Kir4.1/Kir5.1 channel forms the major K⁺ channel in the basolateral membrane of mouse renal collecting duct principal cells

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The cortical collecting duct (CCD) plays a key role in K⁺ secretion and Na⁺ reabsorption (4). In CCD principal cells, K⁺ enters the cell actively through the basolateral Na⁺-K⁺-ATPase and exits across the luminal membrane via renal outer medullary K⁺ (ROMK) channels along the K⁺ electrochemical gradient. Luminal Na⁺ enters the cells by apical epithelial Na⁺ channels (ENaC) and exits by the Na⁺-K⁺-ATPase pump. In this transport scheme, the basolateral K⁺ conductance plays critical roles (10, 22) as it mediates the recycling of the K⁺ carried into the cell across the basolateral membrane by the Na⁺-K⁺-ATPase. It also contributes to setting the resting basolateral membrane potential difference, which may in turn affect the electrogenic luminal processes of K⁺ secretion and Na⁺ reabsorption. Depending on physiological status, it may also determine the direction of the net basolateral flux of K⁺ (19). Thus, when K⁺ conservation is needed, K⁺ carried into the cell by the Na⁺-K⁺-ATPase is recycled across the basolateral membrane to prevent apical K⁺ loss. On the other hand, if there is an excess of K⁺, the net K⁺ flux across the basolateral membrane can be reversed (19), providing an additional route for basolateral K⁺ entry and thus for apical K⁺ secretion.

Despite their important functional role, and efforts to study their properties and regulatory mechanisms, little is known about the correspondence between native K⁺ channels described on the basolateral membrane of CCD principal cells (10, 42, 43, 45) and the variety of K⁺ channel genes expressed in these cells (6). From whole cell clamp studies on mouse CCD cells (21), CCD-IRK3, a 15-pS inwardly rectifying K⁺ channel cloned from the M1 mouse collecting duct cell line, which presents a high degree of homology with human inwardly rectifying K⁺ (Kir) 2.3 channel (46), is thought to be the 18-pS channel described in rat CCD principal cells (45). Using whole cell patch-clamp technique and noise analysis of K⁺ current measurements of rat CCD principal cells, Gray et al. (5) indicated that the native ~30-pS K⁺ channel (45) shares some properties of members of the Kir4/Kir5 subfamily of channels. However, no comparison of the properties at the single-channel level of native K⁺ channels in the basolateral membrane of CCD principal cells with those of cloned channels has been conducted.

The purpose of the present study was to identify the K⁺ channels of the basolateral membrane of mouse CCD principal cells on the basis of the properties of cloned K⁺ channels present in CCD. We also addressed the question of their possible regulation by variations in dietary K⁺ and Na⁺.

MATERIALS AND METHODS

Animal and Tissue Preparation

The experiments were conducted in accordance with the rules of the French Ministry of Agriculture under Permit No. 75-096. Male 15- to 20-g CD1 mice (Charles River Laboratories France, L’Arbresle, France) and male 60-g Wistar rats (Charles River Laboratories France, L’Arbresle, France) were housed in a temperature- and humidity-controlled environment with a 12-h light-dark cycle. Water and food pellets were provided ad libitum. The animals were subjected to a 3-h fasting period prior to killing. The methods were approved by the Experimental Animal Facility of the Biomedical Research Center of Paris. All efforts were made to minimize the number of animals used and their suffering.

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France) were chronically maintained on a standard (0.3% Na\(^+\), 0.6% K\(^+\)) diet (SAFE, Epinay, France) with free access to tap water. When appropriate (see results), the animals were fed for 6 days before the experiment either with a high-potassium diet (8% K\(^+\), 0.3% Na\(^+\)) and free access to tap water or with a low-potassium diet (0% K\(^+\), 0.3% Na\(^+\)) or a low-sodium diet (0.09% Na\(^+\), 0.6% K\(^+\)) and free access to deionized water. On the day of the experiment, one mouse was killed by cervical dislocation and small pieces of renal cortex were treated with collagenase (32).

**Single-Channel Analysis**

Patch-clamp experiments were performed on the basolateral membrane of CCD principal cells. CCDs were microdissected as previously described (26, 32), and principal cells were optically identified as flat and polygonal cells in contrast to the round and protuberant intercalated cells (5, 26).

**Current recordings.** Single-channel currents were recorded with a RK-400 patch-clamp amplifier (Bio-Logic, Claux, France) and stored on either digital audiotapes (DTR-1205, Bio-Logic) or audio CDs (PDR-W839 and DRA-200, Bio-Logic). The reference electrode in the bath was an Ag/AgCl pellet. In the cell-attached configuration, the clamp potential (\(V_c = V_{bath} - V_{	ext{prep}}\)) was superimposed on the cell membrane potential (\(V_m\)). In excised inside-out membrane patches, \(V_c = V_m\). All experiments were conducted at room temperature.

**Data analysis.** Signals were typically low-pass filtered at 1 kHz by an eight-pole Bessel filter (LPBF-48DG; Npi electronic, Tamm, Germany) and digitized at 3 kHz by an analog-to-digital converter with a ROMK sense 5'-GGGTTACCGTTTG-3', antisense 5'-ATTACCGTTACCGCTT-3'; Kir4.1 sense 5'-CCCGATTITACAGAGC-3', antisense 5'-AGATCCTTGAAGTAGAGA-3'; Kir5.1 sense 5'-ATAGCCTTACCGCGT-3', antisense 5'-TGACGAGTTGACGTCTT-3'; Kir2.4 sense 5'-GAGCCCATACAGAGG-3', antisense 5'-GTGAGCTTGTATCGCCA-3'; Kir7.1 sense 5'-ATCCTCCCG-GCAACCTAT-3', antisense 5'-CACCTTGGACACTCG-3'. The primers for the mouse cyclophilin sequence were as previously given (31).

Real-time PCR was performed either with a LIGHTCYCLER System (Roche Diagnostics) and the FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics) as previously described (25, 31) or with a LIGHTCYCLER 480 and the LC 480 SYBR Green Master kit (Roche Diagnostics), on cDNA corresponding to 0.1 mm of tubule in the presence of 4 pmol of primers per 8 μl (LIGHTCYCLER) or 5 pmol per 10 μl (LIGHTCYCLER 480) of final reaction volume. Samples were submitted to either 50 (LIGHTCYCLER) or 70 (LIGHTCYCLER 480) cycles of three temperatures (95°C for 10 s, 60°C for 12 s, 72°C for 20 s). A melting curve was then established in order to check for nonspecific PCR products.

In each experiment, a standard curve was plotted with serial dilutions (1 to 1/500) of a cDNA stock solution obtained from mouse whole kidney RNA, dilution 1 corresponding to the cDNA produced by 10 ng of RNA. For all samples, the amount of PCR product was calculated as a percentage of the RNA standard (arbitrary unit).

After correction for the efficiency of the PCR, the expression of gene 1 relative to that of ROMK gene in a given sample was estimated with the equation

\[
\frac{N_{1}}{N_{\text{ROMK}}} = \frac{E_{C_{\text{ROMK}}}L_{\text{ROMK}}}{E_{1}^{\text{RT}}L_{1}}
\]

where \(N_1\) and \(N_{\text{ROMK}}\) are the numbers of cDNA copies present in the samples before amplification, \(E_1\) and \(E_{\text{ROMK}}\) are the PCR efficiencies, \(C_1\) and \(C_{\text{ROMK}}\) are the crossing points, and \(L_1\) and \(L_{\text{ROMK}}\) are the respective lengths of the amplified products (25).

**Immunohistochemistry**

Tissues were prepared for immunohistochemical studies as previously described (17). Briefly, mice were killed by intraperitoneal injection of pentobarbital sodium. Kidneys were then rapidly removed, frozen in liquid nitrogen, and stored at −80°C until use. Kidney sections (7–9 μm) mounted on glass slides were fixed in ice-cold methanol (8 min) and incubated with either rabbit anti-rat Kir4.1 (7, 12) or rabbit anti-rat Kir5.1 (7, 11) antibodies (1:200 dilution) and then with a Cy3-conjugated goat anti-rabbit Ig (1:100 dilution) (Jackson Immunoresearch, West Grove, PA). Sections were also double-labeled with anti-Kir4.1 or anti-Kir5.1 antibody and a polyclonal antibody directed against the aquaporin-2 water channel (AQP2; kindly provided by J. Loffing, Institute of Physiology, University of Zurich, Zurich, Switzerland), the thiazide-sensitive

\[I_{Mg} = \frac{1}{1 + \exp\left(2FV_0/\deltaK\right)}\]

where \(I_{Mg}\) and \(I_0\) are the currents in the presence and absence of Mg\(^{2+}\), respectively, \(\delta\) is the electrical distance of the binding site from the outside of the pore, and \(K_0\) is the inhibition constant at \(V_c = 0\) mV.

**Real-Time PCR**

Tubular fragments were microdissected at 4°C in medium supplemented with RNase-free BSA (1 mg/ml) and rinsed. Pools of 20–50 microdissected tubules were then directly treated for RNA extraction (31). RT was performed with a first-strand cDNA synthesis kit for RT-PCR (Roche Diagnostics, Meylan, France). The viability of all RNA samples was ascertained by real-time PCR for the housekeeping gene cyclophilin.

The primers for the K\(_\text{ir}\) channels studied here were as follows: ROMK sense 5'-GGGTTACCGTTTG-3', antisense 5'-ATTACCGTTACCGCTT-3'; Kir4.1 sense 5'-CCCGATTITACAGAGC-3', antisense 5'-AGATCCTTGAAGTAGAGA-3'; Kir5.1 sense 5'-ATAGCCTTACCGCGT-3', antisense 5'-TGACGAGTTGACGTCTT-3'; Kir2.4 sense 5'-GAGCCCATACAGAGG-3', antisense 5'-GTGAGCTTGTATCGCCA-3'; Kir7.1 sense 5'-ATCCTCCCG-GCAACCTAT-3', antisense 5'-CACCTTGGACACTCG-3'. The primers for the mouse cyclophilin sequence were as previously given (31).
Na\(^+\)-Cl\(^-\) cotransporter (NCC), or the Tamh-Horsfall protein (Valbiotech, France). Binding was revealed with Cy3- or Alexa Fluor 488-conjugated FAB fragment goat anti-rabbit IgG (1:100, Molecular Probes, Eugene, OR). All tissue sections were examined by confocal laser scanning microscopy (CLSM) (Leica, Wetzlar, Germany).

Kidney sections were also double-labeled with anti-Kir4.1 and anti-AQP2 antibodies in order to quantify the number of AQP2-positive and -negative CCD cells expressing Kir4.1. Quantification of the number of labeled cells was performed on 32 separate tissue sections from 3 different kidneys. The relative fluorescence levels of anti-AQP2 antibodies in order to quantify the number of AQP2-labeled cells was performed on 32 separate tissue sections from 3 different kidneys. The relative fluorescence levels of anti-AQP2 antibodies in order to quantify the number of AQP2-labeled cells was performed on 32 separate tissue sections from 3 different kidneys. The relative fluorescence levels of anti-AQP2 antibodies in order to quantify the number of AQP2-labeled cells was performed on 32 separate tissue sections from 3 different kidneys. The relative fluorescence levels of anti-AQP2 antibodies in order to quantify the number of AQP2-labeled cells was performed on 32 separate tissue sections from 3 different kidneys. The relative fluorescence levels of anti-AQP2 antibodies in order to quantify the number of AQP2-labeled cells was performed on 32 separate tissue sections from 3 different kidneys.

Data Presentation and Statistics

Results are given as means ± SE for n experiments. P values <0.05 (paired or unpaired t-test or Z-test on proportions, when appropriate; SIGMAPLOT, Systat Software, Erkrath, Germany) were taken to represent statistically significant differences. Nonlinear regression analyses were performed with either SIGMAPLOT or ORIGIN (MICROCAL Software, Northampton, MA) software.

RESULTS

Patch-clamp experiments on the basolateral membrane of CCD principal cells of mice maintained on a standard diet revealed a ~40-pS K\(^+\) channel in 63% of the cell-attached membrane patches (52 of 83) and a ~20-pS K\(^+\) channel in 30% of the patches (25 of 83). Throughout this study, the activity of another channel type with a conductance of ~76 pS was also observed. It had an reversal potential of ~56 mV, which suggested the presence of a channel with significant selectivity for K\(^+\), but its low occurrence (8 cell-attached patches) precluded any further analysis. In what follows, we therefore focus on the properties of the 40-pS and 20-pS K\(^+\) channels.

Properties of 20-pS Channel

Typical recordings of 20-pS channel activity on cell-attached membrane patches from a tubule bathed in high-NaCl medium, with the pipette filled with the high-KCl solution, are shown in Fig. 1A. Because this channel was often seen on patches containing several channel types, no confident statistical analysis of its open probability (P\(_o\)) was possible. However, on the patch shown in Fig. 1, channel activity displayed little voltage dependence, because P\(_o\) decreased only slightly from ~0.8 at V\(_c\) = 0 mV to ~0.6 at V\(_c\) = -90 mV. On average, channel number per patch and Np \(\text{in}\) at V\(_c\) = 0 mV were 1.6 ± 0.4 (n = 5) and 1.23 ± 0.31 (n = 3), respectively, yielding a P\(_o\) of 0.68 ± 0.17 (n = 3). The corresponding i/V\(_c\) relationship is shown in Fig. 1B. Inward conductance (G\(_{\text{in}}\)) averaged 22.9 ± 0.89 pS (n = 25) and E\(_{\text{rev}}\) was estimated at 66.6 ± 7.3 mV (n = 10), which was consistent with the presence of a K\(^+\) channel.

After an inside-out patch had been formed and the exposure of the internal face of the membrane to the Mg\(^2+\)- and Ca\(^2+\)-free high-K\(^+\) medium, the channel G\(_{\text{in}}\) averaged 26.3 ± 1.38 pS (n = 7). Outward conductance (G\(_{\text{out}}\)) was 17.3 ± 2.02 pS (n = 4), yielding a G\(_{\text{in}}\)-to-G\(_{\text{out}}\) ratio of 1.13 ± 0.14 (n = 4).

On two patches, we were able to test for the sensitivity of the channel to internal pH (pHi). On both patches, a decrease in pHi from 7.4 to ~6.6 had no detectable influence on channel activity. A further decrease in pHi, reduced channel Np\(_o\), and pK \(_o\) values could be estimated within the range 6.1–6.4.

Properties of 40-pS K\(^+\) Channel in Cell-Attached Membrane Patches

Figure 2A illustrates single-channel activity of the 40-pS channel in a cell-attached membrane patch with high-NaCl medium as the bath solution and high-KCl medium as the pipette solution. From 24 patches where no other channel type was present, an average of 3.2 ± 0.38 channels per patch and a Np \(\text{in}\) of 1.75 ± 0.23 were observed at V\(_c\) = 0 mV, yielding a P\(_o\) of 0.45 ± 0.04. Channel P\(_o\) was quite stable over the range of ~100 mV to 20 mV (n = 8) (Fig. 2B, inset).

The corresponding i/V\(_c\) relationship is shown in Fig. 2B. G\(_{\text{in}}\) averaged 40.6 ± 0.99 pS (n = 52), and currents reversed at 74.6 ± 2 mV (n = 43), which was consistent with the presence of a K\(^+\)-selective channel. To find out whether this channel displays inward rectification, we then established its i/V\(_c\) relationship under depolarizing conditions by bathing tubules in the high-K\(^+\) solution. With the high-K\(^+\) solution in the pipette, single-channel currents reversed at ~0.7 ± 0.9 mV (n = 5), which was consistent with a value of V\(_i\) of nearly zero. As seen in Fig. 2B, this channel clearly behaves as an
inwardly rectifying channel. Thus \( G_{\text{in}} \) averaged 44 ± 1.7 pS (\( n = 6 \)), whereas outward chord conductance, \( G_{\text{chord, out}} \) (as measured between \( E_{\text{rev}} \) and +60 mV), was 12 ± 1.8 pS (\( n = 5 \); \( P = 0.00002 \) vs. \( G_{\text{in}} \), paired \( t \)-test). A \( G_{\text{in}} \)-to-\( G_{\text{out}} \) ratio of 4 ± 0.57 (\( n = 5 \)) was obtained.

Properties of 40-pS Channel in Inside-Out Membrane Patches

We confirmed the high selectivity for potassium of the channel in cell-free patches (not shown). Keeping the high-K\(^+\) solution in the pipette and replacing all but 45 mM KCl by NaCl in the bath shifted the reversal potential of the i\( V_c \) curves by 27.3 ± 0.64 mV (\( n = 6 \)), which corresponds to a \( P_{K}/P_{Na} \) of 23.

Under symmetrical high-K\(^+\) conditions and in the absence of internal Mg\(^{2+}\), single-channel currents reversed at \(-0.7 \pm 0.5\) mV (\( n = 10 \)) and the corresponding \( i/V_c \) relationship was nearly linear (Fig. 3B). Thus \( G_{\text{in}} \) and \( G_{\text{out}} \) were 37.8 ± 1.8 and 34.7 ± 1.5 pS, respectively (\( P = 0.009 \), paired \( t \)-test; \( n = 10 \)), leading to a \( G_{\text{in}} \)-to-\( G_{\text{out}} \) ratio of 1.09 ± 0.03 (\( n = 10 \)). The inward rectification we observed in the cell-attached configuration resulted from a voltage-dependent block of outward currents by intracellular Mg\(^{2+}\) ions. Figure 3A compares the current amplitudes of an inside-out membrane patch, the cytosolic side being exposed either to the Mg\(^{2+}\)-free medium or to a medium containing 1.2 mM Mg\(^{2+}\) (1.5 mM MgCl\(_2\) and 2 mM EGTA) at \( V_c = -80 \) and +80 mV. Addition of internal Mg\(^{2+}\) had little influence on inward currents but reduced the single-channel amplitude of outward currents. The corresponding \( i/V_c \) relationships are shown in Fig. 3B. Addition of 1.2 mM cytosolic Mg\(^{2+}\) had no significant influence on \( E_{\text{rev}} \) (0.2 ± 0.4 mV, \( n = 8 \); \( P = 0.2 \) vs. Mg\(^{2+}\)-free medium) or on \( G_{\text{in}} \) (34.8 ± 1.7 pS, \( n = 9 \); \( P = 0.216 \) vs. Mg\(^{2+}\)-free medium) but greatly reduced \( G_{\text{out}} \) [as defined by \( G_{\text{chord, out}} \)] between \( E_{\text{rev}} \) and +80 mV], which fell to 12.5 ± 1.87 pS (\( n = 7 \)). The resulting \( G_{\text{in}} \)-to-\( G_{\text{out}} \) ratio was 3.17 ± 0.46 (\( n = 7 \)).

These data were analyzed according the Woodhull model (see MATERIALS AND METHODS). The best fit obtained with 1.2 mM Mg\(^{2+}\) (Fig. 3B, dashed line) yielded an inhibition constant at \( V_c = 0 \) mV (\( K_0 \)) of 2.84 ± 0.25 mM Mg\(^{2+}\) and an electrical distance of the binding site from the outside of the pore (\( \delta \)) of 24 ± 0.02 from the inside.

The tetravalent cation spermine (SPM) altered the channel activity of the 40-pS K\(^+\) channel. An illustration of this effect is given in Fig. 4A, where 50 \( \mu \)M SPM nearly abolished channel activity at \( V_c = +60 \) mV in an inside-out patch. The...
As indicated in MATERIALS AND METHODS, channel mRNA investigated by real-time PCR on extracts of CCD segments. In Mouse CCD Cells

Expression of Kir4.1, Kir4.2, and Kir5.1 mRNAs in Mouse CCD Cells

The expression of Kir4.1, Kir4.2, and Kir5.1 mRNAs was investigated by real-time PCR on extracts of CCD segments. As indicated in MATERIALS AND METHODS, channel mRNA expression was quantitated by normalizing Kir4.1, Kir4.2, and Kir5.1 mRNA expression levels to that of the ROMK channel. Both Kir4.1 and Kir5.1 mRNAs were significantly expressed in CCD segments. Although present at levels 25- to 100-fold lower than that of ROMK channel mRNA, Kir4.1 and Kir5.1 mRNA expression were of the same order of magnitude as that of the Kir7.1 channel (13), which has been shown to be present in the basolateral membrane of rat CCD principal cells (27). No significant Kir4.2 mRNA expression was detected in mouse CCD extracts. This negative finding could not be attributed to a lack in the effectiveness of the Kir4.2 primers because Kir4.2 mRNA was detected in proximal convoluted and straight tubules (data not shown), yielding a Kir4.2 pattern of expression consistent with results of serial analysis of gene expression (SAGE) studies in human kidney (1).

Immunolocalization of Kir4.1 and Kir5.1 Channels

The characterization of anti-Kir4.1 and anti-Kir5.1 antibodies by Western blot analysis (immunoblotting of Kir4.1 and Kir5.1 proteins expressed in HEK293T cells and in native tissue, including kidney, and peptide competition study) and Kir4.1 peptide competition study was reported previously (7, 11, 12). Here we carried out the peptide competition assay of anti-Kir5.1 antibody, and, as illustrated in Fig. 7A, preincubation of anti-Kir5.1 antibody with an excess amount of antigenic peptide prevented the detection of protein. We also confirmed that anti-Kir4.1 and anti-Kir5.1 antibodies detected Kir4.1 and Kir5.1 proteins, respectively, in a specific manner and did not cross-react with each other (Fig. 7B).

Figure 8 illustrates the immunolocalization of Kir4.1 and Kir5.1 channels in mouse kidney cortex. For both channels, some but not all tubule sections exhibited positive immunostaining and glomeruli were unstained. Double immunofluorescence studies using antibodies against the DCT-specific thiazide-sensitive NCC or the thick ascending limb (TAL)-specific marker Tamm-Horsfall protein revealed that the two channels are expressed in DCT and TAL segments (Fig. 8A). Some of the Kir4.1- and Kir5.1-positive sections were also labeled with antibodies raised against the AQ2 water channel, known to be localized in intracellular vesicles and apical membrane of CCD principal cells of mouse kidney (16). A first analysis of double immunofluorescence experiments using anti-Kir4.1 and anti-Kir5.1 antibodies and AQ2 antibodies on CCD sections indicated that only AQ2-positive cells exhibited positive Kir4.1 and Kir5.1 staining (Fig. 8B). In contrast, the AQ2-negative intercalated cells of CCD sections were not stained with both anti-Kir4.1 and anti-Kir5.1 antibodies (Fig. 8B). In all cases, Kir4.1 and Kir5.1 proteins appeared localized in basolateral membranes of cells along the distal nephron.

A quantification of Kir4.1 staining among CCD cells is given in Fig. 8C. Thus 742 AQ2-positive cells out of 803 CCD cells (i.e., 92.4%) analyzed from 3 kidneys also showed Kir4.1 staining. Only a small proportion of Kir4.1-labeled cells (3.9%) were negative for AQ2, while the remaining 3.6%
AQP2-negative cells (i.e., intercalated cells) were not labeled with the anti-Kir4.1 antibody. These results on CCD therefore indicate that Kir4.1 is almost exclusively expressed in principal cells.

A quantitative analysis of the labeling intensity of Kir4.1-negative cells and of Kir4.1-positive adjacent DCT cells (Fig. 8C) also revealed that the fluorescence intensity of Kir4.1-positive cells and of Kir4.1-negative cells (87.6 ± 1.9, n = 82) was 40% lower than in AQP2-positive principal cells (118.6 ± 2 fluorescence intensity arbitrary units, n = 118) (33). These data confirmed that Kir4.1 is expressed in both DCT cells and CCD principal cells and indicate that the expression of Kir4.1 predominates in the DCT.

Lack of Effect of K⁺ Diets on 40-pS K⁺ Channel

We compared the effects of low-K⁺ diets with those of high-K⁺ diets on the properties of the 40-pS K⁺ channel. After 6 days of treatment, no statistical difference between low- and high-K⁺ diets was found in the frequency of observation of the channel in the cell-attached membrane patches. Thus the channel was detected on 59.6% of active patches (28 of 46 patches containing K⁺ channels) after a low-K⁺ diet and on 39.5% of active patches (15 of 38 patches) after a high-K⁺ diet (P = 0.264), in the number of channels per patch (P = 0.433), or in the open channel probability (P = 0.331) in cell-attached membrane patches clamped at Vc = 0 mV (Table 1).

Effects of Low-Na⁺ Diet on 40-pS K⁺ Channel

We also tested for the effects of a low-Na⁺ diet on the properties of the 40-pS K⁺ channel. As observed for the high- and low-K⁺ diets, there was no statistical difference between standard and low-Na⁺ diets in frequency of observation of the channel in cell-attached patches. The channel was observed on 63% of active patches after standard diet (see above) and on 82% of active patches (19 of 23 patches) after the low-Na⁺ diet (P = 0.091, Z-test on proportions). Also, as summarized in Table 1, there was no significant change in channel properties of the 40-pS K⁺ channel.
activity was affected by this maneuver, because $P_o$ was significantly increased by ~25% by the low-$Na^+$ diet ($P = 0.005$).

**DISCUSSION**

This study on the basolateral membrane of mouse CCD principal cells revealed the presence of three $K^+$ channel types in situ, with conductance of ~75, 20, and 40 $pS$. The 40-pS channel is the one most frequently expressed.

**Molecular Candidates for Basolateral $K^+$ Channels in Mouse CCD Principal Cells**

The 40-pS $K^+$ channel exhibited a high $P_K/P_{Na}$, little voltage dependence, an intermediate $Mg^{2+}$-induced inward rectification, sensitivity to $pHi$, insensitivity to internal $ATP$, and blockade by internal SPM. In addition, it exhibited strong activity in cell-excised inside-out membrane patches in the absence of internal $Ca^{2+}$ or cyclic nucleotides. These properties rule out the possibility that this channel could be a voltage-gated $K^+$ channel, a $Ca^{2+}$-dependent $K^+$ channel, or a poorly $K^+/Na^+$ discriminating cyclic nucleotide-gated $K^+$ channel.

A real-time PCR study of two-pore domain $K^+$ channels has revealed the expression of TWIK-1, TASK-1, TASK-2, and THIK-1 channel mRNA in the distal nephron of the human kidney (15). It is unlikely that any of these channels can be proposed as a candidate for the basolateral 40-pS $K^+$ channel we describe here. First, the TASK-1 channel is only 14 $pS$ (2). Second, TWIK-1, a 34-pS, weakly inward rectifying and ATP-insensitive $K^+$ channel, appears only intracellularly located in mouse CCD cells (24), with no detectable activity in cell membranes (20). In addition, TWIK-1 is insensitive to $pHi$ in cell-excised inside-out membrane patches of *Xenopus* oocytes (14). Third, the same insensitivity to $pHi$ holds true for THIK-1 (35). And fourth, we failed to detect mRNA of TASK-2, a 60-pS, weakly inwardly rectifying channel (36), in mouse CCD extracts (data not shown).

**Table 1. Effects of low-$K^+$, high-$K^+$, and low-$Na^+$ diets on 40-pS $K^+$ $P_o$, $N$, and $P_o$ on cell-attached membrane patches**

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>$NP_o$</th>
<th>$N$</th>
<th>$P_o$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low $K^+$</strong></td>
<td>18</td>
<td>1.28±0.34</td>
<td>3.6±0.56</td>
<td>0.34±0.007</td>
</tr>
<tr>
<td><strong>High $K^+$</strong></td>
<td>13</td>
<td>1.83±0.32**</td>
<td>4.2±0.42**</td>
<td>0.44±0.06**</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>24</td>
<td>1.917±0.21</td>
<td>3.2±0.38</td>
<td>0.48±0.02</td>
</tr>
<tr>
<td><strong>Low $Na^+$</strong></td>
<td>16</td>
<td>2.524±0.45**</td>
<td>4.4±0.78**</td>
<td>0.626±0.05**</td>
</tr>
</tbody>
</table>

Values are means ± SE for $n$ observations. In the first experimental series, channel properties under high-$K^+$ diet were compared with those under low-$K^+$ diet (ns, not significant vs. low $K^+$). In the second experimental series, channel properties under low-$Na^+$ diet were compared with those under Control diet (ns and *, not significant and $P = 0.005$ vs. control diet, respectively). $NP_o$, normalized channel mean current; $N$, number of channels; $P_o$, single-channel open probability.

**Fig. 8. Immunolocalization of Kir4.1 and Kir5.1 in mouse CCD cells. A:** frozen kidney sections were double-labeled with either anti-Kir4.1 or anti-Kir5.1 antibody (red), anti-$Na^+/Cl^-$ (NCC) transporter, anti-Tamm-Horsfall (TH) protein, and anti-AQP2 antibody (green) and analyzed by confocal laser scanning microscopy. $G$: glomeruli. $B$: a CCD section double-labeled with anti-AQP2 antibody (green) and anti-Kir4.1 and anti-Kir5.1 antibodies (red) shows the cellular colocalization of both channel proteins in principal cells, with no staining in intercalated cells (arrowheads). $C$: illustrations (left) of a kidney section labeled with anti-Kir4.1 (red) and anti-AQP2 antibodies (green) and analyzed by confocal microscopy. $C$: immunohistochemical studies have demonstrated that Kir4.1 and Kir5.1 proteins localize in…

[Image 226x435 to 569x722]
the basolateral membrane of rat CCD principal cells (12, 40). In contrast, no Kir4.2 gene expression was found in CCD of human kidney by SAGE methods (1). Our results in mouse kidney showed the presence of both Kir4.1 and Kir5.1 mRNA, but not that of Kir4.2, in CCD extracts and the colocalization of Kir4.1 and Kir5.1 proteins in AQP2-positive, i.e., principal, CCD cells.

When expressed in heterologous expression systems, homomeric assembly of Kir4.1 subunits forms strong inwardly rectifying channels in the presence of internal Mg$^{2+}$, with an inward conductance of 22 pS, high activity ($P_o$ ~0.9) (48), an insensitivity to changes in pH around physiological values with a pK of ~6 (40), and sensitivity to internal SPM with an EC$_{50}$ of ~5 × 10$^{-8}$ M (3). These properties clearly do not match those of the channel described here, which has a inward conductance of 40 pS with an intermediate inward rectification in the presence of internal Mg$^{2+}$ ($G_{in}/G_{out}$ = 3–4), a moderate activity ($P_o$ ~0.45), and a pK of ~7.25.

On the other hand, the coexpression of Kir4.1 and Kir5.1 units leads to the formation of a 43- to 60-pS K$^+$ channel (34, 49) with an intermediate inward rectification ($G_{in}/G_{out}$ ~4) and a $P_o$ of ~0.4 (49). In addition, channels formed by the Kir4.1/Kir5.1 assemblage are sensitive to pH$_i$ changes around the physiological range, with pK values of ~7.45 (49) and $n_H$ of 2.1 (47). In the light of its properties, i.e., a conductance of 40 pS, a $G_{in}/G_{out}$ ratio of ~3.2, an in situ $P_o$ of 0.45, and sensitivity to pH$_i$ (pK = 7.24, $n_H$ = 1.7), it is clear that the 40-pS K$^+$ channel of the basolateral membrane of mouse CCD principal cells very closely resembles a heteromeric Kir4.1/Kir5.1 channel.

This conclusion may give some indications of the molecular nature of the 20-pS K$^+$ channel. Thus, on the basis of its low conductance, high activity ($P_o$ ~0.7), and sensitivity to acidic pH$_i$ values (pK of 6.1–6.4) on one hand and properties of Kir4.1 channels on the other hand, we postulate that this 20-pS K$^+$ channel may be formed by homomeric Kir4.1 unit association.

Comparison with K$^+$ Channels in Basolateral Membrane of Rat CCD Cells

At the time this work was initiated, K$^+$ channels in the basolateral membrane of CCD principal cells of the mouse kidney were not identified. Some of the K$^+$ channels previously described in the basolateral membrane of rat CCD principal cells may be similar to the 40- and 20-pS K$^+$ channels we observed in mouse cells. Wang et al. (45) identified three types of K$^+$ channels on the lateral membrane of rat CCD principal cells. In particular, a pH-sensitive K$^+$ channel, 45 pS in cell-attached membrane patches, may resemble the 40-pS channel we describe here. However, measurements in cell-excised membrane patches under 140 mM K$^+$ symmetrical conditions gave a conductance of 85 pS (42), markedly higher than the 38 pS we found for the mouse CCD K$^+$ channel under similar conditions. In addition, channel activity in rat CCD cells is voltage dependent in situ, because its $P_o$ increases from 0.2 at rest up to ~0.7 with 60-mV hyperpolarization (45), whereas we observed a nearly constant $P_o$ of ~0.5 over a 120-mV range of clamp potentials in mouse CCD. Finally, the 45-pS K$^+$ channel in rat CCD cells appeared to have comparatively low pH$_i$ sensitivity, because lowering pH$_i$ from 7.4 to 6.7 decreased $P_o$ by 30% in cell-excised membrane patches (42), whereas we can extrapolate from our results that the same maneuver would reduce the $P_o$ of the mouse CCD 40-pS K$^+$ channel by ~60%. This suggests that the basolateral 45-pS channel in rat CCD and the 40-pS channel in mouse CCD may be different channels.

A 30-pS voltage- and ATP-independent K$^+$ channel in both cell-attached and cell-excised inside-out membrane patches has also been described in rat CCD cells (18, 45). However, by its pH insensitivity and high activity in situ with a $P_o$ of 0.8 (45), it also clearly differs from the 40-pS K$^+$ channel we describe here. On the other hand, such a high $P_o$ is reminiscent of the elevated $P_o$ of ~0.7 that we observed in situ for the 20-pS K$^+$ channel. But, because we were unable to provide further information about the properties of the 20-pS K$^+$ channel, the question of whether it may be related to the rat 30-pS channel remains open. The same holds true regarding the 18-pS K$^+$ channel, which has also been identified on cell-excised patches from the basolateral membrane of rat CCD (43).

Our data and the data from the literature therefore indicate that basolateral membranes of mouse and rat CCD principal cells are endowed with distinct populations of K$^+$ channels. The present study showed that a pH-sensitive 40-pS K$^+$ channel predominated in mouse CCD cells, whereas a pH-insensitive, 30-pS K$^+$ channel appeared to be the major K$^+$ channel in rat CCD cells (42).

Channel Regulation After K$^{+}$- and Na$^{+}$-Controlled Diets

The CCD is responsible for part of the final regulation of K$^+$ excretion, because K$^+$ loading stimulates urinary K$^+$ secretion whereas K$^+$ depletion drastically reduces urinary excretion by this segment (4, 22, 44). Numerous studies have demonstrated that an adaptation to dietary K$^+$ intake is, at least partially, achieved by changes in apical K$^+$ conductance through modulation of the ROMK channels and of basolateral Na$^+$/K$^+$-ATPase pump activity in CCD principal cells (44). Regarding the latter, a high-K$^+$ diet stimulates basolateral Na$^+$/K$^+$-ATPase activity, whereas a low-K$^+$ diet reduces it (22). Because of the coupling of basolateral K$^+$ conductance to Na$^+$-K$^+$-ATPase activity (23), parallel changes in basolateral K$^+$ channel properties are expected. However, we failed to observe any change in frequencies, open probabilities, or numbers of basolateral K$^+$ channels per patch of CCD principal cells after low- or high-K$^+$ diets. At first glance, such a lack of effect of high-K$^+$ diet on the basolateral 40-pS K$^+$ channel properties rules out its role in these conditions since an increase in basolateral K$^+$ conductance of CCD principal cells has been reported in DOCA-treated rabbits (37) and in rats maintained on a high-K$^+$ diet (5). Nevertheless, considering the results of morphological measurements of the basolateral membrane surface of CCD principal cells of rat after chronic high-K$^+$ loading (9, 38) or of rabbit after chronic DOCA treatment (41), showing a significant increase in their surface area (38), our results may appear consistent with an increase in the total number of K$^+$ channels after K$^+$ loading.

A low-Na$^+$ diet increases Na$^+$ absorption and K$^+$ secretion by CCD principal cells, by stimulating apical Na$^+$ channel activity and an increase in the driving force for K$^+$ secretion through ROMK channels in rat (29, 30). Na$^+$/K$^+$-ATPase activity should be increased, partly as a result of enhanced Na$^+$
entry (30). We found that keeping mice on a low-Na\(^+\) diet for 6 days significantly increased 40-pS K\(^+\) channel open probability, but not the number of active channels. This indicates that a low-Na\(^+\) diet did not induce any major change in channel expression and that such an adaptation of the basolateral K\(^+\) current is mediated via modulation of channel gating. The mechanism behind which such a regulation takes place has not been investigated here. Nevertheless, it is now established that nitric oxide (NO) and a cGMP-dependent pathway modulate the activity of the basolateral 30-pS K\(^+\) channel of rat CCD principal cells (18) and thus provide the link between apical Na\(^+\) channels and basolateral K\(^+\) channels. Further experiments on the regulation of the mouse 40-pS K\(^+\) channel are obviously needed in order to determine whether the NO pathway may be implicated in the increase in channel activity we observed under a low-Na\(^+\) diet.

The results of this work on basolateral membrane of CCD cells, together with previous data on cortical TAL (CTAL) (32) and DCT (17) cells, provide a new picture of the molecular nature of basolateral K\(^+\) channels along the mouse distal nephron. Thus our results showed that both CTAL (32) and DCT (17) tubule basolateral membranes are endowed with K\(^+\) channels with properties similar to those of the basolateral K\(^+\) channel in C1D cells, indicating that the Kir4/Kir5.1 channel may be a major component of the basolateral K\(^+\) conductance along the mouse distal nephron.

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