Developmental expression of ROMK in rat kidney

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Zolotnitskaya, Anna, and Lisa M. Satlin. Developmental expression of ROMK in rat kidney. Am. J. Physiol. 276 (Renal Physiol. 45): F825–F836, 1999.—The apical secretory K+ (SK) channel in the principal cell represents the rate-limiting step for K+ secretion across the cortical collecting duct (CCD). Patch clamp analysis of mature rat principal cells identifies an increase in number of conducting SK channels after the 2nd week of life [L. M. Satlin and L. G. Palmer. Am. J. Physiol. 272 (Renal Physiol. 41): F397–F404, 1997]. —1 wk after an increase in activity of the amiloride-sensitive epithelial Na+ channel (ENaC) is detected. To correlate the postnatal increase in channel activity with developmental expression of ROMK, the molecular correlate of the SK channel, we used gene-specific probes to show a developmental increase in abundance of renal ROMK mRNA and a ROMK-specific antibody to examine the nephron distribution, localization, and abundance of this protein in developing rat kidney. Using antibodies directed against aquaporin-3 (AQP-3) and Tamm-Horsfall protein (THP), we confirmed that ROMK was expressed along the apical membranes of principal cells and thick ascending limbs of Henle (TALH) in adult kidney. Within the midcortex of the neonatal kidney, ROMK-positive segments revealed weak coincident staining with the TALH-specific antibody but did not colabel with an antibody directed against distal and connecting tubule (CNT)-specific kidney lectin or the lectin Dolichos biflorus agglutinin (DBA), which labels proximal tubules and collecting ducts. In inner cortex and outer medulla of kidneys from 1-wk-old animals, ROMK protein was identified in medullary TALH (MTALH) and DBA-positive collecting ducts. By 3 wk of age, coincident ROMK and DBA expression was detected in midcortical and outer cortical CNTs and CCDs. Immunoblot analysis of plasma membrane-enriched fractions of maturing rat kidney revealed a developmental increase in a ~40-kDa band, the expected size for ROMK. Immunolocalization of α-ENaC showed apical staining of a majority of cells in distal nephron segments after the 1st week of postnatal life. The β- and γ-ENaC subunit expression was routinely detected in a mostly cytoplasmic distribution immediately after birth, albeit in low abundance; γ-ENaC showed some apical polarization. These results suggest that the postnatal increases in a principal cell apical SK and Na+ channel activity are mediated, at least in part, by increases in abundance of ROMK message and protein and ENaC subunit proteins.

GROWING ORGANISMS MAINTAIN a state of positive K+ balance (33). The final renal regulation of K+ homeostasis in the adult occurs in the collecting duct (12). Cortical collecting ducts (CCDs) isolated from adult rabbits and microperfused in vitro at physiological flow rates secrete net K+ at high rates (13, 26, 32). In contrast, net K+ transport is not detected in microperfused CCDs from newborn rabbits until the 3rd week of postnatal life (26). Net Na+ absorption, however, absent at birth, rapidly increases to levels approximating 50% of those observed in the adult after the 1st week of life (26, 34).

K+ secretion in the fully differentiated CCD is accomplished by a two-step process. First, K+ is actively taken up into the principal cell at the basolateral membrane in exchange for Na+, a process mediated by the ubiquitous Na-K-ATPase (12). Thereafter, K+ is secreted through apical K+-selective channels into the tubular lumen by passive diffusion down a favorable electrochemical gradient (12). The electrochemical gradient is determined by the lumen-negative voltage, generated by apical Na+ entry through Na+-selective channels and its electrogenic basolateral extrusion, and the high cell K+ concentration (12). The apical K+ and Na+ channels constitute the rate-limiting steps for K+ secretion and Na+ absorption, respectively.

The apical secretory K+ (SK) channel in the rat principal cell has been shown to be a low-conductance (30–40 pS) inwardly rectifying ATP-sensitive channel (11, 12, 24, 37). Electrophysiological analysis of the apical K+ permeability of the maturing rabbit principal cell revealed a paucity of functional SK channels immediately after birth, with channel activity increasing progressively after the 1st week of postnatal life (28). Yet, in these same cells, the mean number of conducting apical amiloride-sensitive Na+ channels per patch reached the mature level by 2 wk of age (27).

Cumulative functional and biophysical evidence (reviewed in Refs. 12, 25, 38) now identifies ROMK, a cDNA encoding a family of K$_{ATP}$ channels (14, 39), as the molecular correlate of the apical SK channel. ROMK was originally cloned from rat outer medulla where the apical low-conductance K$_{ATP}$ channel in the thick ascending limb of the loop of Henle (TALH) recycles K+ across the apical membrane to ensure an abundant supply of substrate for the Na-K-2Cl cotransporter (36). Three renal ROMK isoforms (ROMK1–3), derived from alternative splicing of the 5′ end of the gene and differing only in the sequence and length of their amino termini, have been identified (2, 14, 39). ROMK mRNA has been found in all nephron segments beyond and including the medullary TALH (MTALH) in rat kidney (2). Immunofluorescence studies of adult rat kidney performed using anti-ROMK carboxy-terminal antibodies revealed that ROMK protein is present along the apical membranes of cortical TALH (CTALH) and MTALH, distal convoluted tubule, connecting tubule (CNT), and principal cells in the CCD and outer medullary collecting duct (OMCD) (18, 22, 38). ROMK...
is not detected beyond the initial portion of the inner medullary collecting duct (IMCD) (38).

The amiloride-sensitive epithelial Na⁺ channel, ENaC, cloned from rat distal colon using a functional expression strategy (4, 5), is a multimeric channel comprising α-, β-, and γ-subunits. Coexpression of the three subunits in oocytes reconstitutes a channel with ion-selective permeability, gating properties, and pharmacological profile similar to the native channel (5). In the kidney of Na⁺-depleted rats, the three subunit mRNAs are coexpressed along the apical membranes of the aldosterone-responsive segments of the distal nephron, including the distal convoluted tubule, CNT, principal cells of the CCD and OMCD, and IMCD (7), a pattern of localization that correlates well with the expression of amiloride-sensitive electrogenic Na⁺ absorption. Vehaskari et al. (35) has shown that steady-state levels of α-, β-, and γ-ENaC mRNA in neonatal rat kidney are comparable to those measured in the adult.

The purpose of the present study was to correlate the functional expression of SK channel activity with appearance of ROMK message and protein in the maturing kidney. To this end, we used gene-specific molecular probes to assess ROMK mRNA abundance and a rabbit polyclonal antibody raised against ROMK to examine nephron distribution, localization, and abundance of this protein in developing rat kidneys by immunocytochemistry and immunoblotting. Immunolocalization of the α-, β-, and γ-subunits of ENaC in rat cortex was also performed to determine the pattern of postnatal expression of Na⁺ channel proteins.

METHODS

Animals. Adult female Sprague-Dawley rats with their litters were obtained from Taconic Farms (Germantown, NY) and raised in the animal facilities at the Albert Einstein College of Medicine or Mount Sinai School of Medicine. Adult animals were maintained on standard rat chow (Purina 5001; Ralston-Purina, St. Louis, MO) and allowed free access to tap water. Newborn animals were raised with and fed by their mothers. Rats were studied at each week during the first 6 wk of postnatal life; adult rats were defined as animals ≥6 wk of age. At least four litters of rat pups were used for each group of studies. Animals were anesthetized or killed by intraperitoneal injection of pentobarbital sodium (30 or 100 mg/kg body wt, respectively).

Preparation of ROMK probe. Northern blot analysis. The ROMK probe was prepared by RT-PCR of rat renal medulla RNA, as described below, using gene-specific primers designed to amplify a highly conserved region (bp 821–1123) of the published ROMK1 sequence (sense: 5'-CAACAGCCTTTCTCCATG-3'; antisense 5'-TGTCTAGCCTCTCCATCTGG-3') (14). The amplification product was subcloned into the pCR2.1 vector (Invitrogen, San Diego, CA). Sequence analysis (Applied Biosystems model 373 fluorescent sequencer) of the insert revealed its identity to the published ROMK sequence (14). The cloned insert was restriction enzyme digested and labeled with [α-³²P]dCTP (Mega-prime DNA-labeling system; Amersham, Arlington Heights, IL).

To generate the probe, RT-PCR was performed using 2 µg of total RNA prepared by the single-step acid guanidinium thiocyanate-phenol-chloroform method (6). The RNA was treated with DNase I (amplification grade, Gibco-BRL; Life Technologies, Grand Island, NY), according to the manufacturer's protocol, to eliminate residual genomic DNA. To 10 µl of the RNA sample were added 2 µl 10× PCR buffer (Gibco), 1 µl 50 mM MgCl₂, 2 µl dNTPs (80 pmol; Pharmacia LKB Biotechnology, Pikachu, NJ), 2 µl deoxyribonucleotide mixture (1.25 mM stock of each nucleotide; Pharmacia), 2 µl 0.1 M dithiothreitol, and 1 µl (200 U/µl) SuperScript II reverse transcriptase (Gibco). Reverse transcription proceeded at 25°C for 10 min, 42°C for 55 min, 95°C for 5 min, and the contents were then cooled to 4°C. The reverse-transcribed cDNA was amplified after a 3-min denaturation at 94°C using 40 cycles of PCR (Perkin Elmer Thermocycler, model 480), each consisting of denaturation for 1 min at 94°C, annealing of primers for 1 min at 55°C, elongation for 1 min at 72°C, and a final extension for 8 min at 72°C. The sample was size fractionated by electrophoresis on a 2% agarose gel to verify that the PCR product was of expected size (303 bp).

Northern blot analysis. Total RNA was extracted from whole kidney homogenates using the method of Chomczynski and Sacchi (6), and the integrity of the RNA was verified spectrophotometrically by measuring absorbance at 260 and 280 nm. Thirty micrograms of total RNA from each age group were size fractionated by electrophoresis on a 1% agarose gel and transferred overnight at 4°C to a Hybond-N filter (Amersham). Each filter was prehybridized for 2 h at 42°C with 50% formamide, 5× Denhardt’s reagent, 5× standard saline citrate (SSC), 40 mM sodium phosphate, pH 6.8, 0.1% SDS, and 200 µg/ml denatured salmon sperm DNA in diethyl pyrocarbonate-treated water. The RNA was hybridized overnight in the same solution with the ³²P-labeled rat ROMK probe. The membranes were washed successively with 1× SSC-0.5% SDS twice at room temperature for 30 min, then 0.1× SSC-0.1% SDS three times at 50°C. The filter was exposed at −80°C for 3 days to Hyperfilm (Amersham). The equality of loading and integrity of RNA were confirmed by reprobing the membranes with a PCR β-actin-generated probe (3). The filter was exposed overnight at −80°C.

The relative intensities of bands were measured by analysis of scanned autoradiographs using an image densitometer (Bio-Rad model GS-670) and Molecular Analyzer software (Bio-Rad, v.2.12). To compensate for differences in quantity of total RNA on each lane of the membrane, each densitometric value for ROMK expression was normalized to its respective value of β-actin.

Tissue preparation for immunofluorescence microscopy. Animals were anesthetized, and their kidneys were perfused either via the aorta (adults) or the heart (newborns) with PBS followed by 2% paraformaldehyde for 10–15 min at a pressure of 150 cmH₂O. Kidneys were immediately removed, sliced into 2-mm sagittal sections, postfixed in 2% paraformaldehyde for 4 h, and immersed overnight at 4°C in 30% sucrose for cryoprotection. Blocks of tissue were then embedded in OCT compound (Sakura Finetek, Torrance, CA), frozen in liquid nitrogen, and cut into 4- to 5-µm sections using a Leica CM3050 cryostat. Sections were collected on poly-L-lysine-coated (Sigma, St. Louis, MO) slides.

Antibodies. Rabbit antiserum directed against a 23-amino acid synthetic peptide corresponding to the carboxy terminus of rat ROMK and thus common to ROMK isoforms 1–3 (22) was generously provided by L. Palmer. Additional tubule-specific probes included an affinity-purified rabbit polyclonal antibody to rat aquaporin 2 (AQP-2) that recognizes principal cells of collecting ducts, kindly provided by G. Fritindt (8); goat anti-human Tamm-Horsfall glycoprotein (THP; uromucoid) antiserum (ICN Capp Pharmaceuticals, Aurora, OH) that recognizes TALH cells (30); and sheep anti-rat kallikrein antiserum that recognizes tissue kallikrein in distal tubules...
and CNTs (gift from J. Chao) (9). A protein-A-purified rabbit polyclonal anti-α,β ENaC antibody was obtained from P. Smith (31); antisera were raised in rabbits and directed against the carboxy-terminal ends of β- and γ-ENaC (7) were provided by C. Canessa. All antibodies have been well characterized and their specificity previously established.

Immunofluorescence microscopy. Sections were hydrated with PBS for 10 min, blocked with FCS for 30 min, and permeabilized using 0.5% saponin in FCS in a 1:1 dilution for 30 min. FCS was replaced by normal goat serum for subsequent labeling with the anti-kallikrein antiserum. Slides were then washed three times in PBS, 5 min each wash, and incubated for 30 min at 37°C with one of the following primary antibodies: anti-ROMK (1:200 dilution) or anti-α-, anti-β-, or anti-γ-ENaC (1:200 dilution). After three successive washes in PBS, the sections were incubated with FITC-conjugated F(ab')2 fragment donkey anti-rabbit IgG (1:200; Jackson Immunochemicals, W. Grove, PA). Colabeling of some sections was performed by the subsequent application of anti-AQP-3 (1:1,250), anti-THP (1:1,250), or anti-kallikrein (1:100) antibodies, visualized with rhodamine-conjugated F(ab')2 fragment donkey anti-rabbit IgG. Lissamine rhodamine-conjugated rabbit anti-goat, or Texas Red-conjugated anti-sheep IgG secondary antibodies, as appropriate (1:200; Jackson Immunochemicals). Other sections were labeled with the proximal tubule- and collecting duct-specific rhodamine-conjugated Dolichos biflorus agglutinin (DBA; 5 µg/ml; Vector Laboratories, Burlingame, CA) (15, 16). Control experiments consisted of either omitting the primary or secondary antibodies, or substituting the primary antibody with anti-ROMK or anti-ENaC antibody preincubated overnight at 4°C with a 50-fold molar excess of peptide against which the antibody was raised. All sections were mounted onto coverslips with the Prolong Antifade Kit (Molecular Probes, Eugene, OR).

Fluorescence microscopy was performed either with an Olympus or Nikon Diaphot inverted microscope equipped with infinity-corrected optics. Images were collected from the Olympus microscope with a Photometrics (Tucson, AZ) cooled charge-coupled device camera driven by I.P. Lab Spectrum software (Signal Analytics, Vienna, VA) running on a Power Macintosh (Apple Computer, Elk Grove, CA). The green and red fluorescence of FITC and rhodamine, respectively, were visualized with Nikon filters (B and G) or, most often, with a Nikon double-filter cassette allowing for simultaneous visualization of fluorescein and rhodamine/Texas Red fluorescence. Photographs were obtained with a Nikon 35-mm camera using Kodak Elite 400 film.

Western blotting. Whole kidney was minced finely and homogenized three times for 15 s each time using a Brinkmann tissue homogenizer (model PT 10/35) in ice-cold isolation solution (250 mM sucrose, 10 mM triethanolamine, pH 7.4), containing 1 mM EDTA, 1 mM EGTA, and the following protease inhibitors: 1 mM Pefabloc, 1 mM leupeptin, 1 mM benzamidine, 1 µg/ml chymostatin, 10 µg/ml pepstatin, and 1 µg/ml aprotinin. Homogenates were spun in a Beckman model LE 80K ultracentrifuge to enrich the fraction in plasma membranes, as previously described (8). The membrane fractions were solubilized at 60°C for 15 min in Laemmli sample buffer. The total protein concentration in spun fractions was measured using a protein assay reagent kit (BCA; Pierce, Rockford, IL).

All aliquots of membrane extracts (40 µg/lane) were separated by SDS-PAGE on 12% polyacrylamide precasted minigels (Bio-Rad, Hercules, CA) and electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were blocked with blot wash buffer (42 mM Na2HPO4, 8 mM NaH2PO4, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) containing 5% nonfat dried milk for 30 min and then incubated with the ROMK antibody at a 1:2,000 dilution overnight. After six washes in blot wash buffer, the secondary antibody, a horseradish peroxidase-conjugated donkey anti-rabbit IgG (Pierce), was applied at a 1:10,000 concentration for 1 h. After washing, antibody binding was visualized by enhanced chemiluminescence (ECL, Amersham) before exposure to X-ray film. Controls were performed by replacing the primary antibody with ROMK antiserum preadsorbed with a 50-fold molar excess of immunizing peptide. The relative intensities of bands were measured by analysis of scanned images using the NIH Image 1.61 software for Macintosh.

Statistics. Developmental comparisons were performed by t-test, ANOVA, and/or linear regression analysis using SigmaStat software (SPSS, Chicago, IL). Values are means ± SE. Significance was asserted for P < 0.05.

RESULTS

Northern analysis. The developmental expression of ROMK mRNA in rat kidney was examined by Northern analysis. As shown in the representative blot depicted in Fig. 1, a single band of expected size (14) for ROMK (~3.1 kb) was detected at all ages. Densitometric analysis revealed a biphasic increase in steady-state ROMK mRNA expression after birth (Fig. 2). This pattern of developmental increase in ROMK mRNA expression was similar to that reported in rabbit (1), the species used for functional (26) and electrophysiological (28) characterization of the ontogeny of net K+ transport and SK channel activity in the CCD, respectively.

Immunofluorescence. Indirect immunofluorescence microscopy of the adult rat kidney labeled with anti-ROMK antibody revealed apical ROMK expression in tubules within medullary rays. Labeling in the outer medulla, localized to MTALH and OMCD (22, 38), was more prominent than that detected in cortex (Fig. 3A). Control experiments were performed using antisera immunoadsorbed with the ROMK-immunizing peptide or secondary antibody alone. No immunofluorescence above background was detected (data not shown), as

![Fig. 1](http://ajprenal.physiology.org/DownloadedFrom/web/journal/ajprenal.physiology.org/article-figures/57609616/f001.jpg)
has been reported by Mennitt et al. (22) using this same antibody.

To confirm the tubular distribution and membrane localization of this protein, double antibody labeling using anti-ROMK and TALH- and principal cell-specific antibodies was performed. As reported by others (22, 38), ROMK staining was heterogeneous in THP-positive CTALH (Fig. 3B) with most, but not all, cells expressing the epitope. ROMK labeling in neighboring tubules (Fig. 3C), devoid of THP antigen and thus not TALHs, was not only restricted to the apical membrane but was apparent in the subapical cytoplasm. The presence of ROMK in only a subset of cells in such segments suggested it likely they represent CNTs or CCDs. In the outer medulla, the majority of ROMK-positive tubules cut in cross section (Fig. 3A) contained anti-TALH antibody (not shown), as has been reported by others (22, 38).

To verify that ROMK is present along the apical membrane of principal cells, other sections were labeled with antibodies against ROMK and AQP-3. Apical ROMK was detected only in AQP-3-positive cells (Fig. 3D), consistent with expression of the channel protein in principal cells. Intercalated cells, which do not express ROMK channels (22, 38), are devoid of ROMK and AQP-3. As controls for these double labeling experiments, anti-AQP-3 or anti-THP antibodies were omitted in the labeling of some cryosections; application of their appropriate secondary antibodies to sections stained with anti-ROMK antibody and its corresponding secondary antibody showed no cross-reactivity (data not shown).

Thus, within the fully differentiated kidney, ROMK is selectively expressed along the apical membranes of TALH and principal cells. This pattern of localization of ROMK expression is identical to that previously described by others using the same (22) or different antibodies raised against the COOH-terminal portion of the protein (18, 38).

Developmental expression of ROMK antigen in maturing rat kidney. Although immunofluorescence studies indicated that ROMK was indeed expressed in the neonatal kidney, our ability to identify the neonatal cortical segments expressing ROMK was hampered by weak colabeling with the anti-THP antibody and almost undetectable staining with the anti-AQP-3 antibody, presumably reflecting low levels of antigen expression. Attempts to increase the concentration of either of the latter two primary antibodies were complicated by generation of high levels of background fluorescence (see Fig. 4D). To circumvent this technical difficulty and identify distal tubular structures, we colabeled sections with anti-ROMK antibody and DBA, a lectin shown to preferentially bind to the brush border of proximal tubules and apical membrane and/or cytoplasm of cells showing basolateral immunoreactivity for Na-K-ATPase in the CCD of rat kidney, i.e., principal cells (15, 16).

Low-power magnification of cryosections cut from 1-wk-old rat kidney colabeled with anti-ROMK antibody and DBA (Fig. 4A) revealed DBA staining of two populations of tubular profiles: one displaying an intense uniform labeling along the luminal membrane, presumably representing proximal tubules (curved arrows), and the other showing a heterogeneous labeling of only some cells, likely representing CNT or collecting ducts, both of which include principal cells (20). CNTs, whether those of juxtamedullary or midcortical nephrons, which form arcades, or those of subcapsular nephrons, which directly join the collecting duct, course laterally through the cortex at some point and thus, in longitudinal section, may appear to be cut in cross section (23), as shown in Fig. 4C. ROMK was absent from the nephrogenic zone and present most often in DBA-negative tubules in middle and inner cortex (CTX; short arrows in Fig. 4A) and outer medulla (OM). Rarely, inner cortical and outer medullary tubules exhibiting a heterogeneous pattern of DBA staining revealed coincident apical ROMK (short arrows in OM; Fig. 4A).

High-power magnification of the midcortex at 1 wk of age revealed apical DBA binding on a discrete population of cells, presumably principal cells within CNTs or CCDs; ROMK was not expressed in these tubules (Fig. 4).

Although colabeling of single sections with anti-ENaC and anti-ROMK antibodies would have provided a definitive identification of CNT/CCD and insight into the temporal pattern of expression of the two channel proteins during postnatal differentiation, we encountered a major difficulty in performing these studies with the available antibody combinations, both raised in rabbit. In preliminary experiments in which single cryosections were sequentially labeled with anti-ROMK antibody followed by its appropriate 2nd antibody and then anti-ENaC antibody visualized by its 2nd antibody, we found that the α-ENaC labeling was no longer specific to the apical membrane of a subpopulation of cells in the CNT/CCD (as shown in Fig. 7). Similarly, cytoplasmic β-ENaC staining could no longer be detected in sections initially labeled with anti-ROMK antibody and its fluorescent 2nd antibody. Based on the loss of selective binding of the second 2nd antibody in these double labeling experiments, in which the two 1st antibodies were raised in the same donor species, we elected to identify collecting ducts by their characteristic heterogeneous apical DBA staining pattern (Fig. 4).
4B). Nor was ROMK immunostaining evident in distal tubules or CNTs expressing kallikrein at this age (Fig. 4C). However, by 1 wk of age, CTALH expressed modest apical staining for ROMK (Fig. 4D).

By 3 wk of age, ROMK was clearly evident in DBA-positive cells in midcortical tubules presumed to be CCDs based on their heterogeneity of staining and linear profiles (Fig. 4E). Occasional outer cortical tubules, likely representing CNTs cut in cross section, also showed coincident labeling with ROMK and DBA at this stage of renal development (Fig. 4F).

In the medulla of the 1-wk-old newborn, ROMK protein was evident along the apical membranes of THP-positive MTALH (Fig. 4G) and occasional collecting ducts exhibiting heterogeneous DBA staining (Fig. 4A).

Western analysis. A representative Western blot of plasma membrane-enriched fractions from maturing rat kidneys using the anti-ROMK antibody is shown in Fig. 5 (left). Four prominent bands were detected at ~76, 50, 45, and 40 kDa. Whereas the 76-kDa band, accompanied by a less intense band at 85 kDa, was present at all ages, a developmental increase in intensity of the 40- (Fig. 6; r = 0.99 by linear regression analysis) and 45-kDa bands, consistent with the predicted and reported molecular weights of core and glycosylated ROMK (22, 38), respectively, was detected (Fig. 6). In contrast, a maturational reduction in intensity of the 50-kDa band was apparent (Fig. 5); the identity of this band is uncertain. Preadsorption of the immune serum with ROMK peptide ablated all bands (Fig. 5, right).
**Developmental Expression of ROMK**

**Fig. 5.** Immunoblot of plasma membrane-enriched protein fractions isolated from maturing rat kidneys. Forty micrograms of protein were loaded in each lane. Left: prominent bands were detected at ~76, 50, 45, and 40 (arrow) kDa. Bands at 40 and 45 kDa, corresponding to the predicted and reported size of core and glycosylated ROMK, increase in intensity with advancing age. The 50-kDa band is also developmentally regulated but is most highly expressed immediately after birth; identity of this band is unknown. The 76-kDa band is present in kidneys of all ages. Right: preadsorption of anti-ROMK antibody with its synthetic peptide resulted in ablation of all bands identified in left.

**Fig. 6.** Densitometric analysis of the 40-kDa ROMK band in membrane fractions isolated from maturing rats. Protein abundance of 1- to 4-wk-old animals was expressed as a percentage of the value measured at 6 wk for each of 3 blots. A significant postnatal increase in protein expression was identified after the 2nd week of postnatal life ($r = 0.99$ by linear regression analysis). Difference in protein abundance between the 1- and 6-wk-old animals did not achieve statistical significance ($P = 0.08$). Values are means ± SE.

**DISCUSSION**

The present study demonstrates a postnatal increase in expression of ROMK protein in the rat kidney after the 2nd week of postnatal life (Fig. 6), closely following the early increase in steady-state ROMK mRNA abundance (Fig. 2). Immunolocalization studies suggest that this increase in protein abundance is likely due to increases in ROMK expression in the TALH and more distal segments, including CNT and collecting ducts (Fig. 4). In the cortex of newborn rats, ROMK was evident in TALH, albeit at low abundance, but was not detected in distal tubules, CNT, and CCDs. By 3 wk of age, labeling of mid-CCDs and occasional outer cortical structures, believed to be CNTs, was clearly evident. In the medulla, ROMK was already present in MTALH and collecting ducts in the 1st week of life.

Our studies confirm the observations, published by others (18, 22, 38), that ROMK antibodies label the apical membranes of TALH and principal cells in adult rat kidney, as expected on the basis of electrophysiological analyses of the apical K⁺ conductances in these two

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**Fig. 4.** Immunolocalization of ROMK protein in renal cortex of young rat. For A–G, ROMK antibody binding is visualized with an FITC-conjugated secondary antibody; kallikrein- and THP-specific antibodies are labeled with rhodamine- or Texas Red-conjugated secondary antibodies. A: low-power photomicrograph of 1-wk-old kidney labeled with anti-ROMK and rhodamine-conjugated Dolichos biflorus agglutinin (DBA). Nephrogenic zone under the kidney capsule (large arrow) is devoid of ROMK antigen. In cortex (CTX), DBA-positive segments (curved arrows) presumably represent proximal tubules; ROMK labeling is identified in DBA-negative tubules (short arrows). Occasional outer medullary (OM) collecting ducts exhibit coincident ROMK and DBA labeling in a heterogeneous pattern (short arrows). B: rhodamine-conjugated DBA labeling of a subset of cells in a tubule, likely a CNT or CCD, devoid of ROMK label in the midcortex of the same cryosection as A. C: at 1 wk of age, ROMK immunostaining is absent in midcortical tubules labeled with antibody directed against kallikrein, with the latter representing distal tubules and CNTs. D: modest coincident staining for both ROMK and THP is detected along the apical membrane of this collapsed CTALH at 1-wk of age; g, glomerulus. E and F: double labeling of midcortex (E) and outer cortex (F) of kidney from a 3-wk-old rat labeled with anti-ROMK and DBA (rhodamine). In E, coincident yellow staining for both ROMK and DBA is evident along the apical membrane of a subset of cells in a CNT/CCD. In F, a similar profile is identified in the outer cortex (arrow); circumferential outline of tubule suggests that it represents a CNT coursing horizontally through the superficial renal parenchyma; large arrow identifies kidney capsule. 6: apical ROMK label is evident in number of THP-positive medullary TALH (arrows) in this section of outer medulla of 1-wk-old kidney. Original magnifications for A and G ×10; B–F, ×500.
segments (11, 12, 28, 36, 37). Because the ROMK antibody used in the present study was raised against the carboxy-terminal region of the protein, common to all alternatively spliced forms, it could not differentiate among the unique isoforms.

The temporal and spatial appearance of ROMK expression after birth correlates well with our patch clamp analysis of the apical K⁺ conductance of the differentiating principal cell in the mid-CCD (28). In the latter study, we identified a developmental increase in apical SK channel activity in the maturing rabbit principal cell. Conducting apical SK channels were not detected in CCDs isolated from animals in the 1st week of postnatal life. The mean number of open SK channels per patch (NPₒ) of principal cell, negligible at 2 wk of age, increased progressively thereafter. The increase in NPₒ appeared to be due primarily to a developmental increase in number (N) of channels; Pₒ remained constant at ~0.5 for all channels identified after the 2nd week of life. To the extent that the program of channel

Fig. 7. Developmental expression of α-ENaC in midcortex. After the 2nd week of life, labeling is evident on the apical surfaces of the majority of cells in distal nephron segments (white arrows). Other cells in the ENaC-positive segments that did not label with the antibody (black arrows) likely represent intercalated cells. Original magnification, ×400.
development in the rat is similar to that in rabbit, the results of the present study provide support for the likelihood that the postnatal increase in SK channel activity is due to an increase in transcription and translation of ROMK message and protein, respectively. As the SK channel constitutes the rate-limiting step for $K^+$ secretion in fully differentiated CCD, we speculate that the limited capacity of the immature CCD for $K^+$ secretion is due, at least partially, to a scarcity of conducting ROMK channels in CNT/CCD.

Apical ROMK was detected in the TALH at an earlier developmental stage than in the collecting duct (Fig. 4). Although functional analysis of the $K^+$ transport pathways in the TALH in the maturing nephron has not been performed, results of micropuncture studies (19) are consistent with functional immaturity of the loop of
Henle early in life. Postnatal increases in diluting capacity (17, 40) and Na-K-ATPase activity (29) in rat TALH have been reported, suggesting that $K^+$ transport pathways in this segment may undergo developmental regulation. Characterization of these pathways and the role of apical ROMK in ion transport in the neonatal segment has yet to be accomplished.

Immunoblotting confirmed a maturational increase in abundance of ~40- and 45-kDa proteins in plasma membranes harvested from maturing rat kidneys; the 76- and 85-kDa bands did not appear to be developmentally regulated. Other investigators using anti-ROMK carboxy-terminal antibodies have reported similar size bands in kidney, all of which were abolished by immunoadsorption with ROMK immunizing peptide. Whereas the 40- and 45-kDa bands are consistent with core and glycosylated ROMK monomer, the high-molecular-mass bands have been suggested to represent ROMK complexed with itself or other proteins that were not dissociated under the conditions used for Western

Fig. 9. Developmental expression of $\gamma$-ENaC in midcortex. Apical and cytoplasmic labeling is evident in the majority of cells in distal nephron segments (white arrows); cells that did not label with this antibody in the ENaC-positive segments (black arrows) likely represent intercalated cells. Original magnification, ×400.
blotting (22, 38). Against this speculation was the observation by Xu et al. (38) that these high-molecular-mass bands were absent in HEK-293 cells transfeected with ROMK1. Our detection of a prominent 76-kDa band in the neonatal kidney, which showed only a trivial 40-kDa band, lends support to the hypothesis that the high-molecular-weight band represents a protein unrelated to, but sharing carboxy-terminal sequence homology with ROMK and does not represent a complex formed by ROMK isoforms. The identity of the developmentally regulated ~50-kDa protein is unknown at this time.

Coexpression of the α-, β-, and γ-subunits is required for maximal and efficient cell-surface expression of ENaC activity (5, 10). Whereas expression of the α-subunit alone allows for conduction of a small amiloride-sensitive current, β- and γ-subunits alone or together do not induce a Na⁺ current (5). Recent studies indicate that synthesis of the α-subunit is the limiting factor in the assembly and targeting of the channel protein to the apical surface (10). The observation of coincident increases in rate of synthesis of the α-subunit and short-circuit current in A6 kidney cells 60 min after addition of aldosterone to the bathing medium suggests that de novo synthesis of this channel subunit may mediate the early increase in sodium transport (21). In the present study, we found that β- and γ-subunits were already present in midcortical distal nephron segments at birth, albeit at low abundance. The temporal relationship between appearance of α-ENaC subunit (Fig. 7) and conducting amiloride-sensitive Na⁺ channels (27) in the apical membrane of the principal cell in the 2nd week of life of the rodent provides further evidence that the α-subunit may be essential for assembly, targeting, and activation of apical amiloride-sensitive Na⁺ channels.

In summary, the results of our investigation suggest that the postnatal increases in number of conducting apical SK and Na⁺ channels in the differentiating principal cell are mediated, at least in part, by increases in abundance of ROMK message and protein and ENaC subunit proteins. The signal(s) mediating these developmental events remains to be identified.

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