Primary structure and functional expression of a cortical collecting duct K$_{ir}$ channel

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Welling, Paul A. Primary structure and functional expression of a cortical collecting duct K$_{ir}$ channel. Am. J. Physiol. 273 (Renal Physiol. 42): F825–F836, 1997.—Maintenance of a negative membrane potential in the cortical collecting duct (CCD) principal cell depends on a small-conductance, inward-rectifying basolateral membrane K$^+$ (K$_{ir}$) channel. In the present study, a candidate cDNA encoding this K$^+$-channel, CCD-IRK$_3$, was isolated from a mouse collecting duct cell line, M1. CCD-IRK$_3$ shares a high degree of homology with a human brain inward-rectifier K$^+$ channel (K$_{ir}$ 2.3). By Northern analysis, CCD-IRK$_3$ transcript (2.9 kb) was readily detected in M1 CCD cells but not in Madin-Darby canine kidney, LLC-PK$_1$, Chinese hamster ovary, or monkey kidney fibroblast cell lines. CCD-IRK$_3$-specific reverse transcription-polymerase chain reaction confirmed bonafide expression in the kidney. Functional expression studies in Xenopus oocytes revealed that CCD-IRK$_3$ operates as strongly inward-rectifying K$^+$ channel. The cation selectivity profile of CCD-IRK$_3$ [ionic permeability values (P$_K$/P$_i$), Ti $>$ Rb $>$ K$^+$ $>$ NH$_4$ $>$ Na; inward-slope conductance (G$_i$/G$_t$), Ti $>$ K$^+$ $>$ NH$_4$ $>$ Na $>$ Rb] is similar to the macroscopic CCD basolateral membrane K$^+$ conductance (G$_i$/G$_t$). CCD-IRK$_3$ also exhibits the pharmacological features of the native channel. Patch-clamp analysis reveals that CCD-IRK$_3$ functions as a high open probability, voltage-independent, small-conductance channel (14.5 pS), consistent with the native channel. Based on these independent lines of evidence, CCD-IRK$_3$ is a possible candidate for the small-conductance basolateral K$_{ir}$ channel in the CCD.

Renal potassium excretion and potassium homeostasis are ultimately dependent on the regulation of the K$^+$ channel activity in the distal nephron. Consider the cortical collecting duct (CCD) principal cells, the major site of K$^+$ excretion in the kidney. In these cells, the vectorial movement of K$^+$ from interstitium to lumen is dependent on the operation of several asymmetrically distributed K$^+$ channels and pumps (27). In the first step, K$^+$ is actively transported from the interstitium into the cell by the basolateral Na-K-adenosinetriphosphatase. Having actively accumulated above electrochemical equilibrium, potassium then passively exits the principal cell either via apically orientated K$^+$ channels or through physiologically distinct K$^+$ channels on the basolateral membrane. In this way, the rate of renal K$^+$ secretion is governed by the relative activity of the different CCD K$^+$ channels, as well as the relative K$^+$ driving forces across each membrane. Normally, both the macroscopic K$^+$ conductance and the driving force on the apical membrane exceed those of the basolateral membrane, favoring K$^+$ secretion (12, 29).

Characterization of native CCD channels by patch-clamp techniques, complimented by recent insights from molecular cloning, has begun to provide some definitive answers to the basis of differential K$^+$ channel regulation in the collecting duct. The apical conductive pathway is the best characterized. K$^+$ exit across this membrane appears to be mediated by a unique intermediate conductance (20–45 pS), mildly inward-rectifying, voltage-independent, high open probability K$^+$ channel (5, 33). Like the ATP-sensitive K$^+$ channels (K$_{ATP}$), first identified by Noma (26) in the cardiac myocyte, a central hallmark of the apical secretory channel is the ability of cytoplasmic ATP (33) to produce channel closure. With a new class of channel proteins characterized by their inward-rectifying properties (K$_{ir}$, Ref. 4), the initial breakthrough discovery of Ho and Hebert (9) has provided some important hints about the molecular structure of the apical channel. These investigators isolated a K$^+$ channel cDNA, called ROMK1, from the inner strip of the outer medulla of the rat kidney, whereas Zhou et al. (40) isolated a splice variant of ROMK1, ROMK2 (40). When expressed in Xenopus oocytes, ROMK channels exhibit many but not all properties characteristic of the apical membrane K$_{ATP}$ channel (24). Moreover, the ROMK family of channels seems to be expressed in the distal nephron (18) exclusively on the apical membrane (S. Hebert, personal communication). These observations strongly suggest that ROMK encodes an important, perhaps not exclusive, functional unit of the secretory K$^+$ channel in the CCD. Certainly the recent discovery that pancreatic ß-islet cell and cardiac myocyte K$_{ATP}$ channels (10, 11) are formed of K$_{ir}$ channel subunits and ATP binding cassette proteins predicts a similar complex heteromultimeric nature for the native CCD K$_{ATP}$ channel.

In comparison with the emerging picture of apical K$^+$ channel structure and function, less is known about the basolateral K$^+$ channels. However, observations that the basolateral and apical membrane K$^+$ macroscopic conductances exhibit different biophysical and pharmacological properties (30) has suggested that particular K$^+$ channels, encoded by different gene products, are specifically targeted to either each membrane to meet different specialized physiological demands. This prediction has been borne out by recent patch-clamp studies of Wang et al. (20, 34) and Hirsch and Schlatter (6–8). As many as three types of ATP-insensitive K$^+$ channels that are significantly different than the K$_{ATP}$ channels observed on the apical membrane have been identified. An inward-rectifying "low-conductance" channel (27–30 pS) appears to be the major determinant of the basolateral membrane K$^+$ conductance (21). In addition, a 67-pS ohmic channel and hyperpolarization-activated, intermediate-conductance channel (50–90 pS) both also appear to contribute to the "resting" principal cell basolateral membrane K$^+$ conductance.
In the present study, an expression cloning strategy, using a CCD cell line as a source of mRNA, was pursued to further elucidate the molecular basis for renal CCD K⁺ channels. Here I describe the molecular identification of a CCD basolateral membrane K⁺ channel candidate by molecular cloning and functional expression.

METHODS

Cell culture. A CCD cell line, M1, described by Stoos et al. (31), was used in the present study as source of CCD RNA. Passages 8–18 cells were grown on 100-mm plastic supports in a serum-free medium (PC1, Ventrex), supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, and exchanged every other day. Cells were maintained in a 5% CO₂-95% air, 37°C atmosphere. Identical conditions have been previously shown to support the growth and development of cells that exhibit many phenotypic properties of the CCD principal cell (13). In some studies, cells were allowed to grow to confluence (7–12 days) in the serum-free, PC1 medium and then placed in Dulbecco's modified Eagle's medium-Ham's F-12 (DMEM/F12) containing insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml) (the PC1 base medium), and 10% fetal calf serum (FCS) for 6 days.

RNA isolation. Confluent monolayers of M1 cells (12–14 days postseeding) were washed three times with cold, sterile, ribonuclease (RNase)-free phosphate-buffered solution. Total RNA was subsequently isolated using the extraction procedure described by Chomczynski and Sacchi (3). Yields were determined spectrophotometrically by measuring absorbance at 260 nm. Total RNA (20 µg) from each sample was fractionated, size-fractionated, and utilized. Tissue culture cell RNA was isolated using a modification of the treatment described by Chomczynski and Sacchi (3). Yields were determined spectrophotometrically by measuring absorbance at 260 nm. Total RNA (20 µg) from each sample was fractionated, size-fractionated, and utilized. Tissue culture cell RNA was isolated using a modification of the treatment described by Chomczynski and Sacchi (3).

Reverse transcription-polymerase chain reaction and Southern blotting. Mouse kidney mRNA (~10 ng) was reverse transcribed using oligo(dT) (15 mer) and SuperScript reverse transcriptase (200 U) at 42°C for 50 min in 15 µl of 20 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 0.5 mM dNTPs, and 5 mM dithiothreitol. After the reverse transcription, the reaction mixtures were then incubated at 37°C for 20 min. The negative control reactions were incubated at 37°C for 20 min. The negative control reactions were incubated at 37°C for 20 min. The negative control reactions were incubated at 37°C for 20 min. The negative control reactions were incubated at 37°C for 20 min. The negative control reactions were incubated at 37°C for 20 min.

Reversed transcription-polymerase chain reaction and Southern blotting. Mouse kidney mRNA (~10 ng) was reverse transcribed using oligo(dT) (15 mer) and SuperScript reverse transcriptase (200 U) at 42°C for 50 min in 15 µl of 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl), pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 0.5 mM dNTPs, and 5 mM dithiothreitol. After the reverse transcription (RT) reaction, RNase H (Boehringer-Mannheim) was added to each reaction tube (0.1 U/µl) and incubated at 37°C for 20 min. The negative control reactions (RT-) were included in the reaction mixtures. The negative control reactions (RT-) were included in the reaction mixtures. The negative control reactions (RT-) were included in the reaction mixtures. The negative control reactions (RT-) were included in the reaction mixtures. The negative control reactions (RT-) were included in the reaction mixtures. The negative control reactions (RT-) were included in the reaction mixtures. The negative control reactions (RT-) were included in the reaction mixtures. The negative control reactions (RT-) were included in the reaction mixtures.

Polymerase chain reactions (PCR) were carried out in 50 µl containing 1 µl of the kidney RT reaction solution, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 0.2 mM dNTPs, 50 µM of the 5’ and 3’ primers, and 1 unit AmpliTaq DNA polymerase. After the addition of the enzyme, the reaction was raised to 94°C for 1 min and then sequentially cycled 18–22 times for 1-min durations at each of the following temperatures: 60°C (annealing), 72°C (extending), and 94°C (denaturing), using a MJ Research Thermal cycler. Two CCD-IRK3-specific primer sets were used to independently and specifically amplify two different regions of the IRK3 cDNA, reversed transcribed from mouse kidney. Oligonucleotides (primer set 1, P1) corresponding to bp 498–514 (sense, 5’ TTGTCCAGTGCTATTG 3’) and bp 844–824 (sense, 5’ GCTGTCCTCGTTGATTTG 3’) were used in one reaction, oligonucleotides (primer set 2, P2) corresponding to bp 855 to bp 844–824 (sense, 5’ GCTGTCCTCGTGGATTCTG 3’) were used in one reaction, and oligonucleo-
to other Kir channel types. However, it appeared that another reaction. Because these primers correspond to sequences of 0.5 to 1.5 M1 when back filled with 3 M KCl. After a stable impalement was attained, such that both electrodes measured the same spontaneous membrane potential (±4 mV), pulse protocols shown below were conducted. Stimulation and data acquisition were performed with a Macintosh Centris 650 computer using an Instrotech ITC16 analog-to-digital, digital-to-analog converter and Pulse software. Data were filtered at 1 kHz and digitized online at 2 kHz to the hard disk using Pulse for latter analysis using Pulsefit.

Cation permeability of CCD-IRK3 relative to K⁺ (Pₚ/Pₑ) was estimated from the change in reversal potential (ΔVₑ) observed when extracellular K⁺ was replaced with an equivalent concentration of test cation using the equation, Pₑ/Pₚ = e⁻²FΔVₑ/(RT). The zero-voltage dissociation constant (Kₒ) and location of binding (δ) for barium block were estimated as originally described by Woodhull (37) using the assumption that barium interacts with a single site within the CCD-IRK3 pore. Specifically, the fractionation of barium-blocked current, Iₓₕ/Iₓₖ, vs. membrane potential data were fit to a linear, logarithmic transformation of a Boltzmann relation (Iₓₕ/Iₓₖ = 1 − ln[(Iₚ/Ke) + δzF(δT)], so that Kₒ and the fraction of the electric field sensed at the barium binding site, δ, could be extrapolated.

For patch-clamp experiments, the vitelline membrane was removed from oocytes following hyperosmotic shrinking. Patch-clamp electrodes, pulled from glass capillary tubes (Corning no. 7052), had resistances of 0.5–10 MΩ when back filled with 140 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, pH 7.4. Cell-attached recordings of single channels were made using an Axopatch 200A. Current records were digitized at a sampling rate of 47 kHz using a VR-108 digital data recorder (Instrutech, Great Neck, NY) and were stored on a videotape. For analysis, recorded currents were transferred on to disk at 2–5 kHz through an analog-to-digital converter and digitally low-pass filtered at 0.5–1 kHz. Single-channel slope conductance and open probability was assessed by measuring single-channel currents at holding potentials ranging from a membrane potential of −100 to 0 mV.

RESULTS

Cloning and primary structure of a CCD Kᵢᵣ. To elucidate the molecular structure of renal CCD Kᵢᵣ channels, particularly the basolateral Kᵢᵣ channels, an expression cloning strategy using the CCD cell line M1 as a source of mRNA was initiated. This approach was prompted by the observation in Xenopus oocytes that M1 cell mRNA reliably induced the expression of Kᵢᵣ channels like those found in the native CCD (35). Accordingly, a unidirectionally cloned cDNA library was constructed from a Kᵢᵣ-enriched 1.2- to 2.5-kb-size fraction of M1 mRNA. cDNA, transcribed in vitro, from two of the five pools of ∼6,000 independently cloned M1 cell cDNAs, induced expression of Kᵢᵣ-like channels in Xenopus oocytes. By measuring barium-sensitive (1 mM) currents in 90 mM K⁺ under voltage clamp at −80 mV to detect the expression of Kᵢᵣ channels in cRNA-injected oocytes and successively subdividing one of the active pools, an ∼1.2 kbp channel cDNA was eventually isolated. Sequencing this clone revealed one long open frame with a predicted primary structure similar to other Kᵢᵣ channel types. However, it appeared that this initial clone (M1F4.11E4B) was prematurely trun-
ated at an extreme 3′ Not I site (see METHODS, M1 cDNA library construction and screening). To isolate an overlapping cDNA with the 3′ end intact, a second M1 cell library, lacking Not I digestion, was subsequently screened by hybridization to a 3′ restriction fragment of the M1F4.11E4B clone under conditions of high stringency. Of six hybridizing clones, the longest clone contained the entire open reading frame (Fig. 1A).

The 1,421-bp cDNA encodes a protein of 445 amino acids with a predicted molecular mass of 49,641 daltons. Like other K<sub>ir</sub> channels, the deduced amino acid sequence predicts two membrane-spanning regions (M1 and M2) that flank a structure exhibiting a high degree of homology with the "pore" or H5 region of voltage-dependent K<sup>+</sup> channels (Fig. 1). Although the CCD K<sub>ir</sub> channel has the predicted topological K<sub>ir</sub> motif, it also displays several unique regions that presumably confer isoform-specific function. A novel glycine/proline-rich region of 17 additional amino acids is predicted to form a longer extracellular loop between M1 and H5. Moreover, hydrophilic NH<sub>2</sub>-terminal and longer COOH-terminal regions, both predicted to be endoplasmic, show weak homology to the classic inward-rectifier, IRK1 (15), the ATP-regulated ROMK K<sub>ir</sub> channel (9), the G-protein-regulated K<sub>ir</sub> channels (16), or K<sub>ATP</sub> channel subunits (10, 11). These putative endoplasmic domains contain a number sites for posttranslational modification, including five consensus sites for protein kinase C phosphorylation, two potential sites for adenosine 3′,5′-cyclic monophosphate/guanosine 3′,5′-cyclic monophosphate (cAMP/cGMP)-dependent kinase phosphorylation and one possible site for tyrosine kinase phosphorylation (Fig. 1A) but no consensus sites for ATP binding.

Fig. 1. A: nucleotide and predicted amino acid sequence of a cortical collecting duct, inward-rectifying, basolateral membrane K<sup>+</sup> channel (CCD-IRK<sub>3</sub>). Both the proposed membrane spanning regions (M1 and M2), deduced from hydropathy analysis (B), and the H5 region are underlined. Putative endoplasmic domains contain 5 consensus sites for protein kinase C phosphorylation (■), 2 potential sites for cAMP/cGMP-dependent kinase phosphorylation (●), and 1 possible site for tyrosine kinase phosphorylation (▲). Not I site (*), which truncated initial clone, is shown. B: hydropathy plot of CCD-IRK<sub>3</sub> was deduced by the method of Kyte and Doolittle with a 9 amino-acid window.
Based on the identity (\(\approx 96\%\)) of the CCD K\(_i\), with an inward-rectifier K\(^+\) channel cDNA, recently isolated from human brain (23, 28) while this study was in progress, this clone was named CCD-IRK\(_3\) (mKir 2.3 by current nomenclature).

Expression of IRK\(_3\) mRNA in CCD cell culture and kidney. In Northern analysis, a CCD-IRK\(_3\) probe strongly hybridized to a \(\approx 2.9\)-kb transcript in M1 CCD (Fig. 2A). Absolute expression of the CCD-IRK\(_3\) mRNA in M1 CCD cell culture is not an unusual result of tissue culture conditions employed to generate the cDNA library; either the serum-free PC1 medium or the base DMEM/F12 medium supplemented with 10% FCS supports the expression of the CCD-IRK\(_3\) transcript (Fig. 2A). The 1.7-fold increase in the abundance of the CCD-IRK\(_3\) mRNA in the FCS medium compared with the PC1 medium (\(n = 3\)) does, however, raise the possibility that CCD-IRK\(_3\) mRNA abundance is under hormonal control. The observation that the CCD-IRK\(_3\) transcript could not be detected in Northern blot analysis using the same amount RNA from Madin-Darby canine kidney (MDCK), LLC-PK\(_1\) (pig kidney cell line), Chinese hamster ovary (CHO), or monkey kidney fibroblast (COS) cell lines grown under identical conditions (Fig. 2B) provides strong evidence that CCD-IRK\(_3\) expression is not a universal consequence of cell culture. The specific physiological role of CCD-IRK\(_3\) in the kidney is, however, supported by RT-PCR analysis (Fig. 2C). CCD-IRK\(_3\) mRNA was readily amplified from mouse kidney (\(n = 4\)). As resolved by agarose gel

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**Fig. 2.** CCD-IRK\(_3\) mRNA is expressed in the kidney and in CCD cell culture. A: Northern analysis using a CCD-IRK\(_3\) probe and M1 CCD RNA. Either PC1 medium (lane 1) or base DMEM/F12 medium + 5% fetal calf serum (lane 2) support the expression of 2.9-kb IRK\(_3\) transcript in CCD cell culture. Both lanes contain 25 \(\mu\)g of total RNA. Blots were washed at a final stringency of 0.1\(\times\) SSPE, 0.2% SDS at 65°C. B: In contrast, CCD-IRK\(_3\) transcript could not be detected in Northern blot analysis using an identical amount RNA from Madin-Darby canine kidney (MDCK), M1-CCD, LLC-PK\(_1\), monkey kidney fibroblast (COS), or Chinese hamster ovary (CHO) cells. C: reverse transcription-polymerase chain reaction (RT-PCR) analysis of IRK\(_3\) expression in the mouse kidney. Reaction products of predicted size were observed using mouse kidney first-strand cDNA (RT+) as a template and two different IRK\(_3\)-specific primer sets (P1 and P2). Southern blots are shown with internal IRK\(_3\)-specific oligonucleotides (P1 and P2). mRNA (RT–) was used as a negative control template.
electrophoresis and visualized by ethidium-bromide staining, reaction products of predicted size were observed using mouse kidney first-strand cDNA as a template and two different IRK-specific primer sets (18–20 amplification cycles). Southern blots with internal IRK-specific oligonucleotides confirmed CCD-IRK3 identity (Fig. 2B). The observation that no amplification product could be detected when mRNA rather than cDNA was used as a template (i.e., RT−), rules out spurious genomic amplification and verifies bona fide IRK3 mRNA expression in the kidney.

Functional expression in Xenopus oocytes. To begin to elucidate the physiological counterpart of CCD-IRK3 in the kidney, a detailed functional analysis of cloned CCD-IRK3 was conducted. Recombinant RNA transcribed in vitro from the CCD-IRK3-pSP64T cDNA induced the expression of inward-rectifying K+ channels in Xenopus oocytes [CCD-IRK3 currents measured under the 2-microelectrode voltage clamp had a peak amplitude of 10.54 ± 1.69 mV in 45 mM K+ and 45 mM NMDG (n = 30)]. No such channel activity was observed in oocytes injected with water.

CCD-IRK3 is strongly inward rectifying (Fig. 3) and exhibits slight inactivation at extreme hyperpolarizing potentials. From a 0-mV holding potential, hyperpolarizing test pulses evoked much larger K+ currents than those observed at depolarizing test pulses. The large inward CCD-IRK3 currents display very rapid activation kinetics (<10 ms). Similar to native inward rectifiers (19), the rate of activation is influenced by membrane potential and external K+, being augmented by further hyperpolarization or an increase in the K+ concentration (Fig. 3A). After rapid activation, CCD-IRK3 exhibits some degree of inactivation. Although not apparent at voltage steps more positive than $E_K = -60$ mV, larger hyperpolarizing steps caused inactivation; at these potentials, peak CCD-IRK3 currents relaxed over the 400-ms test pulse to a quasi-steady-state value ≥80% of the maximum. More predominant than the closely related IRK1 channel (15) but less than IRK2 (32), inactivation cannot be explained by external cat-ionic block (1) since K+ is the only charge carrier present in these studies (45 mM K+ + 45 mM NMDG). The most remarkable feature of CCD-IRK3 is the strong rectification. In contrast to the large inward currents, outward K+ currents are not easily detected in oocytes injected with 250 ng of cRNA. If more RNA is injected (1 ng) to increase expression, then small but significant outward currents are easily detected (Fig. 4).

Cation selectivity studies are also consistent with a complex permeation mechanism similar to the native CCD basolateral membrane macroscopic K+ conductance (Fig. 5). In these experiments, extracellular K+ (5 mM K+ + 85 mM NMDG) was replaced by an equivalent concentration of either Tl, Rb, NH₄, or Na, and the bi-ionic reversal potential and steady-state inward-slope conductance were measured. Substitution of K+ with Tl caused a 12.6 ± 0.9 mV (n = 6) depolarization, indicating that $P_K/P_T$ is 0.61. The value is in close agreement with the increase in inward-slope conductance observed with Tl replacement ($G_K/G_T = 0.71$). In contrast to the high permeability of CCD-IRK3 for Tl, the channel is relatively impermeable to Na or NH₄. Yielding a $P_K/P_{Na}$ of 9.5 and a $P_K/P_{NH_4}$ of 3.16, substitution of Na or NH₄ for K+ shifted the reversal potential by −55.7 ± 4 mV (n = 6) and −28.5 ± 2.4 mV (n = 6), respectively. Because the contaminating influence endogenous channels are more problematic when the CCD-IRK3 current becomes small, the relative permeability coefficients for Na and NH₄ only reflect minimum estimates. Nonetheless, both relative permeabilities are in close agreement with the relative inward-slope conductance measurements; $G_K/G_{Na}$ was found to be 4.1, and $G_K/G_{NH_4}$ is estimated to be 3.53. Studies with Rb, on the other hand, are more consistent with a pore blocking action than permeation. Certainly, the discrepancy between the low relative ionic permeability value ($P_K/P_{Rb} = 0.8$, n = 6) and high relative inward-slope conductance ($G_K/G_{Rb} = 8.31$) suggests Rb binds more tightly within the CCD-IRK3 pore than K+. In any case, the cation selectivity profile of CCD-IRK3 ($P_K/P_{Na}$, $P_K/P_{NH_4}$) closely parallels that of the macroscopic CCD basolateral membrane K+ conductance ($G_K/G_{Na}$, $G_K/G_{NH_4}$) (30).

Pharmacology. The pharmacological profile of CCD-IRK3 is also similar to the CCD basolateral membrane small-conductance K+ channel (W. Wang, personal communication). As shown in Fig. 6, external barium blocked CCD-IRK3 conduction in a voltage- and concentration-dependent manner. Assuming a 1:1 stoichiometry, barium binds at a site 18 ± 1.4% within the electric field with an inhibitor constant K, (0 mV) of 364 ± 117 μM (n = 7, 45 mM K+ + 45 mM NMDG). Furthermore, CCD-IRK3 is sensitive to quinine (Fig. 7); 1 mM quinine inhibited 52% (n = 3) of the inward CCD-IRK3 current at −100 mV (45 mM K+ + 45 mM NMDG). No detectable changes in the functional properties of CCD-IRK3 were noted with external tetraethylammonium (TEA, 10 mM; n = 6) or glibenclamide (250 μM, n = 6).

Single-channel properties of CCD-IRK3. As noted with the small-conductance basolateral K+ channel in the CCD, patch-clamp studies with Xenopus oocytes in the cell-attached configuration reveal that CCD-IRK3 (Fig. 8) exhibits a high and voltage-independent open probability ($P_o = 0.78 ± 0.03$ at −100 mV, n = 6). Compatible with the inward-rectifying nature of CCD-IRK3 channels observed macroscopically, single currents were detected at potentials more negative that $E_K$.
in the cell-attached configuration but not at more positive holding potentials (Fig. 6). With the unitary inward current-voltage relation, the inward single-channel slope conductance is estimated to be \(14.5 \pm 1\) pS \((n = 6)\), close to the values reported for the small-conductance basolateral channel in the CCD. Furthermore, the observation that CCD-IRK3 spontaneously inactivates or runs down on patch excision from the cell suggests that channel activity is dependent on soluble cytosolic factors, as have been demonstrated for the native CCD channel (8, 21, 34).

### DISCUSSION

\(K^+\) homeostasis is ultimately dependent on \(K^+\) channel activity in the renal CCD, the major site of \(K^+\) secretion in the kidney. To be sure, functionally disparate \(K^+\) channels, expressed on either the basolateral or apical membrane, play specific roles in \(K^+\) secretion. The apical membrane \(K_{ATP}\) channel, characterized by a near linear current-voltage relationship (weak inward-rectifying properties), allows avid \(K^+\) efflux and efficient \(K^+\) secretion (5, 33). In contrast, the two types of \(K^+\) channels on the basolateral membrane, an inward-rectifying \(K^+\) channel.
rectifier and a hyperpolarization-activated channel (6, 7, 34), carry less current in the outward direction. Subsequently, the basolateral channels maintain membrane potential and ensure a favorable driving force for the electrogenic K\textsuperscript{+} secretory process (and mineralocorticoid-dependent Na reabsorption) without significantly recycling K\textsuperscript{+} to the interstitium. In addition such channels, designed to carry K\textsuperscript{+} more efficiently into the cell than out of the cell, are ideally suited for their roles as conduits of K\textsuperscript{+} uptake in hypermineralocorticoid states when the basolateral membrane potential actually becomes more negative than the K\textsuperscript{+} equilibrium potential (29).

Although recent observations strongly suggest that ROMK (K\textsubscript{ir} 1.1) encodes an important but not exclusive functional unit of the secretory K\textsubscript{ATP} channel in the CCD (9), the molecular nature of the two different types of basolateral membrane K\textsuperscript{+} channels has remained undetermined. In the present study, a functional expression cloning strategy, unbiased toward homologous selection as are traditional hybridization-based screening methods, was employed to isolate a K\textsuperscript{+} channel cDNA, CCD-IRK\textsubscript{3} (K\textsubscript{ir} 2.3), from a CCD cell line. Based on its origin and the functional resemblance to one of the CCD K\textsuperscript{+} channels, CCD-IRK\textsubscript{3} is a possible candidate for the small-conductance basolateral K\textsubscript{ir} channel, the major determinant of the CCD principal cell K\textsuperscript{+} conductance (21).

Fig. 4. Despite its strong inward-rectifying nature, CCD-IRK\textsubscript{3} can carry small outward currents K\textsuperscript{+} currents. Steady-state currents were measured from a +50 mV or -60 mV holding potential (V\textsubscript{H}) on voltage pulses from -60 to +50 mV for 400 ms in 5 mM extracellular K\textsuperscript{+} (K + NMDG = 90 mM, n = 6). Bell-shaped current-voltage (I-V) relationship presumably reflects the voltage-dependent binding of intracellular polyamines and Mg, causing a negative slope conductance at voltages more positive than -20 mV. Barium acetate (5 mM) completely blocked the newly expressed current. Oocytes shown here were injected with 4 times more cRNA than the oocytes shown in Fig. 3.

Fig. 5. CCD-IRK\textsubscript{3} is highly K\textsuperscript{+} selective. Inward currents through CCD-IRK\textsubscript{3} channels, measured by 2-microelectrode voltage clamp in CCD-IRK\textsubscript{3} injected oocytes, are dependent on the ionic nature of the charge carrier. Shown are currents relative to those measured in 5 mM K\textsuperscript{+} + 85 mM NMDG (-I\textsubscript{K,ATP}/I\textsubscript{ATP}) with respect to voltage. In these studies, 5 mM K\textsuperscript{+} was replaced with 5 mM of either Na (△), NH\textsubscript{4} (●), Rb (■), or Tl (▲). Arrowheads indicate reversal potentials measured with signified extracellular cation (means ± SD, n = 6).

Fig. 6. Barium blocks CCD-IRK\textsubscript{3} in a voltage and concentration-dependent manner. Steady-state K\textsuperscript{+} currents through CCD-IRK\textsubscript{3} channels, measured by 2-microelectrode voltage clamp in CCD-IRK\textsubscript{3} injected oocytes (45 mM K\textsuperscript{+} + 45 mM NMDG), are shown as a function of voltage and extracellular barium (○, 0 μM Ba; ●, 3 μM Ba; □, 30 μM Ba; ■, 300 μM Ba; △, 3,000 μM Ba). K\textsubscript{d} (0 mV) and K\textsubscript{d} were estimated from linear regression fits of fractionation of barium-blocked current, I\textsubscript{ATP}/I\textsubscript{ATP} = 1, vs. membrane potential data to a logarithmic transformation of a Boltzmann relation, i.e., I\textsubscript{ATP}/I\textsubscript{ATP} = 1 - ln[(Ba/K\textsubscript{d}) + 1/1 + ZF/RT (inset).
As determined by sequence homology, CCD-IRK₃ appears to be a mouse homolog of a human inward-rectifier K⁺ channel recently isolated from brain (HIR, Ref. 28; HRK₁, Ref. 23), IRK₃ (25), or Kir₂.3 (4). Like other Kir channels, the deduced amino acid sequence of CCD-IRK₃ predicts two membrane-spanning regions that flank a structure exhibiting a high degree of homology with the “pore” or H₅ region in voltage-dependent K⁺ channels. As recently demonstrated in a related Kir isoform (39), the assembly of four CCD-IRK₃ “subunits” is presumably required for the formation of functional CCD-IRK₃ channels. With the precedence for heteroligomeric assembly of other Kir isoforms, particularly the G protein-gated K⁺ channels (14), it remains to be determined whether the native CCD small-conductance Kir channel is formed of heteroligomeric or homooligomeric CCD-IRK₃ complexes. In any case, although the CCP Kir channel shares the basic topological Kir motif (4), it does display several unique regions that presumably confer isoform-specific function. Most notably, a novel glycine/proline-rich region of 17 additional amino acids, predicted to form a longer extracellular loop between M₁ and H₅, may play a unique role in conduction or extracellular protein-protein interaction. The two putative endoplasmic domains, least conserved among the Kir channels, contain a number sites for posttranslational modification, as might be predicted from known avenues of CCD basolateral K⁺ channel regulation (8, 21, 34), including five consensus sites for protein kinase C phosphorylation, two potential sites for cAMP/cGMP-dependent kinase phosphorylation, and one possible site for tyrosine kinase phosphorylation.

Although initial Northern blot analysis suggested the human Kir₂.3 channel is primarily expressed in excitable tissue (28), a specific physiological role in the kidney, particularly in the CCD, is supported by these studies. First, the CCD-IRK₃ cDNA was isolated from a CCD cell line, M1. Derived from a single CCD of a mouse transgenic for the SV40 large T antigen, M1 CCD cells exhibit phenotypic properties of the principal cell, including K⁺ secretion and amiloride-sensitive, electronegative Na⁺ reabsorption (13, 31). The isolation of a K⁺ channel cDNA that has many functional characteristics of native CCD basolateral K⁺ channel and is abundantly expressed in M1 cell culture is consistent with the predominant basolateral K⁺ conductance found in M1 cells (13). Second, absolute expression of CCD-IRK₃ mRNA in M1 cells is not an unusual result of tissue culture conditions used to derive the cDNA library. Although the cells for cDNA library construction were grown in a serum-free medium (PC1 medium, Ventrex) as before (13), the base medium, DMEM/F12, containing FCS also supports the expression of CCD-IRK₃ mRNA. The increased expression of CCD-IRK₃ in the FCS-supplemented medium does, however, raise the intriguing possibility that CCD-IRK₃ mRNA abun-

Fig. 7. Quinine blocks CCD-IRK₃. Steady-state I-V relationships are shown in the presence (●) and absence (○) of 1 mM quinine (5 mM K⁺ + 85 mM NMDG).

Fig. 8. CCD-IRK₃ is a small-conductance channel. A: single CCD-IRK₃ currents in a cell-attached Xenopus oocyte patch with 140 mM K⁺ in the pipette and bath. B: open probability (Pₒ) voltage relationships of channel. C: single channel records, shown at bottom, were low-pass filtered at 1 kHz.
Molecular Identification of a CCD K⁺ Channel

Inward rectification as under hormonal control as might be expected for a channel expressed in corticosteroid hormone-regulated principal cell (29). Third, the observation that the CCD-IRK₃ transcript is not expressed in MDCK, LLC-PK₁, CHO, or COS cells grown under identical conditions (PC1) demonstrates that CCD-IRK₃ is not ubiquitously expressed in cell culture, providing compelling evidence for a specific role of CCD-IRK₃ in the native collecting duct. Furthermore, although kidney IRK₃ mRNA falls below the detection limits of Northern analysis, it is readily observed by RT-PCR. Finally, the role as a basolateral membrane channel is supported by preliminary studies from our group. Using an epitope-tag approach to follow subcellular expression of CCD-IRK₃ in a IRK₃-transfected epithelial cell line, we have found that this channel is targeted specifically to the basolateral membrane (17).

The functional similarities and differences that are exhibited between CCD-IRK₃ and the small-conductance K⁺ basolateral channel in the rat CCD provide some insight into a possible physiological role of the recombinant mouse channel in the CCD. Extrapolation of a mouse donor to the native rat tissue seems appropriate since the rat homolog of IRK₃ has been doned and shown to have essentially the same functional features (2) as reported here for the mouse channel.

Although both the recombinant and native CCD channels exhibit inward rectification, the extent of rectification is different. Studied in the cell-attached mode, CCD-IRK₃ is strongly inward rectifying. The native small-conductance basolateral Kᵢᵢ channel detected by Wang et al. (34), on the other hand, has been reported to be mildly inward rectifying in the presence of Mg. Unfortunately, the rectifying properties of the native channel have not been systematically studied in cell-attached patches as they have for CCD-IRK₃, precluding an accurate comparison. Considering the mechanistic basis for inward rectification, the experimental configuration can have dramatic effects on the degree of rectification. In strong inward rectifiers, like mouse Kᵢᵢ 2.1 (38) and human Kᵢᵢ 2.3 channels (20), inward rectification is brought out by voltage-dependent occlusion of the ion conduction pore by intracellular polyamines and Mg, rather than intrinsic gating process. Subsequently, these Kᵢᵢ channels lose their strong rectifying properties on excision into polyamine-free solutions.

Using site-directed mutagenesis, Yang et al. (38) determined that the high-affinity polyamine and Mg binding site in the mouse Kᵢᵢ 2.1 channel is created by an aspartic acid residue in the M2 segment and a glutamic acid at 44 amino acids away from the M2 domain in the hydrophilic COOH terminus. The presence of these critical amino acids at the corresponding positions in CCD-IRK₃ is consistent with a similar labile inward-rectification mechanism. Subsequently, the discordance in the rectification properties of the two channels may reflect differences in the experimental configuration. Certainly further study is required before it is known whether extent of inward rectification of the native and recombinant channel are similar when measured under identical conditions. Unfortunately, the rapid rundown process of CCD-IRK₃ on patch excision from the membrane makes this comparison technically arduous with the oocyte expression system.

The parallels between CCD-IRK₃ and the native CCD basolateral K⁺ channel at the single channel level offer additional, albeit correlative, evidence that the two channel may be related. The "small-conductance" basolateral K⁺ channel in the native CCD has been reported by Hirsh and Schlatter (6, 7) to have a inward-slope conductance of 67 pS, whereas Wang (21, 34) has reported a value of 27–30 pS. At present, it is not known whether this actually represents two different channels in the rat CCD or different experimental differences. Nevertheless, when measured under similar ionic conditions as Wang et al. (21, 34) employed for native channel, CCD-IRK₃ has a unitary inward single-channel conductance of 14 pS at room temperature. The value is close but slightly smaller than the 27–30-pS conductance of the native channel at 37°C. While small, disparity may reflect differences in the temperature of the two experimental preparations as would predicted by the temperature dependence of aqueous diffusion (Q₁₀ = 1.3–1.8). Furthermore, like the native small-conductance channel described by Wang et al. (21, 34), IRK₃ exhibits a voltage-independent high open probability. Besides these similarities in the single-channel biophysical fingerprints of the native basolateral K⁺ channel and CCD-IRK₃, CCD-IRK₃ also appears to be regulated by identical, albeit somewhat generic, processes. On patch excision from the cell, both channels also exhibit a rapid inactivation process, suggesting that activity of both the native (6, 8, 21) and recombinant channel is maintained by soluble cytosolic factors. Furthermore, preliminary studies from this lab indicate that elevation of cytosolic Ca suppresses CCD-IRK₃ activity (36) by a similar, membrane delimited process as the small-conductance K⁺ channel on the basolateral membrane (7).

CCD-IRK₃ shares several other generic biophysical and pharmacological features with the small basolateral membrane channel. Like the native channel (Ref. 7 and W. H. Wang, personal communication), IRK₃ is inhibited by barium and quinine but is insensitive to TEA and glibenclamide. Although the selectivity of the native channel has not been systematically studied, the cationic selectivity sequence (Pᵢᵢ/Pᵢᵢ, Tl > Rb > K > Na > NH₄ > Na > Rb) of CCD-IRK₃ is similar to the macroscopic CCD K⁺ conductance on the basolateral membrane (Gᵢᵢ/Gᵢᵢ, K > NH₄ > Rb; Pᵢᵢ/Pᵢᵢ, Rb > K > NH₄) (30). Although indirect, the similarity in the selectivity profile is consistent observations that the small-conductance channel is the major determinant of the macroscopic conductance (21).

In summary, these studies provide correlative evidence that CCD-IRK₃ may encode the CCD small-conductance basolateral membrane Kᵢᵢ channel. The molecular identification of the major determinant of the CCD principal K⁺ conductance offers a crucial step...
toward elucidating the molecular mechanisms of differential K⁺ channel targeting, expression, and regulation in the CCD and understanding the molecular basis for K⁺ homeostasis.

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