Localization of Inward Rectifier Potassium Channel Kir7.1 in the Basolateral Membrane of Distal Nephron and Collecting Duct

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Potassium channels in the kidney maintain cell-negative potential, K+ recycling at the cell membrane, K+ secretion, and cell volume (1,2). Patch-clamp methods have identified a variety of K+ channels, including an inwardly rectifying K+ channel (Kir), an ATP-sensitive K+ channel, a Ca2+-dependent maxi K+ channel, and a voltage-gated K+ channel (1,2). Some of these K+ channels have been cloned, and their amino acid sequences and molecular structures have been clarified (3). The amino acid sequence of the Kir family reveals two pore-forming regions (H5). Kir does not possess a voltage sensor (S4) or the inactivation ball motif seen on voltage-gated K+ channels (Kv) (4,5) but does form a tetramer to make a putative K+ pore (6,7).

Kir channels are now recognized as forming a superfamily of seven members (Kir1 through Kir7) with distinct cellular and subcellular locations, patch-clamp properties, and physiologic functions (3,8–10). The most recently identified member, termed Kir7.1, has 360 amino acid residues with a less than 40% similarity to other Kir channel proteins and exhibits unique pore properties (11–14): The permeability of Kir7.1 is almost independent of external K+, which is in marked contrast to other members of the Kir family. Northern blot analysis of human and rat tissues with a selective cDNA probe for Kir7.1 revealed a major transcript of 3.4 kb that is expressed in the small intestine, stomach, kidney, and brain (11–14). We have also independently cloned human and rat Kir7.1 (accession numbers AB013889 and AB013890) from the kidney and raised a specific antisera (14). The distribution, function, and regulation of expression of Kir7.1 in the kidney are unknown. Therefore, the aims of this study were (1) to localize Kir7.1 along the nephron, (2) to study its subcellular expression using immunoelectron microscope techniques, and (3) to determine the levels of Kir7.1 mRNA in the kidney under low- and high-K diets.

Materials and Methods

Animal Preparation

For immunohistochemical studies, six male Sprague-Dawley rats (180 to 200 g) were fed a standard rat chow and given tap water to drink. Rats were anesthetized with pentobarbital (100 mg/kg intraperitoneally). The kidneys were perfused with phosphate-buffered saline (PBS) through a cannula inserted into the abdominal aorta to flash out the blood, and then followed by perfusion with periodate-lysine-paraformaldehyde (PLP) solution (0.01 M NaIO4/0.075 M lysine/2% paraformaldehyde/0.0375 M phosphate, pH 6.2). Kidney slices were immersed in PLP solution overnight at 4°C. For immunogold studies, 1-mm3 blocks of tissue were embedded in Lowicryl.
The remaining tissue was processed for the pre-embedding immunoperoxidase method. To provoke hypokalemia, seven male Sprague-Dawley rats, approximately 5 wk of age (120 to 130 g), were placed on a K⁺-deficient diet (8 mg K/100 g; Funabashi Nojoh, Chiba, Japan) for 15 d and compared with seven control rats fed a normal diet (860 mg K/100 g). In the other seven rats, hyperkalemia was induced by a K⁺-deficient diet with 4% KCl drink. Three rats of each group were used for the immunohistochemistry, and four rats of each group were used for the RNase protection assay. All animal experiments were conducted in strict accordance with the Prime Minister’s Office guidelines and were approved by the Animal Studies Committee of the Research Center for Experimental Biology, Tokyo Institute of Technology.

**Western Blotting**

As described previously (15,16), kidneys were removed immediately after perfusion with ice-cold saline and cut into cortex and inner medulla. The tissue was homogenized on ice with a Teflon glass tissue homogenizer (Iwaki, Chiba, Japan) in 4 ml of buffer A containing 50 mM Tris-HCl (pH 7.4), 0.2 mM ethylenediaminetetraacetate, 0.2 mM ethyleneglycol-bis(β-aminoethylether)-N,N′-tetraacetic acid, 2 mM leupeptin, 50 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 12,000 × g for 20 min, and the supernatants were centrifuged at 100,000 × g for 1 h. The membrane pellet was solubilized in buffer A with the addition of 1% Triton X-100. It was centrifuged at 12,000 × g for 20 min. The supernatant was diluted in sodium dodecyl sulfate (SDS) sample buffer (0.5 M Tris-HCl pH 6.8/10% glycerol/2% SDS/5% β-mercaptoethanol/0.05% bromophenol blue). Samples containing 50 μg of protein were separated by SDS/polyacrylamide gel electrophoresis on a 10% gel and electroblotted to nitrocellulose membranes. After incubation with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h, membranes were incubated with a polyclonal antibody for Kir7.1 at a 1:1,000 dilution overnight. The rabbit antibody to Kir7.1 was raised against the C-terminal peptide (GC)INGQSIDNFQIDE (amino acid residues 343 to 360 of the human Kir7.1 sequence with additional N-terminal glycine and cysteine) (14). The extra glycine and cysteine residues were used to couple the peptide to a carrier protein, keyhole limpet hemocyanin, before immunization. After being rinsed in TBST, membranes were incubated for 1 h with HRP-conjugated anti-rabbit Ig antibody at a 1:1,000 dilution (Dako, Glostrup, Denmark). The blots were detected by 0.8 mM dianisobenzidine (Dojin Laboratories, Kumamoto, Japan) with 3 mM nickel chloride and 0.3% hydrogen peroxide.

**Microdissection of Tubule Segments**

The kidneys from male Sprague-Dawley rats that weighed 120 g were processed for microdissection study as described previously (17,18). Three young, germ-free rats were used to facilitate tubule microdissection. In brief, the kidneys were perfused with 10 ml of cold dissection buffer containing 135 mM NaCl, 5 mM KCl, 1 mM NaH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, and 5.5% glucose. Thin sagittal kidney slices were incubated in dissection buffer with 0.1% collagenase at 37°C for 30 min. The tubule segments were microdissected in cold dissection buffer without collagenase under a stereomicroscope. Each 20 segments of proximal tubule, distal convoluted tubule (DCT), connecting tubule (CNT), cortical collecting duct (CCD), outer medullary collecting duct (OMCD), inner medullary collecting duct (IMCD), and glomeruli were microdissected, separated, and dissolved in the SDS sample buffer as mentioned above. The tubule samples were sonicated three times before Western blotting.

**Light and Electron Microscopic Immunoperoxidase Procedures**

Sections (50 μm) of each kidney were cut on a vibratome and processed for pre-embedding electron microscopic (EM) immunoperoxidase as described previously (15,16,19). They were washed with 50 mM NH₄Cl in PBS and incubated in TBST for 30 min, followed by incubation with the primary antibody against Kir7.1 at 1:200 dilution overnight at 4°C. For a negative control, other sections were incubated with TBST alone. After rinsing with TBST, the sections were incubated with HRP-conjugated secondary antibody against rabbit Ig (Dako, Glostrup, Denmark) for 2 h, rinsed, and fixed with 1% glutaraldehyde in PBS for 1 h and rinsed again. The HRP labeling was detected by incubation with 0.8 mM dianisobenzidine. The sections were postfixed in 2% osmium tetroxide in 0.1 M PBS buffer for 1 h at 4°C and embedded in epoxy resin. Sections (1 μm) were cut and stained with toluidine blue for light microscopy. Ultrathin sections for electron microscopy were cut on an ultramicrotome, stained with lead citrate, and observed on a transmission electron microscope (Hitachi H-7000, Tokyo, Japan).

**Postembedding Immunogold Procedure**

Ultrathin sections of the Lowicryl blocks were cut and mounted on collodion-coated nickel grids and then processed for immunogold labeling as described previously (15,16). Briefly, the sections were incubated with 0.1 M NH₄Cl for 1 h and rinsed with buffer solution (0.02 M Tris-HCl pH 7.2/0.15 M NaCl/0.05% Tween 20) for 15 min. The sections were incubated with the following primary antibodies at 4°C overnight: rabbit polyclonal antisera against Kir7.1 (1:200) and mouse monoclonal antisera against α1 subunit of Na⁺, K⁺-ATPase (Wako, Osaka, Japan; 1:50). After the sections were washed with the buffer solution, they were incubated with the following secondary antibodies at a dilution of 1:50 for 2 h: 25 nm gold-labeled goat anti-rabbit IgG antibody (Aurion, Wageningen, The Netherlands) for visualization of Kir7.1 and 10 nm gold-labeled goat anti-mouse IgG antibody (Aurion) for Na⁺, K⁺-ATPase. The sections were washed with the buffer solution, incubated with 2% glutaraldehyde in PBS for 30 min, rinsed with distilled water, counterstained with uranyl acetate and lead citrate, and examined under the electron microscope.

**Northern Blot Analysis and RNase Protection Assay**

To determine the changes in the Kir7.1 mRNA expression during the diet-induced hyperkalemia and hypokalemia, poly(A)-rich RNA were isolated from the kidneys of each group and analyzed by Northern hybridization as described previously (14,20). Message levels of the Na⁺,K⁺-ATPase α1 subunit were determined similarly using a PCR-amplified cDNA probe (nucleotides 406 to 938, accession number M28647). The same blots were probed sequentially for β-actin mRNA, which served as an internal control.

RNase protection analyses of total RNA preparations were performed according to the published method (20). Briefly, single-stranded antisense RNA probes were generated from the linearized plasmids containing portions of the rat Kir7.1 sequence (477 bp) and the above-mentioned Na⁺, K⁺-ATPase α1 subunit sequence (533 bp) using T3/T7 polymerase in the presence of [α-³²P] UTP. Total RNA (10 μg) was hybridized with the antisense RNA probes (1 × 10⁵ cpm/sample) at 42°C overnight. After RNase digestion, the protected fragments were electrophoresed on urea-denaturing gels and exposed
to an imaging plate. Quantification ($n = 4$) was performed with a Fujix BAS2000 Bioimage Analyzer (Fuji Film) using photostimulated luminescence values.

**Results**

**Protein Expression for Kir7.1**

Western blotting, using the previously characterized antiserum (14), detected Kir7.1 immunoreactive protein expression in membrane preparations of the rat kidney with a molecular mass of 52 kD (Figure 1A). A specific band for Kir7.1 at the same molecular size as identified in extraction of kidney cortex was observed in the tubule segments microdissected from the DCT, CNT, and CCD. Weak band for Kir7.1 was also observed in the nephron segments from the thick ascending limb (TAL) of loop of Henle and in the OMCD and IMCD. Kir7.1 was not observed in the glomeruli, but a very faint band was observed in the proximal convoluted tubules (Figure 1B).

**Light Microscopic Distribution of Kir7.1**

Light microscopic observation of 1-μm sections using pre-embedding methods demonstrated clear immunostaining for Kir7.1 in the rat kidney. Kir7.1 was prominent in the TAL of loop of Henle, DCT, and CCD (Figure 2a). The proximal tubule, macula densa cells, glomeruli, or renal vasculature were not stained (Figure 2a). Using a high magnification, staining for Kir7.1 was observed in the basal areas of cells in the DCT and principal cells in the CCD (Figure 2, b and c). Immunostaining for Kir7.1 was also weakly expressed in the TAL of the loop of Henle (Figure 2d) and in the basal membrane of the OMCD (Figure 2d) and the IMCD (Figure 2e). The negative controls showed no staining of any structures in the kidney (Figure 2f).

**EM Distribution of Kir7.1 Immunoreactivity**

The pre-embedding EM method revealed that Kir7.1 was detected predominantly in the basal area of the DCT but not in the apical membrane (Figure 3A). In high magnification, Kir7.1 was located exclusively on the membrane of basolateral invagination. There was no immunostaining on the nucleus, mitochondria, or cytoplasm (Figure 3B). The basolateral membranes of the principal cells of the CCD were stained with the antibody for Kir7.1, but neither type A nor type B intercalated cells were stained (Figure 3, C and D).

Using double-labeling EM immunogold methods, we revealed that both Kir7.1 (labeled with large particles) and Na$^+$, K$^+$-ATPase (small particles) were located on the basolateral membranes of a principal cell in the CCD (Figure 4). The number of gold labels was less in Kir7.1 than in Na$^+$, K$^+$-ATPase, and they were located independently on the basal membrane. The distribution of Kir7.1 is summarized in Table 1.

**Effects of High-K and Low-K Diets on Kir7.1 Levels**

Northern blot analysis using poly(A)-rich RNA from kidneys of rats on a potassium-depleted diet showed a dramatic reduction in the Kir7.1 message level. To confirm this initial result, we next performed the following series of experiments using total RNA and RNase protection analysis, which is much more sensitive and accurate than Northern analysis.

After 2 wk on a low-K$^+$ diet, the rats exhibited a reduced plasma level of potassium from $4.57 \pm 0.47$ to $2.60 \pm 0.36$ mM ($n = 3$ for each group). There was a marked decrease in the Kir7.1 mRNA (Figure 5A) with a concomitant decrease of the immunoreactive protein for the Kir7.1 protein (Figure 5B(a)). However, 4% KCl added to the drinking water of rats that were given the low-K$^+$ diet reversed the plasma potassium concentration ($4.82 \pm 0.44$ mM), the Kir7.1 mRNA expression (Figure 5A), and immunoreactivity (Figure 5B(b)). These results indicate that the expression of Kir7.1 in the kidney depends on plasma K$^+$ concentration and suggest that Kir7.1 may have a role in maintenance of K$^+$ homeostasis. Compared

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**Figure 1.** Western blot analysis of membrane fraction of kidney cortex (A) and microdissected nephron segments (B). A prominent band for Kir7.1 is observed at the molecular weight of ~52 kD. Kir7.1 was detected in the segments of distal convoluted tubule (DCT), connecting tubule (CNT), and cortical collecting duct (CCD). There was weak band for Kir7.1 in the thick ascending limb of loop of Henle (TAL), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD) and only faintly in proximal tubule (PT) but not in glomeruli (Glm).
Discussion

The main new finding of this study is that a novel inwardly rectifying K\textsuperscript{+} channel, Kir7.1, is expressed on the basolateral membrane of cells from the DCT, CNT, and CCD of rat kidney, and its expression changes in response to plasma K\textsuperscript{+} concentration. Kir7.1 is also weakly expressed in the TAL of Henle and in OMCD and IMCD. The localization of Kir7.1 was confirmed by Western blot of microdissected tubule segments. The pre-embedding EM method identified that the cells with the large changes in Kir7.1, only slight changes were observed in the message levels of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase (Figure 5A).

Figure 2. Light micrographs from 1-\textmu{}m sections of pre-embedding electron microscopic samples for Kir7.1 in the rat kidney. Immunostaining for Kir7.1 was observed in the TAL of loop of Henle (∗ in a and d), DCT (▲ in a and b), CNT (△ in c), and CCD (○ in c). Kir7.1 was weakly stained in the IMCD (●) and very faintly in the OMCD (○ in d). Some cells in the CNT and CCD were not stained (arrowhead in c). There was no immunostaining for Kir7.1 in the PT and macula densa (arrow in a). In the negative control, no immunostaining for Kir7.1 was observed in any of these structures (f). Magnifications: a, ×250; b through f, ×400.
Figure 3. Electron micrograph illustrating Kir7.1 immunostaining using the pre-embedding immunoperoxidase procedure. Cells of the DCT (A and B) and principal cells in the CCD (PC in C and D) were immunoreactive for Kir7.1 in the basal area of cells. High magnification of DCT showed that Kir7.1 is exclusively present on the membrane of basolateral invaginations, and not in the cytoplasm or organelles (B). The type A (αIC in C) and type B (βIC in D) intercalated cells were negative for Kir7.1. N, nucleus; M, mitochondria. Magnifications: A, ×5700; B, ×12,000; C, ×6000; and D, ×5000.
that express Kir7.1 in the CCD are principal cells, and no immunostaining was detected in types A or B intercalated cells. Kir7.1 has discrete sites of localization that are summarized in Table 1. The localization of Kir7.1 is similar to Kir4.1, which is expressed over the basolateral membranes of the DCT, CNT, and CCD (21).

As for the K⁺ channels that have been demonstrated in the basolateral membranes of renal tubule cells, there are currently five channels, including Kir7.1 and Kir4.1, whose structures are known. The other three channels have been identified as ATP-insensitive K⁺ channels by electrophysiologic measurements and have not been cloned yet: an inwardly rectifying low-conductance channel (27 to 30 pS), a 67-pS ohmic channel, and hyperpolarization-activated, intermediate-conductance channel (50 to 90 pS) (22–27). The possible identity of Kir7.1 with these electrophysiologically identified ATP-insensitive K⁺ channels should be addressed by future studies, but it may deserve consideration here. The lower conductance, ATP-insensitive, basolateral K⁺ channels are candidates for a hypothetical low-conductance K⁺ channel that is considered to play a major role in the basolateral potassium conductance. In the basal state, it allows K⁺ efflux and thus maintains cell interior-negative membrane potential. After stimulation by mineralocorticoid, the basolateral membrane potential becomes hyperpolarized because of increased activity of the electrogenic Na⁺,K⁺-ATPase. Under these conditions, K⁺ enters the cell via the inwardly rectifying conductance channels in the basolateral membrane. This provides K⁺ for apical secretion during stimulation with mineralocorticoid. Similar situations occur when a high-potassium diet is ingested and plasma K⁺ concentration increases, which in turn directly stimulates the mineralocorticoid production in the zona glomerulosa cells of the adrenal gland. Recently, CCD-IRK3 was also identified in the basolateral membrane of a CCD cell line (28). This is another candidate for the small conductance basolateral K⁺ channel. It has a high open probability, a voltage independence, and a small conductance (14.5 pS) (28). However, renal expression of CCD-IRK3 remains to be demonstrated. The Kir4.1 is a candidate for the low-conductance K⁺ channel expressed in

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a PT, proximal tubule; DL, thin descending limb of loop of Henle; TAL, thick ascending limb of loop of Henle; MD, macula densa; DCT, distal convoluted tubule; CNT, connecting tubule; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct.

Figure 4. Electron micrographic immunogold labeling for Kir7.1 and Na⁺, K⁺-ATPase in the DCT cell. Kir7.1 was labeled with 25 nm of immunogold and Na⁺, K⁺-ATPase, with 10-nm particles. Most of the labels for Kir7.1 and Na⁺, K⁺-ATPase are seen on the membrane of the basal infoldings. Magnification, ×53,000.
the distal nephron. The Kir4.1 channel is expressed over the basolateral membrane of the distal nephron, although the type of cell has not been defined (21). Kir4.1 has moderately strong inward rectification via a high- and low-conductance pathway of 36 and 21 pS (29). However, several distal segments do not express Kir4.1 protein; moreover, the role of basolateral intermediate conductance of Kir4.1 is not clear. Our finding that Kir7.1 is expressed in the basolateral cell membranes of specific cells in the distal nephron and collecting duct provides an additional candidate K⁺ channel protein to mediate basolateral K⁺ exchange and membrane potential at these sites.

Kir7.1 has a very low single-channel conductance (~50 fS). It is relatively insensitive to blockade by external Ba²⁺ or Cs⁺ (11,13). The low conductance has been ascribed to a methionine at position of 125 in the pore region where other Kir channels express an arginine (11,13). The electrical conductance of above three K⁺ channels that have so far been defined electrophysiologically in the renal basolateral membrane does not show the functional properties of the Kir7.1 channel (22–27). Kir6.1/ßKATP-1 and Kir6.2/BIR increase their conductance and develop ATP sensitivity only when combined with a sulfonylurea receptor member of the ATP-binding cassette superfamily (30–32). Therefore, the conductance properties of Kir7.1 may also offer some insight into its physiologic function. Kir7.1 was expressed in the principal but not in the intercalated cells of the CCD. The intercalated cells function primarily to secrete acid or base, whereas the principal cells are the primary sites for K⁺ secretion. Furthermore, the levels of Kir7.1 underwent marked changes depending on the plasma K⁺ levels, and the changes in the expression of Kir7.1 was paralleled with those in Na⁺, K⁺-ATPase. Thus, Kir7.1 in the principal cells is involved in the transcellular K⁺ secretion with a functional association with Na⁺, K⁺-ATPase and contribute to achieve fine adjustment of plasma K⁺ within a very narrow range.

In conclusion, the Kir7.1 inwardly rectifying K⁺ channel is expressed strongly in the basolateral membrane of the DCT and CNT and the principal cells of the CCD and also weakly in the TAL of loop of Henle and the medullary collecting duct. The expression of Kir7.1 is regulated depending on physiologic requirements. Its levels are lowered under hypokalemia and restored with potassium supplementation. This novel K⁺ channel may function to regulate basolateral K⁺ conductance and membrane potential of the distal nephron and collecting duct.

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References

4. Ho K, Nichols CG, Lederer WJ, Lytton J, Vassilev PM, Kana- 