Accelerated Publication

Spatially Distributed Alternative Splice Variants of the Renal Na-K-Cl Cotransporter Exhibit Dramatically Different Affinities for the Transported Ions*

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Three splice variants of the renal Na-K-Cl cotransporter (NKCC2 F, A, and B) are spatially distributed along the thick ascending limb of the mammalian kidney. To test whether NKCC2 splice variants differ in ion transport characteristics we expressed cDNAs encoding rabbit NKCC2 F, A, and B in Xenopus oocytes and determined the ion dependence of bumetanide-sensitive 86Rb influx. The three splice variants of NKCC2 showed dramatic differences in their kinetic behavior. The medullary variant F exhibited 3–4-fold lower affinity than variants A and B for Na⁺ and K⁺. Chloride affinities also markedly distinguish the three variants (KmF = 111.3, KmA = 44.7, and KmB = 8.9 mM Cl⁻). Thus, the kinetic properties of the NKCC2 splice variants are consistent with the spatial distribution of the variants along the thick ascending limb as they are involved in reabsorbing Na⁺, K⁺, and Cl⁻ from a progressively diluted fluid in the tubule lumen. Variant B also showed an anomalous inhibition of rubidium influx at high extracellular Na⁺ concentrations, possibly important in its highly specialized role in the macula densa. The adaptation of the kinetic characteristics of the NKCC2 variants to the luminal concentrations of substrate represents an excellent example of functional specialization and diversity that can be achieved through alternative mRNA splicing.

The absorptive isoform of the Na-K-Cl cotransporter (NKCC2) is restricted in its distribution to the apical membrane of the thick ascending limb of Henle’s loop (TAL) in the vertebrate kidney. Transport of Na⁺ and Cl⁻ via the NKCC2 comprises a critical component of total renal salt reabsorption (1), which is the central element in whole body fluid and electrolyte balance. Improper function of this process is at the origin of human diseases such as Bartter syndrome (2–4) and hypertension (5, 6). Efficient operation of this transport system must overcome the unusual problem that the luminal concentration of Na⁺ and Cl⁻ decreases about 5-fold over the length of the TAL as salt is reabsorbed.

Intriguingly three exons encoding a 96-base pair region of NKCC2 are alternatively expressed, giving rise to three different protein sequences in the second transmembrane domain. Each of the three variants (F, A, and B) of NKCC2 shows a specific distribution along the TAL. The F variant is expressed only in the outer medulla, the A variant is found in the outer medulla and cortex, and the B variant is found only in the region of the macula densa (see below, Fig. 1a; Refs. 7–9). Because the membrane-embedded regions of transport proteins are most involved in interactions with the transported ions, we have proposed that the three variants of NKCC2 may act with different affinities in transporting ions (7). This hypothesis has been indirectly supported by the finding that the second transmembrane domain plays a major role in determining Na⁺ and K⁺ affinity in the secretory Na-K-Cl cotransporter NKCC1 (10).

Here we show that the three alternatively spliced variants of NKCC2, which are known to be axially distributed along the tubule, are individually specialized for optimal transport. We found that, when expressed in Xenopus oocytes, the three variants differ dramatically in their affinities for Na⁺, Rb⁺, and Cl⁻. In addition the NKCC2B splice variant that is localized in the macula densa region exhibits unique behavior, consistent with an important role in tubuloglomerular feedback (9, 11). The current findings present a remarkable example of molecular specialization underlying physiological specificity in the mammalian kidney. An abstract of these findings has been previously presented (12).

EXPERIMENTAL PROCEDURES

cRNA Preparation—To generate three full-length splice variants of rabbit NKCC2, a 280-bp fragment (SphI-BglII) from the rbNKCC2A was replaced by the same fragment from the rbNKCC2F and rbNKCC2F clonal vector. The three cDNAs are thus identical to one another except for the alternatively spliced 96-bp exon. The cDNAs were subcloned into the EcoRI site of a modified oocyte expression vector between the untranslated regions of Xenopus β-globin (13), and the cRNA for each construct was synthesized with T7 RNA polymerase (mMessage-mMachine, Ambion). After lithium chloride precipitation, cRNA was dissolved in diethyl pyrocarbonate-treated water at a concentration of 1.5–2 μg/μl.

NKCC2 Functional Expression in Xenopus Oocytes—Xenopus laevis oocytes were injected with 50 nl of cRNA/oocyte and maintained at −17 °C in medium containing 96 mM NaCl, 2 mM KCl, 0.9 mM MgCl₂, 1.8 mM CaCl₂, 0.04 mM furosemide, and 10 mM HEPES (pH 7.4 at room temperature). Three days after injection, NKCC2 activity was determined in 86Rb influx assays. Oocytes were preincubated for 20 min at room temperature in a low chloride (3 mM) hypotonic (160 mosM) solution to inhibit the activity of the Xenopus NKCC1 (14) and incubated for 40 min in an influx medium containing 100 mM NaCl, 2 mM RbCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM NaHPO₄, 2 mM Na₂SO₄, 0.1 mM ouabain, 3 μCi/ml 86RbCl, 5 mM HEPES (pH 7.4 at room temperature), and optionally 0.15 mM bumetanide in 0.1% MeSO. The dependence of “Rb⁺ influx on cotransported ions was determined by substitution of N-methyl glycine for Na⁺ or K⁺ and of glurionate for Cl⁻. Influx was stopped by three rinses with an ice-cold solution containing 100 mM potassium gluconate, 2 mM sodium gluconate, 1 mM MgCl₂, 1 mM CaCl₂,
Functional Specialization of NKCC2 by Alternative Splicing

RESULTS AND DISCUSSION

Here we report the functional characterization of the three splice variants of NKCC2 in X. laevis oocytes. cRNAs for rabbit NKCC2 were prepared, differing only in the sequence of the 96-bp region encoding the second transmembrane domain (Fig. 1b). Injection of each NKCC2 cRNA led to appropriate synthesis of the transporter protein (Fig. 2b), and the transport activity of each variant, measured as bumetanide-sensitive 86Rb influx, was at least 6–7-fold higher than endogenous activity (Fig. 2a). In comparing the behavior of the three variants we found large differences in affinities for the transported ions (Fig. 3a–c); these differences can be seen to provide a functional rationale for alternative splicing.

Variant F of NKCC2 exhibits the lowest affinity for each of the cotransported ions (Fig. 3, data in blue). This variant is found in the initial segment of the TAL where it is the only form among NKCC2s. In embryonic mouse kidney illustrated in a superimposition (center panel) of three variant-specific in situ hybridizations with NKCC2B (red), NKCC2A (green), and NKCC2F (blue) from our previous data in Ref. 8; the phase contrast image is shown in the left panel. A sketch of the nephron is illustrated on the right with corresponding color-coding of NKCC2. a, amino acid sequences for the three alternative cassettes. The thick underline highlights the proposed second transmembrane domain of the protein, and color-coding highlights residues varying among NKCC2s.

1 mM Na₂HPO₄, 2 mM Na₂SO₄, 5 mM HEPES, 0.25 mM bumetanide, and 0.1 mM ouabain. All solution transfers were executed by transferring oocytes from one well to another in a 48-well plate. 86Rb in individual oocytes was determined by Cerenkov radiation in a scintillation counter. Data were fit by least square analysis using the Simplex algorithm (PLOT)² to a one binding site model (Michaelis-Menton equation) or to a two binding site approximation (Hill equation, n = 2; see Ref. 15). Results are presented as mean ± S.E. of three to seven experiments with four to six oocytes per experimental condition. For Western blot analysis, oocytes (five to eight per group) were solubilized in 100 μl of homogenization buffer (1% Triton X-100), and samples containing 50 μg of protein were run on SDS gels and transferred to Immobilon-P (Millipore).

Why is the higher affinity isoform, NKCC2A, not deployed throughout the TAL? One possibility is that the transporter with higher affinity transport has a lower capacity, for example, if NKCC2A were unable to unload ions on the intracellular side as efficiently as NKCC2F. This idea is analogous to a proposal of “dynamic matching” of substrate- and product-containing complexes in evolutionary optimization of the catalytic effectiveness of enzymes (16). In this present case, however, the flux determined for NKCC2F under control conditions in oocytes is lower than that of NKCC2A (Fig. 2a), and from the measured Kₘ values we estimate that at the high salt concentrations of the tubule lumen the two isoforms would exhibit similar transport rates. Thus transport efficiency does not appear to be at the heart of the issue.

An alternative hypothesis is that with a reduced affinity for Na⁺ and Cl⁻, the F isoform serves to buffer the luminal concentration of ions in the medullary segment, thus more equally partitioning salt transport between medulla and cortex (17, 18). A medullary/cortical distribution of reabsorption can be expected to enhance several features of renal function. 1) NaCl uptake in the medulla takes place against a large gradient; salt recycling and oxygen consumption are minimized by limiting reabsorption in this region. 2) Reduction of medullary diluting power ensures flow-dependent salt delivery to the macula densa and thus enables tubuloglomerular feedback. 3) Medullary/cortical partitioning provides a basis for regional control of regulation of salt reabsorption; as a result, antidiuretic hor-

2 Software program by B. Forbush.
proximate range of ion concentrations in the tubule lumen in the curve.

Horizontal bars
squares

/H11001

than variant F. The Na
variants,
B
Rb
/H11001

effect of varying extracellular ion concentrations is shown for Na
), or NKCC2F (blue
NKCC2B (red circles

FIG. 3.
Dependence of 86Rb influx on cotransported ions. The effect of varying extracellular ion concentrations is shown for Na+(a), Rb+(b), and Cl−(c). Normalized 86Rb uptake by oocytes injected with NKCC2B (red circles), NKCC2A (green triangles), or NKCC2F (blue squares) is presented as the mean ± S.E. of at least six experiments per curve. Horizontal bars at the bottom of the graphs represent the approximate range of ion concentrations in the tubule lumen in the portion of the mammalian TAL in which each variant is present using the same color code. Kinetic constants were calculated from these data (mM): Km(Na+): B = 20.65 ± 2.4, A = 16.45 ± 1.9, F = 66.72 ± 5.8; Km(K+): B = 0.89 ± 0.17, A = 0.78 ± 0.08, F = 2.93 ± 0.48; Km(Cl−): B = 8.95 ± 1.3, A = 44.65 ± 3.87, F = 111.3 ± 13.4, †, statistically different from B (p < 0.01). +, statistically different from B and A (p < 0.01). mone (or vasopressin) is able to enhance the concentration gradient for water reabsorption by altering the ratio of medullary to cortical salt reabsorption in the mouse TAL without substantially changing net salt reabsorption (19).

Variant B is seen to have the highest Cl− affinity of the variants, ~3-fold higher than variant A and 10-fold higher than variant F. The Na+ and Rb+ affinities are similar to NKCC2A (Fig. 3). Since the distribution of NKCC2B is restricted to the distal-most portion of the TAL (Fig. 1), the splicing phenomenon results again in an apparent adaptation of an NKCC2 variant to the decreasing luminal salt concentrations. On the other hand, the Na+ and Rb+ affinities are not significantly different between A and B variants except for an anomalous inhibition of Rb+ influx seen at higher Na+ concentrations.

The punctate distribution of NKCC2B seen in Fig. 1c and in Ref. 8 underscores the specific localization of the B variant to the macula densa cells of the TAL (9). Two important regulatory mechanisms are initiated in these specialized salt-sensing cells, tubuloglomerular feedback and regulation of renin secre-

It is not immediately obvious why variant B is particularly well suited to a luminal Cl−-sensing role in the macula densa since its high ion affinities would ensure operation near kinetic saturation, a situation that would decrease its fidelity as a signaling mechanism. We propose that the unique aspect of transport at the level of the macula densa is that net cotransporter ionic distributions are at or near equilibrium across the apical membrane due to the very low luminal ion concentrations (22). Under these conditions, net apical salt transport may be determined by gradient rather than kinetic parameters with the overall transcellular transport limited primarily by Na+ pump performance at limiting intracellular [Na+]i. In this circumstance, variant A could perform as well as variant B, and we suggest that variant B significantly differs in another, as yet unidentified, property. One possibility, suggested by the inhibition of Rb+ influx at high Na+ concentration (Fig. 3a), is that the B variant has a different transport stoichiometry, perhaps allowing Na+ to occupy the K+ site under some conditions; this would allow uptake of Cl− against a larger gradient. A second possibility is that NKCC2B varies in some aspect of its regulation, perhaps through a Cl−-sensing role of the transporter itself.

In this work, we have reported the functional properties of NKCC2 along the TAL. These properties vary with the axial distribution of NKCC2 splice variants in successive regions of that tubule segment. Indeed the variants are shown to have very distinct sets of ion affinities, mirroring the decrease in luminal concentrations of Na+, K+, and Cl− in the corresponding regions. A similar pattern of axial specialization of the renal Na+ pump has been recently reported based on functional differences conferred by four structurally different γ subunits of Na,K-ATPase (23). The functional arrangement of renal splice variants is similar to the tonotopic distribution of Ca2+-activated K+ channel splice isoforms in hair cells of the vertebrate cochlea where variants that are more sensitive to Ca2+ have been mapped to the high frequency end of the organ (24, 25). These are excellent illustrations of the fine tuning that alternative splicing can confer on the utilization of the limited set of genes in the mammalian genome.

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REFERENCES

1. Greger, R. (2000) Am. J. Med. Sci. 319, 51–62
2. Simon, D. B., and Lifton, R. P. (1998) Curr. Opin. Cell Biol. 10, 450–454
3. Simon, D. B., Karett, F. E., Hamdan, J. M., DiPietro, A., Sanjad, S. A., and Lifton, R. P. (1996) Nat. Genet. 13, 183–188
4. Takahashi, N., Chernavaevsky, D. R., Gomez, R. A., Igarashi, P., Gitelman, H. J., and Smithies, O. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5434–5439
5. Glioros, N., Filigheddu, F., Trofia, C., Soro, A., Parpaglia, P. P., Tsikoudakis, A., Myers, R. H., Herrera, V. L., and Ruiz-Opaño, N. (2001) Hypertension 38, 204–209
6. Herrera, V. L., Lopez, L. V., and Ruiz-Opaño, N. (2001) Mol. Med. 7, 125–134
7. Payne, J. A., and Forbush, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4544–4548
8. Igarashi, P., Vanden Heuvel, G. B., Payne, J. A., and Forbush, B. (1995) Am. J.
Physiol. 269, F405–F418
9. Yang, T., Huang, Y. G., Singh, I., Schnermann, J., and Briggs, J. P. (1996) Am. J. Physiol. 271, F901–F909
10. Isenring, P., Jacoby, S. C., and Forbush, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7179–7184
11. Schnermann, J. (1988) Am. J. Physiol. 274, R263–R279
12. Gimenez, I., Isenring, P., and Forbush, B. (1996) J. Am. Soc. Nephrol. 10, 32A (abstr.)
13. Caron, L., Rousseau, F., Gagnon, E., and Isenring, P. (2000) J. Biol. Chem. 275, 32027–32036
14. Suvitayavat, W., Palfrey, H. C., Haas, M., Dunham, P. B., Kalmar, F., and Rao, M. C. (1994) Am. J. Physiol. 266, C284–C292
15. Isenring, P., and Forbush, B. (1997) J. Biol. Chem. 272, 24556–24562
16. Burbaum, J. J., Raines, R. T., Albery, W. J., and Knowles, J. R. (1989) Biochemistry 28, 9293–9305
17. Burg, M. (1982) Kidney Int. 22, 454–464
18. Reeves, W. B., Molony, D. A., and Andreoli, T. E. (1988) Am. J. Physiol. 255, F1145–F1154
19. Hebert, S. C., and Andreoli, T. E. (1984) Am. J. Physiol. 246, F745–F756
20. Schlatter, E., Salomonson, M., Persson, A. E. G., and Greger, R. (1989) Pfluegers Arch. 414, 286–290
21. Gagnon, E., Caron, L., Forbush, B., Batchvarov, R., and Isenring, P. (1999) J. Am. Soc. Nephrol. 10, 32A (abstr.)
22. Lapointe, J. Y., Laamarti, A., and Bell, P. D. Kidney Int. (1998) 54, S58–S64
23. Arystarkhova, E., Donnet, C., Asinovski, N. K., and Sweadner, K. J. (2002) J. Biol. Chem. 277, in press
24. Ramanathan, K., Michael, T. H., Jiang, G. J., Hiel, H., and Fuchs, P. A. (1999) Science 283, 215–217
25. Jones, E. M., Gray-Keller, M., Art, J. J., and Fettiplace, R. (1999) Ann. N. Y. Acad. Sci. 868, 379–385
26. Lytle, C., Xu, J. C., Biemesderfer, D., and Forbush, B. (1995) Am. J. Physiol. 269, C1496–C1505
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