Developmental regulation of ENaC subunit mRNA levels in rat kidney

V. Matti Vehaskari, James M. Hempe, Jennifer Manning, Diego H. Aviles, and Mary Catherine Carmichael

Developmental regulation of ENaC subunit mRNA levels in rat kidney. Am. J. Physiol. 274 (Cell Physiol. 43): C1661–C1666, 1998.—To assess the role of distal nephron apical Na channel (ENaC) gene expression in Na wasting by the immature kidney, ENaC α-, β-, and γ-subunit mRNA levels were examined in the rat by RT-PCR. In microdissected nephron segments, all three ENaC subunit mRNAs were detected in the distal convoluted tubule, connecting tubule, cortical collecting duct, and outer medullary collecting duct. The inner medullary collecting duct and all other nephron segments were consistently negative. The mRNA levels were quantified in kidneys at different developmental stages by multiplex RT-PCR with “primer dropping,” with endoplasmic reticulum-specific cyclophilin mRNA as an internal standard. All three ENaC mRNA levels were low or undetectable on gestational day 16 and only slightly higher 3 days before birth. A sharp rise was observed between 3 days before and 1–3 days after birth; the levels at postnatal days 1–3 were already similar to those of adult kidneys. The results suggest that ENaC subunit gene expression is not a limiting factor in the full-term newborn rat kidney, but low levels of expression may limit distal Na absorption in more immature kidneys, such as those of very premature human infants.

Sodium transport; collecting duct; renal ontogeny; multiplex reverse transcription-polymerase chain reaction; capillary electrophoresis with laser-induced fluorescent detection

Premature infants have a limited ability to regulate Na homeostasis, partly because of renal Na wasting, which may lead to the so-called late hyponatremia of prematurity (1, 23). The renal site of inappropriate Na loss has not been unequivocally determined, but there is indirect evidence to suggest that distal nephron Na reabsorption may be inadequate (2, 19). We previously showed by in vitro microperfusion that rabbit cortical collecting ducts (CCD) have a low Na transport rate during the first 2 wk of life (24), suggesting that the functional characteristics of the CCD may contribute to renal Na wasting by immature kidneys. Furthermore, patch-clamp studies have demonstrated a paucity of functional Na channels in the apical membrane of the CCD of the newborn rabbit (20).

The apical low-conductance, highly selective amiloride-sensitive Na channel is believed to be the rate-limiting step in CCD Na transport. This channel, termed ENaC, was recently cloned from rat colon and shown to consist of at least three subunits, α, β, and γ, all of which are required for full Na conductance in the oocyte expression system. The subunits share ~35% identity and appear to be products of different but related genes, which are part of a new gene family (4, 5, 14, 15). The presence of ENaC has been confirmed in kidney, colon, lung, and salivary and sweat glands (7, 18). Although it is found predominantly in the distal nephron, the exact extent of ENaC gene expression along the collecting duct has not been unequivocally established (6, 7, 25).

The current study was undertaken to investigate the role of ENaC gene expression in the maturation of distal nephron Na transport. When studied in microdissected rat nephron segments by RT-PCR, the three ENaC subunit mRNA species colocalized exclusively to the distal nephron segments from the distal convoluted tubule (DCT) to the outer medullary collecting duct (OMCD). Although relative quantification by multiplex RT-PCR revealed low mRNA levels of all three subunits in late fetal kidney, adult expression levels were already reached within 24–72 h after birth. These findings suggest that the ENaC subunit gene expression is a potentially limiting factor in Na transport only in the very immature kidney.

METHODS

Animals and tissue preparation. Sprague-Dawley rats were used for all experiments. For microdissection of nephron segments, rats weighing 120–160 g were anesthetized with pentobarbital sodium (50 mg/kg ip), and left kidneys were perfused retrograde via the abdominal aorta with dissection solution (see below) containing 2 mg/ml collagenase (Boehringer Mannheim) and 1 mg/ml BSA. Sagittal kidney sections were incubated for 15–30 min at 37°C in the same solution with the addition of 0.2 mg/ml protease ( Pronase E, Sigma Chemical) during continuous bubbling with 100% O2. Single nephron segments were dissected freehand in ice-cold dissection solution containing in mM 135 NaCl, 5 KCl, 1 Na2HPO4, 3 sodium acetate, 1.2 Na2SO4, 2.5 CaCl2, 1.2 MgSO4, 5 HEPES, 5.5 glucose, and 10 vanadyl ribonucleoside complex (VRC; Life Technologies), a potent RNase inhibitor. VRC was centrifuged and filtered before use to remove precipitates. The pH of the solution was adjusted to 7.4 with NaOH. After dissection, nephron segments were rinsed twice in the same solution without VRC and transferred in 2-μl aliquots to tubes containing 6.6 μl of 2% Triton X-100 (Sigma Chemical) to permeabilize the cells, 1.4 U/μl placental RNase inhibitor (Boehringer Mannheim), and 5 mM dithiothreitol (Sigma Chemical). A total tubule length of 2–3 mm (usually 2 segments) or two to four glomeruli were placed in each tube. The tubes were frozen on dry ice until they were used for RT within 2 h.

Whole kidneys at different developmental stages were harvested for RNA extraction. Fetal kidneys were obtained from timed pregnancies on gestational day 16 (E16) or 19 (E19). The mothers were anesthetized with pentobarbital sodium, and fetal kidneys were quickly removed and snap frozen in liquid nitrogen. Postnatal kidneys were obtained on day 16.
postnatal days 1–3 (D1–3) and 14 (D14) and from adult rats (120–160 g body wt). The kidneys were quickly removed under anesthesia without perfusion and snap frozen in liquid nitrogen. Tissue was stored at −80°C until RNA isolation.

Primer design. Rat ENaC (rENaC) α-, β-, and γ-subunit PCR primer sets were designed with the aid of computer software (Wisconsin Sequence Analysis Package; Genetics Computer Group) from published rENaC subunit cDNA sequences (GenBank) and were selected from the nonhomologous regions on the basis of product size and optimal annealing temperature (56–59°C). The α-, β-, and γ-subunit primers spanned base pairs 1424–1915 (PCR product size 492 bp), 1295–1685 (391 bp), and 341–610 (270 bp), respectively, and were synthesized commercially (Integrated DNA Technologies). When tested with rat genomic DNA (Clontech Laboratories) as a template, the only PCR product obtained with any of the primers was an occasional large-molecular-weight (>1,000-bp) DNA, indicating that the primers spanned an intron. RT-PCR with each primer pair, with rat kidney RNA as a template, yielded a single DNA product of expected size that was sequenced to confirm its identity. The DNA sequencing was performed commercially with an automated sequencer by use of dye deoxy terminator cycle sequencing chemistry (ACGT). Primers for cDNA of a housekeeping gene, the endoplasmic reticulum-specific cyclophilin (10), were used to produce an internal standard; these primers (Ambion) spanned base pairs 295–510 and produced the expected 216-bp DNA product. In preliminary experiments, cyclophilin mRNA in the kidney showed little variation with developmental stage of the kidney (data not shown). The use of cyclophilin as an internal normalizing standard for quantitative RT-PCR has been validated (3, 16).

RT-PCR of microdissected nephron segments. The method of Moriyama et al. (17) with slight modifications was used for qualitative localization of rENaC subunit mRNAs. The tubules were frozen in permeabilizing solution were thawed, and RT was carried out at 42°C for 50 min in 20 µl of solution containing 11.5 U of avian myeloblastoma virus reverse transcriptase (Stratagene), 0.25 µg of oligo(dT) primer, 50 nmol of each deoxynucleotide triphosphate (dNTP), 50 U of reverse transcriptase (Stratagene), 10 nmol of each dNTP, and the proprietary buffer (Stratagene). An initial melt was carried out for 2 min at 94°C, followed by 40 cycles with the following sequence: 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C. The DNA polymerase was added before the first extension ("hot start"). A final extension consisted of a 7-min incubation at 72°C. The PCR products were size fractionated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. Two negative controls, one without template and the other with reverse transcriptase omitted, were included to rule out contamination and amplification of genomic DNA, respectively. Rat whole kidney mRNA (Clontech Laboratories) was used as a positive control template; it yielded consistently positive results with each of the four primer pairs.

Isolation and RT of whole kidney RNA. For quantitative analysis, total RNA was extracted from fetal and postnatal kidneys. Fetal kidneys from each pregnant animal (usually from 6 or 7 fetuses) were pooled, and the two kidneys from each newborn and 14-day-old animal were pooled; single kidneys were taken from adult rats. Total RNA was isolated with the Ultraspec RNA system (Biotecx Laboratories) and treated with DNase I (Boehringer Mannheim), 0.25 U/mg tissue, for 15 min at 37°C. DNase I was removed by phenol-chloroform-isoamyl alcohol extraction before ethanol precipitation of total RNA with 0.5 M ammonium acetate for 15 min at −20°C. After centrifugation the pellet was washed with 70% ethanol, air dried, and resuspended in water. RNA concentration was measured by ultraviolet absorbance. The DNA concentration was determined by capillary electrophoresis with laser-induced fluorescent detection (CE-LIF). There was an approximate log-linear relationship below 30 cycles for each template.

Fig. 1. Relationship between PCR cycle number and yield. RFU, relative fluorescence units. Primers for rat epithelial Na channel (rENaC) α-, β-, and γ-subunits as well as for cyclophilin (CP) were used in separate PCR reactions with equal amounts of rat kidney cDNA. Of total PCR volume of 100 µl, 20 µl were removed after 25, 30, 35, and 40 cycles; each aliquot was quantified by capillary electrophoresis with laser-induced fluorescent detection (CE-LIF). There was an approximate log-linear relationship below 30 cycles for each template.

Multiplex PCR with "primer dropping." To minimize problems inherent in the quantification of mRNA with RT-PCR, primer-dropping multiplex PCR with an internal standard was employed (26). Preliminary experiments were conducted to determine the optimal number of PCR cycles for rENaC α-, β-, and γ-subunits, as well as for the internal standard cyclophilin. Figure 1 illustrates the relationships between cycle numbers and product yield, showing that below 30 cycles the plateau (saturation) was not reached with any of the primers. The PCR products, even at these relatively low cycle numbers, were easily detected by the method used (see below). Furthermore, adding a single cycle increased the yield approximately twofold as predicted (data not shown). On the basis of the calculated slopes and intercepts in Fig. 1, it was estimated and confirmed experimentally that, with adult rat kidney cDNA as the template, comparable quantities of DNA were produced with 26 PCR cycles for rENaC α-subunit, 24 cycles for rENaC β-subunit, 28 cycles for rENaC γ-subunit, and 20 cycles for cyclophilin. These cycle numbers were therefore used in all subsequent PCR reactions when all four primer pairs were included in the same reaction tube (multiplex PCR). To achieve the intended number of cycles, each primer pair was added individually during the PCR at its primer pair was added individually during the PCR at its...
melt at 94°C for 3 min, thermal cycling was carried out for 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C, with a final extension at 72°C for 7 min.

Detection and quantification of PCR amplicons by capillary electrophoresis with laser-induced fluorescence detection. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was based on the principle described by Fasco et al. (8). Before analysis by CE-LIF, each PCR sample was desalted by dry dialysis in deionized, distilled (DD) water for 30 min with use of 0.025-mm V5 filters (Millipore). Amplicons were separated and quantified using a P/ACE 5000 capillary electrophoresis system equipped with a laser-induced fluorescence detector (argon ion, 488/520 nm excitation/emission) and System Gold software (Beckman Instruments). The instrument was operated at constant temperature (25°C) in reverse polarity with cathode and anode at the inlet and outlet reservoirs, respectively. Run buffer containing TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA), 0.5% (wt/vol) hydroxypropylmethylcellulose, and 0.005% (vol/vol) SYBR Green I (Molecular Probes) was used as the catholyte and the anolyte. Before each run the 100-µm-ID 27-cm-long coated capillary (DB-1, J & W Scientific) was rinsed with run buffer at high pressure (20 psi) for 1 min. Sample was introduced by electrokinetic injection for 10 s at 3.0 kV. Double-stranded DNA fragments were separated by electrophoresis at 8.1 kV (300 V/cm) for 8 min while fluorescence was recorded at 520 nm. The capillary was rinsed with methanol and DD water for 0.25 min each between samples. A double-stranded DNA standard containing 100-, 200-, 350-, and 500-bp fragments (Bioventures) in DD water was analyzed with each run to verify optimal instrument performance. To quantify mRNA, the amount of each different amplicon (cyclophilin, 216 bp; rENaC-γ, 270 bp; rENaC-β, 391 bp; rENaC-α, 492 bp) in each PCR sample was measured by integrating the fluorescence peak areas. The relative amount of each rENaC subunit mRNA in each sample was then determined as the ratio of the subunit amplicon peak area to the ampiclon peak area of cyclophilin.

Statistical analysis. Values are means ± SE. The levels of each specific rENaC subunit mRNA, expressed relative to cyclophilin mRNA, were compared between the groups with ANOVA, followed by the Tukey-Kramer multiple comparison test. P < 0.05 was considered significant.

RESULTS

Localization of rENaC subunit mRNA species. The following microdissected segments were studied by RT-PCR in adult rats: blood vessels, glomerulus, proximal convoluted tubule (PCT), proximal straight tubule (PST), thin limbs, medullary and cortical thick ascending limbs, DCT/CNT, CCD, OMCD, and IMCD. Blood vessels were dissected as bundles without separation of arteries and veins. Ascending and descending thin limbs were not separated. Because it was not possible to identify a clear transition from DCT to CNT, these segments were treated as one; the dissected DCT/CNT usually included a branch at the distal end. Inner and outer stripes of OMCD were not separated. Because controversy exists regarding the presence of ENaC in IMCD (6, 7, 25), the papilla was carefully excised and the dissected papillary IMCDs were considered separately from the more proximal initial IMCDs, which were dissected from the proximal one-third of the total IMCD length. At least four different specimens of each nephron segment were studied (Fig. 2). All primer pairs produced a single DNA band of the expected size. All segments were positive for cyclophilin, rENaC-α, β, and γ-subunit mRNAs co-localized consistently to the following nephron segments: DCT/CNT, CCD, and OMCD. The initial and papillary IMCDs, as well as all other nephron segments and blood vessels (not shown), were negative for all subunits, except an occasional (<10% of specimens) very faint band with a single subunit.

rENaC subunit mRNA levels during development. mRNAs for rENaC-α, β, and γ-subunit mRNA levels were determined in kidneys at five different stages of maturation: E16, E19, D1–3, D14, and adult. Figure 3 shows a typical CE-LIF recording from each age group. The peaks of all three rENaC subunits from the fetal E16 and E19 kidneys were small compared with those from the newborn kidneys (D1–3). There was no further increase during the postnatal period after D1–3. There appeared to be no differences in the developmental patterns between the subunits, except the rENaC β-subunit was not detected in any of the samples at E16. For statistical analysis, each specific rENaC subunit mRNA peak area was normalized to the cyclophilin peak area obtained from the same multiplex PCR reaction. Figure 4 illustrates the relative abundances of the rENaC α-subunit mRNA. Although the mRNA was detected in all samples, the levels appeared very low in the prenatal samples; however, the difference did not reach statistical significance. The rENaC β-subunit mRNA levels are shown in Fig. 5. At E16 there was no detectable mRNA, and at E19 the level was still significantly lower than at D1–3 or D14. The rENaC γ-subunit mRNA is illustrated in Fig. 6. The
level was measurable but very low at E16 and significantly lower at both prenatal ages than at any of the postnatal ages. All three rENaC subunit mRNA levels in the immediate newborn period (D1–3) were already comparable to the levels in adult kidneys. At D14 the levels were numerically higher than in adults, but only the \(\beta\)-subunit mRNA reached a statistically significant higher value. When the rENaC subunit mRNA abundances were analyzed without normalization to cyclophilin, as the peak area of specific mRNA per microgram of total RNA, a similar developmental pattern for all subunits was observed (data not shown).

**DISCUSSION**

Distal nephron Na reabsorption is critical for Na homeostasis and the target for regulation by mineralocorticoids. The rate-limiting step in transepithelial Na transport is believed to be the entry of Na into the cell via the apical Na channel, ENaC. The present studies were undertaken to investigate the role of ENaC gene expression in the developmental regulation of distal nephron Na transport in the rat. The main findings were 1) colocalization of rENaC \(\alpha\), \(\beta\), and \(\gamma\)-subunit mRNAs exclusively to the distal nephron, 2) sharp developmental increase in the mRNAs of all subunits between 72 h before birth and 24–72 h after birth, and 3) higher \(\beta\)-subunit mRNA level was lower at E19 than at D1–3. At D1–3, level was not different from adult value, but, at D14, level was higher than in adults (P < 0.05, significance not shown).
Rat kidney ENaC subunit mRNA levels are clearly developmentally regulated, as shown in Figs. 3–5. In the mature animal, ENaC α-, β-, and γ-subunit genes may be differentially regulated (18, 25). In contrast, we found the developmental profiles for all three subunits in the current study to be similar (although the values for the α-subunit did not reach statistical significance). The mRNA levels in the fetal kidney were undetectable or low at E16 and only slightly higher 3 days before birth, at E19. A sharp increase appears to occur during the last 2 prenatal days and/or the first days of life. The signal for this increase is unknown. Further studies are necessary to determine whether it is related to the physiological changes occurring during the transition from intrauterine to extraterine life, such as increase in renal blood flow and glomerular filtration rate. Another important finding was that during the first 24–72 h of life, the rENaC mRNA levels were already as high as those in mature kidneys. They remained at the levels of mature kidneys (or higher) at 14 days of age, suggesting that the genes were not just transiently turned on during the perinatal period.

Na transport rate in the newborn rat distal nephron has not been directly measured; however, in the newborn rabbit CCD, we demonstrated very low active Na transport rates by in vitro microperfusion during the first 2 wk of life (24), and Satlin and Palmer (20) were unable to find many open Na channels by patch clamping. Rat and rabbit kidneys are relatively immature at birth, with nephrogenesis continuing well into the postnatal life. If it is assumed that distal nephron Na transport characteristics in the newborn rat and rabbit are similar, our results suggest that lack of ENaC gene expression is not responsible for the inefficient Na reabsorption. Possible underlying mechanisms include impaired translation of the mRNAs, impaired incorporation of synthesized proteins into the apical membrane, and effects of various regulatory pathways. The current study did not address the question of ENaC subunit protein synthesis. In the mature kidney, regulation of the channel activity may occur independently of changes in mRNA levels (18). ENaC regulatory mechanisms may involve recycling of channel subunits between the apical membrane and the subapical compartment (11) or regulatory degradation of the subunits via the ubiquitination pathway (22). Further studies are needed to determine whether the immature kidney lacks mechanisms for inserting ENaC proteins into the apical membrane and/or retaining inserted channels in the membrane. It is also possible that the channel is present in the apical membrane in the closed state, hence, undetectable by patch clamping, due to absence of proregulatory elements or presence of inhibitory factors.

Detailed information on the developmental pattern of ENaC gene expression in the human kidney is not available. Human kidneys are relatively well developed at birth, with cessation of new nephron formation at about gestational week 36. Only premature infants born before gestational week 35 appear to be at risk of developing renal Na wasting (1). It is conceivable that,
at that stage of maturation, human kidneys resemble late prenatal rat kidneys and that low levels of ENaC gene transcription limit renal Na conservation. If, on the other hand, the described perinatal surge in mRNA levels is induced by birth regardless of gestational age, the premature human kidney may resemble the newborn rat kidney with adequate ENaC mRNA levels; a discrepancy between collecting duct Na transport and ENaC expression similar to that postulated for newborn rat and rabbit would then have to be assumed to be present.

In summary, we have shown that the ENaC subunit gene expression in the rat kidney is localized to the distal nephron and is developmentally regulated, with a sharp rise in all subunit mRNAs from low prenatal levels to adult levels during the perinatal period. The low levels of expression in late fetal life may be of importance in the development of renal Na wasting and hyponatraemia in premature infants.

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Address for reprint requests: V. M. Vehaskari, Dept. of Pediatrics, LSU Medical Center, Box T8-1, 1542 Tulane Ave., New Orleans, LA 70112.

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