asterisk). (K) NCC is located luminal in the DCT. (L) Anti-THIK-2 shows basolateral immunoreactivity in cTAL and DCT. (M) Merge, G marks the glomerulus. (N-P) Transition (bar) between DCT (asterisk) and connecting tubule (cross; CNT). (N) Apical NCC ends at the beginning of CNT. (O) Anti-THIK-2 staining continues from DCT into CNT with same intensity. (P) Merge. (R-T) Transition (bar) between CNT (cross) and cortical collecting duct (double cross; CCD). (R) Anti-calbindin D_{28k} localization ends at the transition to the CCD. (S) Basolateral anti-THIK-2 signal continuous from CNT into CCD with somewhat less intensity. (T) Merge. (U) Anti-THIK-2 labeling after pre-incubation with specific GST-fusion protein shows no signal. (V and W) Localization of THIK-2 mRNA by in situ hybridization on mouse kidney. (V) THIK-2 mRNA is expressed in profiles of the proximal tubule S3 segment (asterisks). (W) Strong THIK-2 mRNA signals are observed in CNT (cross) and CCD (double cross). Bar (A-C) = 25 μ m, bar (D-T) = 50 μ m, bar (U) = 100 μ m and bar (V and W) = 50 μ m.

pression was detected in distal segments, in CNT, and in CCD (Fig. 7W). Overall mRNA and protein expression of THIK-2 were in agreement.

Localization of THIK channels in human kidney

To compare the expression pattern found in mouse and rat kidneys RT-PCR analysis of microdissected parts of human nephrons was performed. For both THIK pro-

teins, a specific signal was found in PT, TAL and CCD but not in glomeruli (Fig. 8). Control GAPDH expression was found in all four samples (shown only for glomeruli in Fig. 8). PCR signal for human THIK-1 was strong in all three tubular portions tested, whereas bands for human THIK-2 were strong in CCD but weak in PT and TAL. Accordingly, these patterns were in good agreement with our findings in rodent kidneys.

Fig. 8. RT-PCR analysis of THIK expression in human kidney. Microdissected nephron segments of human glomeruli (Glo), proximal tubule (PT), thick ascending limb (TAL) and cortical collecting duct (CCD) were used for production of cDNA. PCR products of 290 bp for human THIK-1 and 358 bp for human THIK-2 were amplified. In addition a negative control of isolated RNA from CCD without reverse transcription and a positive control (GAPDH expression) for RNA samples isolated from glomeruli are shown.

Discussion

K^+ -channels are involved in the most basic processes such as cell proliferation, excitability of muscle, heart and nerves, pH and volume regulation as well as the maintenance of the cell resting potential. The kidney is the major organ that regulates K^+ -balance, and thus prevents severe disturbances of basic body functions. Even after significant nephron loss, K^+ -homeostasis is still kept stable [36].

So far, a lot of different K^+ -channels have been described in the kidney with electrophysiological and molecular biological methods [1, 37, 38]. Only for some of them a physiological role and function has been clearly determined, such as for the ROMK-channel in the thick ascending limb and cortical collecting duct [1]. It serves as a K^+ -recycler and essential K^+ -deliverer for the NKCC-2 in the TAL [39, 40] and as the main K^+ -secreting channel and fine regulator for the K^+ -transport in the cortical collecting duct [41]. Another functionally characterized channel is the Ca^{2+} -dependent BK-channel, which is expressed in nearly every cell. In the kidney, its physiological role was unclear until several groups demonstrated its role as an essential volume regulator [42-44]. K^+ -channels located in the basolateral membranes of nephron cells are considered to be housekeeping channels that are functionally coupled to the Na^+/K^+ -ATPase [45-48].

The two novel K^+ -channels, THIK-1 and THIK-2, that are expressed in the kidney belong to the family of K_{2p} -channels [28]. K_{2p} -channels are found throughout

Fig. 9. Schematic representation of THIK expression in the mammalian kidney. The distribution of THIK-1/-2 along the mammalian nephron and collecting duct system is shown. Both antigens have the same localization but slight differences in intensity and subcellular distribution were observed.

the nephron, from glomeruli to collecting duct [18, 20, 22, 23, 27]. As shown in the present study, this is also true for THIK-1 and THIK-2, with the exception of the glomeruli. THIK-1 shows strong immunohistochemical staining from proximal tubule to the collecting duct, whereas THIK-2 shows strongest expression towards distal parts of the nephron, namely TAL, DCT, CNT and CCD. A strong signal for both K^+ -channels was also obtained in MD cells. In mice, THIK-2 is localized to the basolateral membrane whereas in rat THIK-1 and THIK-2 were mainly localized in membrane vesicles of PT, distal tubule and CCD. Such intracellular localizations are not uncommon among transport proteins. Especially in the CCD such a mechanism is well known from the secreting K^+ -channel ROMK, the amiloride-sensitive Na^+ -channel ENaC and the water channel aquaporin-2 [49-53]. The minor variation in the expression pattern observed between rat and mouse is not surprising because species-specific differences occur quite frequently. Even expression differences in the kidney between two strains of the same species are known [54].

Compared to the other K_{2p} channels much less is known about the physiological regulation and function of THIK channels. A few reports indicate that THIK-1 currents are potentiated by arachidonic acid [28] and

inhibited by hypoxic conditions [29]. Considering O_2 -sensitivity, the expression of THIK channels in the PT and TAL is of particular importance as they might contribute to the energy efficiency by decreasing Na^+ - and Cl^- -transport in these segments under hypoxic conditions.

Even though our localization data of the THIK-channels does not offer answers to the physiological role and function of these channels, it provides us with two interesting points that could be the foundation for future studies:

1) Considering the distribution pattern of THIK-1 in the nephron, it becomes apparent that it matches exactly that for ROMK [55] even though a direct interaction can be excluded. However, of interest is the distribution of THIK-1 in MD cells, which are responsible for the tubuloglomerular feedback (TGF) mechanism [56]. MD cells possess NKCC-2 and at least one K^+ -channel responsible for the K^+ -recirculation on the apical side of the membrane [57]. Whereas ROMK has been shown to fulfill this task in the TAL, one could assume that ROMK is responsible for the K^+ -recirculation in MD cells as well. However, the only electrophysiological description and regulatory patterns of a K^+ -channel described in MD cells so far do not fit the characteristics of ROMK [57]. This intermediate-conductance K^+ -channel is inhibited by calcium ions, independent of ATP concentration and can not be activated by cAMP or forskolin [57]. It has to be determined whether THIK channels contribute to this conductance.

2) So far, several basolateral K^+ -currents of the nephron have been described [45, 58-63] and two have been identified in principal cells of the cortical collecting duct [64, 65]. These basolateral K^+ -currents show regulatory properties that could easily explain the absence of THIK-2 currents when THIK-2 protein is expressed in an artificial expression system such as *Xenopus laevis*. The K^+ conductances were highly active in cell-attached patches but showed immediate rundown when the basolateral membrane was excised indicating the loss of structural proteins or regulators [61]. These necessary

regulators include cGMP and PKG II, the membrane-bound cGMP-dependent protein kinase [66]. When Ca^{2+} was added to the internal solution, channel activity shuts down immediately [61]. In nearly 70% of 200 attempts only silent patches were detected. Like most K^+ -channels in the kidney these channels were pH-sensitive [64]. Because of its localization, THIK-2 is a likely candidate for this K^+ -channel, responsible for the recycling of K^+ across the basolateral membrane. In addition, THIK-2 could be directly coupled to the activity of the Na^+/K^+ -ATPase, since inhibition of the pump by ouabain leads to an increase in cellular Na^+ -concentration. Consecutively the activation of a Na^+/Ca^{2+} -exchanger leads to an increase in intracellular Ca^{2+} . As a result, an inhibition of the basolateral K^+ -channel would occur [61].

Conclusions

The detailed comparative localization study of the expression of THIK-1 and THIK-2 in mouse, rat, and human kidney presented here, adds two more players to the diversity of renal K^+ -channels. We showed that both K^+ -channels are widely distributed throughout the nephron from PT to CCD. The localization of these channels gives us some interesting clues, especially concerning their function in MD cells and principal cells of the CCD. However, further studies need to confirm the physiological role for these two novel K_{2p} channels in the kidney.

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