Immunolocalization of an inwardly rectifying K^+ channel, K_{AB}-2 (Kir4.1), in the basolateral membrane of renal distal tubular epithelia

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Abstract Immunolocalization of K_{AB}-2 (Kir4.1), an inwardly rectifying K^+ channel with a putative ATP-binding domain, was examined in rat kidney where expression of K_{AB}-2 mRNA was previously shown. Anti-K_{AB}-2 antibody was raised in rabbit and then affinity-purified. An immunohistochemical study revealed that K_{AB}-2 immunoreactivity was detected specifically in the basolateral membrane of distal tubular epithelia. Therefore, K_{AB}-2 is the first K^+ channel shown to be localized in the basolateral membrane of renal epithelia. The finding suggests that K_{AB}-2 may contribute to supplying K^+ to the Na^+-K^+ pump, which is abundant in the basolateral membrane of distal tubular epithelia, as well as to maintenance of the deep negative membrane potential of these cells.

Key words: Potassium channel; Basolateral membrane; Kidney tubule, distal; Immunohistochemistry; Immunogold electron microscopy

1. Introduction

Potassium (K^+) channels in renal tubular epithelia have several important functions. They set the negative membrane potential of tubular cells to regulate passive fluxes of other ions, and recycle potassium ions (K^+) across apical and basolateral membranes to support the Na^+-K^+-$2Cl^-$ cotransporter and Na^+-K^+ pump, respectively. In distal tubules, excess extracellular K^+ is secreted into urine through apical membrane K^+ channels. Therefore, renal K^+ channels, particularly in distal tubules, play essential roles in the maintenance of K^+ homeostasis [1]. The mechanism of K^+ transport across the distal tubular epithelia has been investigated with various physiological techniques. In the apical membrane, two types of K^+ channels, a ~30-pS inwardly rectifying K^+ channel regulated by intracellular ATP and protons, and an ~100-pS Ca^{2+}-dependent one, have been described electrophysiologically [2,3]. Recently, two cDNAs encoding inwardly rectifying K^+ channels, ROMK1 (Kir1.1) and RACTK1, have been isolated from rat and rabbit kidneys, respectively. By immunohistochemical analyses, these K^+ channels were localized to the apical membrane of cortical collecting duct epithelial cells [4–8]. It is indicated that ROMK1 corresponds to the 30-pS apical K^+ channel, which may be responsible for K^+ secretion [4–6]. In contrast, K^+ channels in the basolateral membrane of epithelial cells in distal nephron segments have been less well characterized and no molecular counterpart has been identified.

We have previously cloned an inwardly rectifying K^+ channel, K_{AB}-2 (Kir4.1), which is characterized with a putative ATP-binding domain in its carboxyl (C-) terminus from rat brain cDNA library ([9], see also [10]). The deduced amino acid sequence of K_{AB}-2 showed 53% identity with that of ROMK1. Northern blot analysis revealed the expression of K_{AB}-2 mRNA in kidneys as well as in the central nervous system [9]. In the present study, we examined the localization of the K_{AB}-2 channel in rat kidney by immunohistochemical methods and immunoelectron microscopy, and found that the K^+ channel is specifically localized to the basolateral membrane of distal tubular epithelia.

2. Materials and methods

2.1. Purification of anti-K_{AB}-2 antibody

Anti-K_{AB}-2 antibody was raised in rabbit against a synthetic peptide corresponding to amino acids 366–379 (EKEGSALSVRISNV) in the C-terminus of rat K_{AB}-2. The antiserum was affinity-purified through protein A Cellulofine (Seikagaku Corp., Tokyo, Japan) and antigenic peptide-coupled Sulfolink resin (Pierce, Rockford, IL). The concentration of the antibody was determined by the absorbance at 280 nm.

2.2. Immunoblot analysis

C-terminal regions of rat K_{AB}-2 and mouse GIRK1 (Kir3.1), G protein-gated muscarinic K^+ channel [11], were expressed as fusion proteins with glutathione *S*-transferase (GST). The cDNAs encoding nucleotides 688–1137 of K_{AB}-2 and 538–1503 of GIRK1 were amplified by polymerase chain reaction, inserted into pGEX-2T vector (Pharmacia, Uppsala, Sweden) and transformed into the DH5α strain of *E. coli*. The fusion proteins were extracted from cells and purified with glutathione Sepharose 4B (Pharmacia) as described previously [11]. GST-K_{AB}-2C and GST-GIRK1C, 50 ng/lane each, were electrophoresed in SDS-polyacrylamide (12%) gels and transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-K_{AB}-2 antibody. After incubation with horseradish peroxidase-conjugated anti-rabbit antibody, immunoreactive proteins were detected with an enhanced chemiluminescence immunostaining kit (Amersham, Bucks, UK).

2.3. Immunohistochemistry of thick sections

Sections of renal tissue were immunostained using the avidin-biotin complex (ABC) method. Briefly, male Wistar rats weighing 100–200 g were deeply anesthetized with pentobarbital sodium (50 mg/kg i.p.),

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and kidneys were fixed by perfusion with 4% (w/v) paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.3). Kidneys were dissected, stored in 30% (w/w) sucrose in phosphate-buffered saline (PBS) at 4°C overnight, and cut into 2–4 mm sections. The sections were pretreated with 3% (w/v) H_2O_2 in methanol at 4°C for 3 h to inhibit endogenous peroxidase activity, and then with buffer A (PBS with 5% (w/v) nonfat skim milk powder and 0.1% (w/v) Triton X-100) at room temperature for 1 h to reduce nonspecific immunostaining. To block endogenous biotin, Histofine (Nichirei, Tokyo, Japan) was used. The sections were incubated with anti-K_{AB}-2 antibody (0.15 μg/ml) in buffer A at 4°C overnight. Detection of K_{AB}-2 was accomplished using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Sections were incubated with biotinylated secondary antibody diluted 1:2000 in buffer A at 4°C overnight, washed 5 times in buffer A for 30 min each, then incubated with ABC reagent at 4°C overnight and washed. The localization of K_{AB}-2 was visualized using diaminobenzidine-H_2O_2 solution.

2.4. Immunohistochemistry of cryostat sections

After perfusion fixation with 4% PFA and sucrose infiltration, the renal tissue was frozen in O.C.T. compound (Miles Inc., Elkhart, IN) and cut into 5-μm sections. The sections were pretreated with 0.3% H_2O_2 in ethanol at 4°C for 15 min, and then with PBS containing 5% (w/v) bovine serum albumin (BSA) and 0.1% Triton X-100 at room temperature for 30 min. The sections were incubated with anti-K_{AB}-2 antibody (0.15 μg/ml) in PBS containing 0.1% BSA, 1% (v/v) normal goat serum and 0.1% Triton X-100 at 4°C overnight. Incubation with secondary antibody and color development were carried out using the Vectastain Elite ABC kit. The localization of K_{AB}-2 was visualized with diaminobenzidine-H_2O_2 solution. Negative control sections were processed in the same way using the anti-K_{AB}-2 antibody preabsorbed with excess antigenic peptide. Nuclei were counterstained with methyl green. Photomicrographs were taken using a Zeiss Axioskop with a Nomarski system (Carl Zeiss Inc., Oberkochen, Germany). For confocal imaging, the sections were incubated first with the primary antibody and then with fluorescein isothiocyanate (FITC)-labelled anti-rabbit antibody (1:2000), and examined with a Zeiss LSM410 system.

2.5. Immunogold electron microscopy

After perfusion fixation with 0.1 M sodium cacodylate buffer containing 4% PFA and 0.1% (w/v) glutaraldehyde, renal tissue was cut into 1×1 mm blocks and post-fixed at 4°C for 4 h in the fixative to which 0.1% (w/v) $CaCl_2$ was added. Then, the blocks were dehydrated in ethanol, infiltrated in LR white (London Resin, London, UK) at 4°C overnight and embedded in new LR white. Ultrathin sections were cut with a diamond knife and mounted on formvar-coated nickel grids. The sections were pretreated with PBS containing 5% BSA at room temperature for 30 min, and incubated first with the primary antibody at 4°C overnight and then with 10-nm gold conjugated anti-rabbit antibody (1:20) (Zymed Laboratories, Inc., San Francisco, CA). The sections were then fixed with 2% glutaraldehyde in PBS, stained with aqueous uranyl acetate and lead citrate, and examined with a transmission electron microscope (H7100TE, Hitachi, Tokyo, Japan).

3. Results

The affinity-purified polyclonal antibody against the C-terminal region of K_{AB}-2 was checked for specificity (Fig. 1). On SDS-polyacrylamide gels, the fusion proteins, GST-K_{AB}-2C and GST-GIRK1C, were detected at 42 and 58 kDa, respectively, by staining with Coomassie Blue (Fig. 1A). By immunoblot analysis, the anti-K_{AB}-2 antibody recognized only GST-K_{AB}-2C as a single band of protein (Fig. 1B). Preincubation of the antibody with antigenic peptide prevented the detection of GST-K_{AB}-2C (Fig. 1C). The findings indicate that the anti-K_{AB}-2 antibody specifically immunoreacted with the C-terminus of the K_{AB}-2 protein.

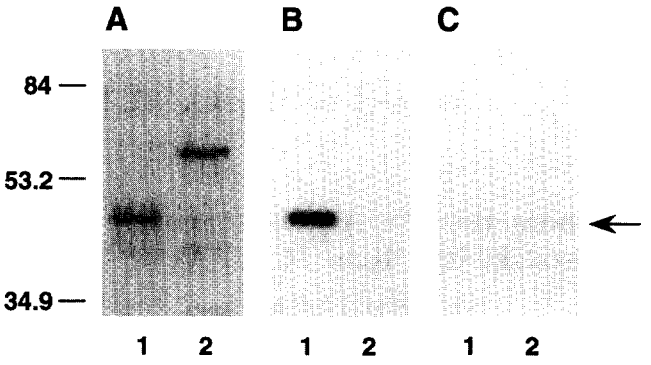

Fig. 1. Characterization of anti-K_{AB}-2 antibody. Purified fusion proteins, GST-K_{AB}-2C and GST-GIRK1C, (lanes 1 and 2, respectively; 0.5 μg each for A and 50 ng each for B and C) were stained with Coomassie Brilliant Blue R-250 (A), affinity-purified anti-K_{AB}-2 antibody (B), and anti-K_{AB}-2 antibody preabsorbed with antigenic peptide (C). Molecular mass markers in kDa are shown on the left (Bio-Rad, Hercules, CA). The arrow on the right indicates the position of GST-K_{AB}-2C.

Fig. 2 shows the distribution of K_{AB}-2 immunoreactivity in a thick section of rat renal tissue. Specific stainings were detected only in the cortical area, while the medulla remained unstained (Fig. 2A). The staining appeared to be localized to specific segments of each nephron, because worm-like tubular stainings, 50–200 μm long, were scattered uniformly in the cortex. At higher magnification, convoluted tubular stainings were clearly visualized (Fig. 2B). Negative control sections treated with nonimmunized rabbit IgG showed no staining (data not shown).

In Fig. 3, the localization of K_{AB}-2 immunoreactivity was examined in a cryosection of renal cortex. The immunoreactivity was detected only in the basolateral membrane of distal tubular epithelial cells (Fig. 3A), which were recognized by a few microvilli and closely neighboring nuclei (Fig. 3B). These epithelial cells showed broad, rather than thin linear, staining on the basolateral side, where infoldings of the membrane were noticeable (Fig. 3B; see also Fig. 4). Consistent with the extensive invaginations of the basolateral membrane, the nuclei of the positive cells were displaced apically (Fig. 3B). Using the FITC immunofluorescence technique, essentially the same results were obtained (Fig. 3C). The luminal membrane of the same segment was unstained (Fig. 3A–C). In some of the distal tubular epithelial cells, no immunoreactivity was detected on both sides of the membranes (arrows, Fig. 3C). Negative cells were smaller than positive ones, and had basally located nuclei (Fig. 3B,C).

The epithelium at the segment around the macula densa was unstained, suggesting that K_{AB}-2 was not present in the thick ascending limb of Henle, but specifically localized in distal convoluted tubule, connecting tubule, and initial collect-

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Fig. 3. Localization of K_{AB}-2 in rat renal cortex. Cryosections of renal cortex immunostained with anti-K_{AB}-2 antibody. Stains were detected using the ABC method (A,B,D) or FITC-labelled secondary antibody (C). The antibody preabsorbed with antigenic peptide was used for negative control (D). Photomicrographs were taken using the Nomarski system (A, ×200; B, ×1000, high magnification of the marked region in (A); D, ×200) or confocal system (C, ×800). MD, macula densa; p, proximal tubule; d, distal tubule; Bars=40 μm.

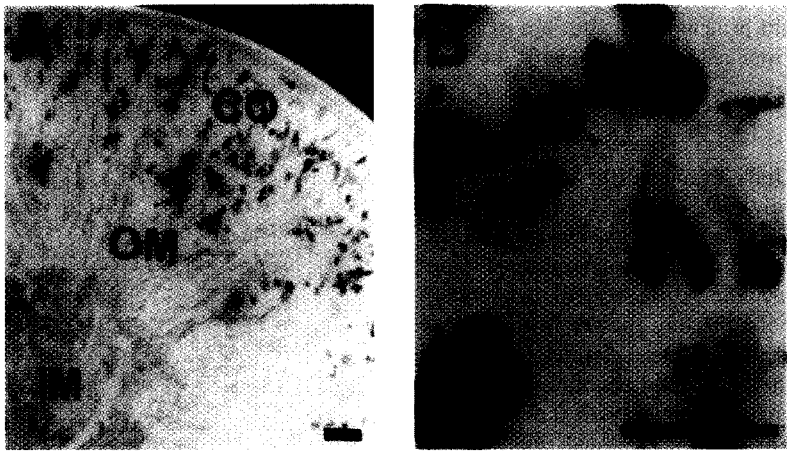
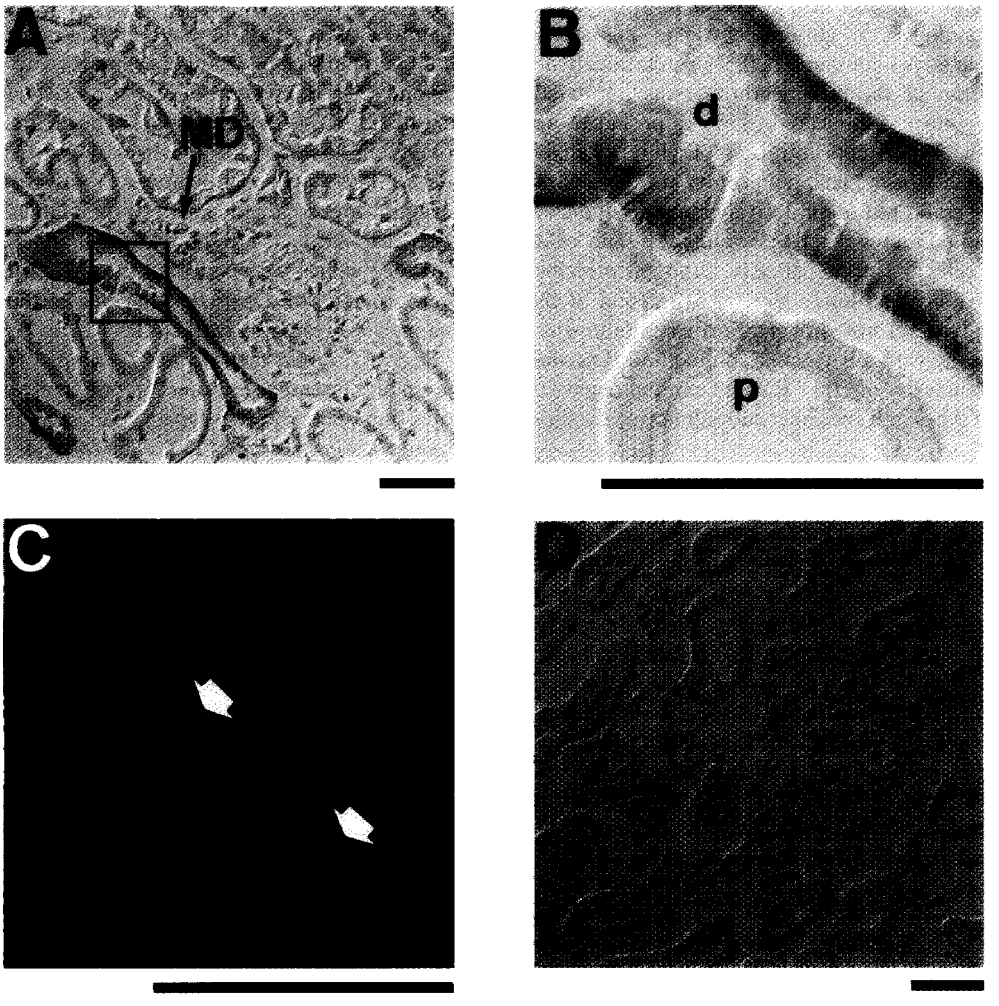


Fig. 2. Distribution of K_{AB}-2 in rat kidney. Thick section of rat kidney immunostained with anti-K_{AB}-2 antibody using the ABC method. (A) ×20; (B) ×80. CO, cortex; OM, outer medulla; IM, inner medulla; Bars=200 μm.

ing tubule (Fig. 3A). Negative control sections treated with nonimmunized rabbit IgG showed no staining of the epithelial membrane (data not shown). Staining was abolished after preabsorption of anti-K_{AB}-2 antibody with antigenic peptide (Fig. 3D).

The subcellular localization of K_{AB}-2 was further examined with immunoelectron microscopy of ultra-thin sections (Fig. 4). The positive cells showed poorly developed microvilli on the apical membrane and extensive infoldings of the basolateral membrane, which confirms that these cells belong to distal tubular epithelia (Fig. 4A). Furthermore, in the particular cell shown in Fig. 4, the mitochondria between the infolded membranes are lamella-like shaped and densely stuffed. These characteristics strongly suggest that it is a distal convoluted tubule cell [12]. At higher magnification, immunogold labellings were detected only on the invaginations of the basolateral membrane of the epithelial cell (arrowheads, Fig. 4B). Positive labellings were also detected on the basolateral side



of connecting tubule cells and principal cells, but not in intercalated cells, in the same segment (data not shown).

4. Discussion

In the present study, we have examined the localization in rat renal tissue of an inwardly rectifying K^+ channel, K_{AB}-2. Immunohistochemical analysis using a specific anti-K_{AB}-2 antibody located the K^+ channel in the basolateral membrane of distal tubular epithelia including distal convoluted tubule, connecting tubule, and initial collecting tubule. In these segments, it appears that the epithelial cells, characterized with extensive invaginations of the basolateral membrane and apically located nucleus, were mainly stained with the antibody. These were possibly either of distal convoluted tubule cells, connecting tubule cells or principal cells [13]. No K_{AB}-2 immunoreactivity was detected in some of the epithelial cells. The morphologic characteristics of the unstained cells, i.e. relatively small cell size and basally located nucleus, are compatible with those of intercalated cells, which are electrophysiologically known to have much lower basolateral K^+ conductance than other types of epithelial cells [14].

Immunoelectron microscopy further indicated that K_{AB}-2 immunoreactivity was localized on the infoldings of the basolateral membrane of distal convoluted tubule cells, connecting tubule cells and principal cells, but not of the intercalated cells. Preservation of the cell membrane was, however, unsatisfactory and the association of immunogold labelling with the basolateral membrane was not well visualized, since we used fixative containing 0.1% glutaraldehyde in this study. When we used fixative containing 0.5% glutaraldehyde, although preservation of the cell membrane was improved, positive labelling almost disappeared. This is probably because fixation with a high concentration of glutaraldehyde affected the antigenicity of the channel protein in the preparation. Therefore, we could not quantitatively compare the level of K_{AB}-2 expression among distal convoluted tubule cells, connecting tubule cells and principal cells, although principal cells seemed to have a much lower level of expression of the channel in this study (data not shown). Further immunoelectron microscopic study with a better fixation method is needed for quantitative analysis of the K_{AB}-2 immunoreactivity among various cell types and the exact subcellular localization of the channel.

Since K^+ channels in the basolateral membrane of distal tubules are difficult targets for patch-clamp studies because of the basement membrane, their properties have been less well studied than those of apical K^+ channels in the same segments or those of K^+ channels in proximal tubules and the loop of Henle [2]. Despite this difficulty, there are several reports on basolateral K^+ channels in distal tubules: In the basolateral membrane of rabbit distal convoluted tubule, a K^+ channel with two different single-channel conductances of $\sim$50 and $\sim$60 pS was recorded in the cell-attached configuration (hereinafter, conductance values measured with 150 mM extracellular K^+ are quoted) [15]. It was not reported whether this channel is inwardly rectified. A flickering, 77-pS K^+ channel was also observed at low occurrence [15]. In rat cortical collecting tubule, a basolateral K^+ channel which showed linear and large (150–200 pS) conductance was described. A 67-pS K^+ channel was also seen in this preparation but has not been characterized sufficiently [16]. In rabbit cortical collecting tubule, an inwardly rectifying 20-pS K^+ channel was found [17]. These data indicate that basolateral K^+ channels in renal distal tubules are divergent.

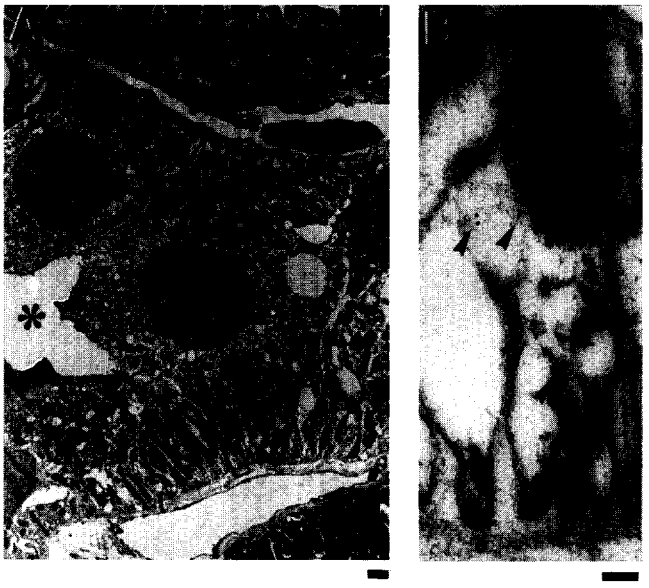


Fig. 4. Immunogold detection of K_{AB}-2 in ultrathin sections of rat renal cortex. (A) Distal convoluted tubule cell. Asterisk indicates the lumen. (B) High magnification of marked region in (A). (A) ×3200, bar=1 μm; (B) ×66000, bar=0.1 μm.

K_{AB}-2 belongs to the superfamily of inwardly rectifying K^+ channel clones, which have a common molecular motif of two putative membrane-spanning segments and one pore-forming region [9,18]. Expression of K_{AB}-2 in *Xenopus* oocytes resulted in a highly K^+-selective channel. The channel has two distinct conducting states, both of which show strong inward rectification. The slope conductances of inward currents through high and low conductive states are 36 and 21 pS, respectively [9]. Thus, although basolateral K^+ channels with similar conductance values were reported, no definitive information has been provided to decide whether one of those basolateral K^+ channels corresponds to K_{AB}-2.

Recently, a new method was developed to selectively patch-clamp the lateral membrane of principal cells of rat cortical collecting duct, and two types of K^+ channels, 27- and 45-pS channels, were recorded [19]. This type of electrophysiologic approach, together with immunoelectron microscopic study on the subcellular localization of K_{AB}-2, may enable us to identify the physiological K^+ channel corresponding to K_{AB}-2.

Because some of the epithelial cells in the distal tubules did not express K_{AB}-2 and electrophysiological studies indicate that the basolateral K^+ channels are divergent, unidentified K^+ channels other than K_{AB}-2 may also contribute to the basolateral K^+ conductance in these segments. Therefore, further studies are needed to isolate and characterize cDNAs of these K^+ channels in order to elucidate the molecular mechanism of regulation of K^+ transport in renal distal tubules.

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