Rare mutations in the human Na-K-Cl cotransporter (NKCC2) associated with lower blood pressure exhibit impaired processing and transport function

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RARE MUTATIONS IN THE HUMAN Na-K-Cl cotransporter (NKCC2) belongs to the family of electroneutral cation-chloride cotransporters (SLC12A) which also contains the Na-Cl and K-Cl cotransporters; these are all members of the amino acid polyamine cotransporter (APC) superfamily. By homology to other recently crystallized APC transporters, NKCC2 is a 12-transmembrane-helix transport protein whose transport domain is composed of two structurally homologous 5-helix domains with inverted symmetry across the membrane (Fig. 1). The cytosolic N-terminal domain of NKCCs contains the binding sites for the regulatory kinase (5), whereas the large intracellular C terminus is an alternating β-strand α-helix bundle (28) involved with dimerization of the protein (19) and probably with regulation of transport activity as well.

Within the thick ascending limb (TAL) of Henle’s loop, NKCC2 carries out the coupled movement of 1 Na+, 1 K+, and 2 Cl− ions across the cell membrane, thereby providing the major salt reabsorption pathway in the kidney. As such, NKCC2 is the target for widely prescribed loop diuretic drugs, and homozygous loss-of-function mutations in NKCC2 are responsible for Bartter’s syndrome type 1, a rare autosomal recessive disorder characterized by salt wasting and arterial hypertension (25). Given the critical role of NKCC2 in renal salt handling and its link with a disease that features arterial hypertension, the possibility exists that more prevalent heterozygous mutations in NKCC2 are contributing to blood pressure variation and affecting the risk for the development of arterial hypertension. To examine this, a recent study screened ~3,000 members of the Framingham Heart Study for variations in several genes encoding renal ion transport proteins including SLC12A1 (NKCC2), SLC12A3, (NCC), and KCNJ1 (ROMK) (14). Using two selection criteria (high sequence conservation and low allele frequency), Ji et al. (14) identified 30 different mutations in 49 subjects, 9 of which were missense mutations in NKCC2. When adjusted for age and sex differences, 80% of the mutation carriers had long-term systolic and diastolic blood pressures that were below the cohort mean and were also found to be significantly protected from the development of hypertension. Because they affect phylogenetically conserved residues, it was inferred that the majority of the NKCC2 mutations were likely to impair protein function, thereby providing a mechanism for reduced renal salt reabsorption and lower than normal blood pressure (14); however, the direct functional consequences of the individual mutations remained to be determined.

The aim of the present investigation is to examine the functional implications of the rare heterozygous mutations in NKCC2 identified by Ji et al. (14), testing the hypothesis that mutations in highly conserved NKCC2 residues would have functionally deleterious consequences likely to contribute to blood pressure differences in the human population. To accomplish this, we expressed human (h) NKCC2A and the nine missense mutants in Xenopus laevis oocytes as well as in a human cell line (HEK-293) and assessed protein function,
expression, localization, regulation, and ion transport kinetics. Our results demonstrate that six of the nine studied mutations impair the net transport function of hNKCC2A by disrupting protein processing, transport turnover rate, regulation, and ion transport kinetics.

EXPERIMENTAL PROCEDURES

hNKCC2A constructs. Nine mutations in the protein-coding region of NKCC2 were functionally characterized in this study (Fig. 1). Full-length cDNA encoding human NKCC2A was obtained from the ORFeome collaboration (Open Biosystems) and was cloned into an oocyte expression vector (9). hNKCC2A was selected as the representative isoform because of its relatively high expression in the both the cortex and medulla of the thick ascending limb of the human kidney (3). For detection of NKCC2 in X. laevis oocytes and HEK-293 cells, DNA encoding a Kozak sequence, a FLAG epitope tag, and a SanDI site for yellow fluorescent protein (YFP) insertion was cloned at the 5'-end of wild-type hNKCC2A using T7 DNA polymerase (a native SanDI site having been previously removed from hNKCC2A by QuikChange site-directed mutagenesis; Roche). QuikChange site-directed mutagenesis was subsequently performed to generate nine mutants of wild-type FLAG-YFP-tagged hNKCC2A (see Supplemental Materials for primer sequences; all supplemental material for this article is available on the journal web site). All mutations were confirmed by sequencing. For expression in HEK-293 cells, constructs were subcloned into pcDNA3.1(+) using KpnI and XbaI restriction sites.

Functional expression of hNKCC2A mutants in X. laevis oocytes. cRNA was synthesized from cDNA templates previously digested with T7 RNA polymerase (Ambion) and injected into stage IV-VI defolliculated X. laevis oocytes (15 ng of RNA/oocyte). Transcript integrity was confirmed on agarose gels, and cRNA concentration was determined by absorbance at 260 nm on a Nanodrop spectrophotometer (Thermo Scientific). cRNA was stored in frozen aliquots at −80°C until use. Oocytes were incubated for 3–5 days at 17°C in ND96 medium supplemented with 275 μg/ml Na-pyruvate, 90 μg/ml theophylline, 100 μg/ml gentamicin, 25 μg/ml tetracycline, and 2.5% horse serum. All procedures were performed in accordance with institutional regulations (Yale University Animal Care and Use Committee).

Transporter activity was determined by measuring radioactive 86Rb influx into individual oocytes using an automated 96-well plate assay (5). For the determination of basal flux levels, oocytes were preincubated for 30 min in isotonic media containing the following (in mM): 96 NaCl, 2 RbCl, 1 MgCl2, 1.8 CaCl2, and 5 HEPES, pH 7.5. Basal flux was then measured in a 40-min 86Rb uptake period in isotonic media in the presence or absence of 250 μM bumetanide. Flux solutions contained 0.1 mM ouabain to reduce 86Rb uptake from endogenous Na-K-ATPase. For examination of transporter regulation, oocytes were preincubated for 30 min in hypotonic, isotonic, or hypertonic media. Hypotonic medium contained the following (in mM): 73 NaCl, 2 RbCl, 1 MgCl2, 1.8 CaCl2, and 5 HEPES, pH 7.5. Isotonic and hypertonic solutions consisted of 50 and 100 mM sucrose, respectively, in hypotonic medium. Flux was then measured in a 40-min 86Rb uptake period in hypotonic, isotonic, or hypertonic media containing 0.1 mM ouabain.

Functional expression of hNKCC2A mutants in HEK-293 cells. HEK-293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. All cells were maintained at 37°C with 5% CO2. At ∼70–80% confluence, cells were transfected with wild-type and mutant hNKCC2A proteins in a pcDNA3.1(+) expression vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Stable cell lines were selected by growth in the presence of 1 mg/ml of Geneticin (G418) and 250 μM furosamide.

For 86Rb influx assays, confluent cells were split into 96-well poly-l-lysine-coated microplates (BD Biocoat), grown for 24 h at 37°C, and subsequently transferred to 25°C for ∼24 h before flux experiments. As has been reported for NKCC1 constructs (20), we noted stronger membrane staining and higher 86Rb influx for all hNKCC2A constructs when cells were cultured at a low temperature. Before each flux assay, cells were incubated for 45 min in solutions with various compositions to lower intracellular Cl concentrations. This was followed by a 2-min 86Rb uptake period determined in basal media containing as follows (in mM): 135 NaCl, 5 RbCl, 0.5 CaCl2,

Fig. 1. Human Na-K-Cl cotransporter (hNKCC2A) mutants. A: 2-dimensional representation of the structure of hNKCC2, based on homology modeling described in B. The locations of the 9 mutations identified by Ji et al. (14) are shown. A FLAG epitope and a yellow fluorescent protein (YFP) were cloned at the N terminus for detection of hNKCC2A protein in Xenopus laevis oocytes and HEK-293 cells. Colors illustrate similarity to moth NKCC (Manduca sexta, accession Q25479), with red indicating high similarity or identity. B: estimate of the 3-dimensional structure of NKCC2, based on homology modeling using Modeller (23) using homology to other APC superfamily transporters (8, 24) and to the structure of the C terminus of a prokaryotic member of the cation-chloride cotransporter family (28). Orange beads indicate positions of the 9 mutations studied here. Insertions found in the vertebrate NKCCs are predicted to be largely unstructured; the position, but not the size of these is indicated by light green ribbon.
0.5 MgCl₂, 0.5 Na₂HPO₄, 1 Na₂SO₄, and 15 HEPEs, pH 7.4, plus 0.2–1.0 µCi [⁸⁶Rb]m1l and 0.1 mM ouabain. Cell protein content for each construct was determined after lysis of cells in 1% SDS using the D₆₅ protein assay (Bio-Rad).

**Immunoblotting.** For Western blot analysis, confluent cells were split into 12-well culture plates and grown for 24 h at 37°C. Before sampling, cells were incubated overnight at 37°C or 25°C. Cells were solubilized in 500 µl of homogenization buffer containing 1% Triton X-100, and homogenates were spun at 14,000 rpm at 4°C for 10 min. Supernatants were diluted in Laemmili buffer containing 100 mM DTT and also saved for protein determination using the BCA protein assay (Pierce). Approximately 2.5 µg of protein was run on a 7.5% Tris·HCl gel, transferred to polyvinylidene difluoride membranes, and blocked in 5% milk in PBS-T. Immunoblots were incubated with anti-green fluorescent protein (GFP; Rockland) antibody diluted 1:2,000 for 1 h at room temperature or overnight at 4°C, washed, and incubated with the appropriate horsedradish peroxidase-conjugated secondary antibody diluted 1:5,000 for 1 h at room temperature. Antibody binding was detected using chemiluminescent reagents (Pierce) and high-performance autoradiography film (GE Healthcare) in the linear range of sensitivity, and band intensity was quantified using ImageJ version 1.37 (National Institutes of Health).

**Confocal microscopy.** For confocal analysis, cells were plated onto poly-L-lysine-coated 12-mm round glass coverslips and grown for 24 h at 37°C. Cells were rinsed briefly in PBS and fixed in ice-cold methanol for 5 min. Cells were then washed in PBS and blocked with PBS containing 1% BSA for 30 min at room temperature. Cells were incubated in anti-FLAG (Sigma) primary antibody diluted 1:500 in 1% BSA/PBS overnight at 4°C, washed, and incubated with Alexa 488 secondary antibody diluted 1:500 in 1% BSA/PBS for 1 h at room temperature. For the visualization of nuclei, cells were incubated for 5 min in To-Pro-3 (Invitrogen) diluted 1:5,000 in PBS. Cells were then mounted in Vectashield and examined at a magnification of ×63 with a Zeiss LSM 510 Meta confocal laser-scanning microscope.

**Measurement of apparent ion affinities.** To determine the co-ion concentration dependence of Na-K-Cl cotransporter function, HEK-293 cells were preincubated for 45 min in 30 mM Cl to activate the exogenous NKCC2 cotransporter without appreciably activating the endogenous HEK cell cotransporter. [⁸⁶Rb] influx was subsequently determined in basal flux medium in which Cl was replaced with gluconate, and Na and Rb were replaced with N-methylglucamine to achieve indicated ion concentrations. K冕 values were obtained by fitting individual experiments with the Michaelis-Menton equation for Na and Rb, or the Hill equation for Cl (Hill coefficient fixed at 1.6) (12), minimizing least squares differences using the simplex algorithm. We point out that the term “affinity” is used here to represent “apparent affinity,” the inverse of the Michaelis constant (K冕), a quantity that is related to real binding site affinity by an undetermined set of kinetic constants.

**Structural analysis.** The structural model shown in Fig. 1B and in cartoon form in Fig. 1A is based on homology to the APC superfamily transporters AdiC (8) and Apc (24) and to the cytoplasmic domain of MaCCC (28), a prokaryotic cation-chloride cotransporter. Initially, primary sequence alignments were obtained using ClustalX and PSIPRED (supplemental materials); these alignments are in good agreement with previously published analyses (24, 28). The homology model in Fig. 1B was prepared from these alignments using Modeller (23) and the PDB structures 3NCY, 3GIA, and 3G40 with thorough optimization (model-changeopt.py). It is interesting to note that a number of features of the hypothetical homology model were surprisingly well predicted by earlier secondary structure analysis (10); these features include the 12 transmembrane domains with especially long TM3 and TM8, 8 of the 10 β strands in the C-terminal cytoplasmic domain, and large sequence insertions with little predicted tertiary structure [predicted by PONDOR (17), DisEMBL (18)].

**Statistical analysis.** Data are expressed as mean ± SE; n represents the number of experimental replicates unless otherwise noted. Differences between means were evaluated with Student’s t-test when two groups were compared or ANOVA followed by Dunnett’s multiple comparison test when multiple groups were compared with control. P < 0.05 was considered statistically significant.

**RESULTS**

hNKCC2A mutants. We investigated the functional consequences of nine rare heterozygous mutations in human NKCC2 previously described but not functionally characterized (14). The localization of the mutations within hNKCC2A is shown in Fig. 1. In the analysis of Ji et al. (14), these mutations were subselected from identified coding sequence variants on the basis of phylogenetic conservation; this conservation is illustrated in Fig. 1A by color coding the similarity of human and moth NKCCs. The majority of the mutations are located within the central transmembrane domain, while Y1070C and P1083A are within the large intracellular C terminus. For the purpose of hNKCC2A detection using Western blot and confocal analyses, a FLAG epitope and a YFP were added at the N terminus. Previous work from our laboratory has demonstrated that insertion of YFP at the N terminus of the closely related cotransporter, NKCC1, has no detectable effect on protein function (20), and in control experiments we also found no effect of the YFP tag on NKCC2 function (data not shown).

**Functional analysis of hNKCC2A mutants in X. laevis oocytes.** To determine the consequences of these mutations on transport function, we examined [⁸⁶Rb] influx in *X. laevis* oocytes. As seen in Fig. 2A, oocytes injected with wild-type and mutant hNKCC2A proteins exhibited robust bumetanide-sensitive [⁸⁶Rb] influx compared with water-injected controls, with the level of [⁸⁶Rb] influx varying among mutants. With combined data from three separate experiments employing different frogs, we see that four of the nine mutants exhibit significantly different transport function compared with wild-type (Fig. 2B). [⁸⁶Rb] influx was 68, 36, 54, and 72% of wild-type in the T235M, R302W, L505V, and P569H mutants, respectively. In other experiments (not shown), we examined transport regulation in response to changes in osmotic volume: within the error of our experiments, we did not detect significant differences between the wild-type and the variants after preincubation in isotonic, hypertonic, and hypertonic media.

**Functional analysis of hNKCC2A mutants in stable HEK-293 cell lines.** As a more appropriate test of human NKCC2A function, we sought to characterize the nine mutants in a human cell line. To date, only a few studies have been successful in achieving functional expression of NKCC2 in mammalian cell lines (1, 11, 29). In our own laboratory, we have found that rabbit NKCC2 is very difficult to express and mammalian cell lines (1, 11, 29). In our own laboratory, we have found that rabbit NKCC2 is very difficult to express and maintain in mammalian cell culture unless fused to the NKCC1 N terminus (2, 13).

Here, we transfected HEK-293 cells with FLAG-YFP-tagged hNKCC2A and isolated stably transfected lines; these efforts proved to be very successful, with bumetanide-sensitive [⁸⁶Rb] transport rates ~18-fold above the basal HEK cell flux (Fig. 3A). This is the first report of functional expression of the human ortholog of NKCC2 in a mammalian cell line. Consistent with earlier findings using other orthologs and expression systems (10, 21), and in striking contrast to the behavior of NKCC1 (13), the constitutive activity of hNKCC2A in the basic isotonic medium is very high: >50% of the maximally
activated flux achieved in low-Cl/hypotonic medium (here with and without calyculin A) (Fig. 3A).

Examining the hNKCC2A variants in the HEK-cell system, we found significantly impaired function in the same four mutants highlighted in the oocyte experiments: $^{86}$Rb influx was 44, 15, 27, and 66% of wild-type in the T235M, R302W, L505V, and P569H mutants, respectively (Fig. 3A). In addition, the N399S and P1083A mutants also exhibited significantly lower flux in HEK cells, 57 and 55% of wild-type (P < 0.05, Dunnett’s post hoc test).

Expression and localization of hNKCC2A mutants in stable HEK-293-cell lines: is transport impairment due to differences in protein abundance and localization? Utilizing an anti-GFP antibody for Western blot analysis of tagged hNKCC2A, we detected a ~190-kDa band representing the glycosylated mature form of the cotransporter and a band at ~150 kDa which presumably represent multimeric forms of the cotransporter (1, 11, 29). Qualitatively, the proportion of cotransporter appearing in the ~150-kDa band appeared lower when cells were incubated at 37°C compared with 25°C, suggesting that processing is more complete or that immature NKCC2 is rapidly degraded at higher temperatures (Fig. 4A).

In an examination of the hNKCC2A variants, the R302W and L505V mutants clearly showed diminished production of complex-glycosylated mature NKCC2A protein, as indicated by reduced expression of the ~190-kDa band compared with wild-type at both temperatures; however, NKCC2A abundance was reduced to a greater extent when cells were incubated at 37°C (Fig. 4, A and B). Interestingly, upon incubation at 25°C, the L505V mutant appears to have the normal ratio of immature to mature cotransporter (150:190 kDa), whereas the R302W mutant exhibited a large amount of protein in the ~150-kDa band and also displayed unusual proteolytic cleavage (band at ~40–50 kDa, data not shown). These results suggest that the two mutants are recognized as defective at different steps in the maturation process or that their misfolded products are handled differently.
Visualizing wild-type hNKCC2A distribution by confocal microscopy, when cells were incubated at 37°C, we found a strong pattern of staining at the plasma membrane, demonstrating the correct targeting of hNKCC2A to the cell membrane under the condition of modest expression achieved in stable cell lines (Fig. 4C). Unlike the wild-type transporter, the R302W and L505V mutants appear to be absent from the plasma membrane and instead appear restricted to an intracellular distribution (Fig. 4, F and I). Together with the results of Western blot analysis, these results demonstrate that the severe loss of functional activity in the R302W and L505V mutants results from a processing defect, which leads to a dramatic reduction of mature cotransporter at the plasma membrane.

Consistent with an intermediate level of transport activity (Fig. 3B), the protein abundance and cellular distribution of NKCC2A in the T235M mutant are seen to be intermediate between that of wild-type and the severely impaired mutants, suggesting that the principal defect in T235M is also one of protein processing (Fig. 4, B and D). Supporting this, NKCC2A abundance in the T235M mutant was reduced to a greater extent when cells were incubated at 37°C, similar to the R302W and L505V mutants which also showed impaired protein processing (Fig. 4A and B). On the other hand, the N399S, P569H, and P1083A mutants exhibit robust NKCC2 abundance and plasma membrane expression despite significantly reduced 86Rb influx. The most likely explanation of this behavior is that these mutants have intrinsically lower rates of membrane transport turnover. Finally, the P254A, P348L, and Y1070C mutants exhibit robust NKCC2 abundance and plasma membrane expression and have basal transport activity levels that are statistically indistinguishable from wild-type.

Is transport impairment due to altered regulatory response of the hNKCC2A variants? As shown in Fig. 5, activation of mutant transporters in response to changes in intracellular Cl− concentration exhibits a similar profile compared with wild-type hNKCC2A, consistent with similar volume-regulatory responses observed in oocyte experiments. However, two mutants have significantly steeper profiles than wild-type: P254A and N399S are almost fully active when maximally stimulated by low intracellular Cl− concentration in the presence of the phosphatase inhibitor calyculin A, but exhibit lower levels of constitutive activity compared with wild-type. For wild-type NKCC2, we observed a 52 ± 0.08% constitutive activity level whereas this was significantly lower in the P254A and N399S mutants, being 45 ± 0.07 and 40 ± 0.06%, respectively (P = 0.033 and P = 0.019, Student’s t-test, n = 5). We propose that these two residues, situated near the cytoplasmic ends of TM3
RARE MUTATIONS IN HUMAN NKCC2 IMPAIR TRANSPORT FUNCTION

Fig. 5. Functional regulation of hNKCC2A mutants in HEK-293 cells. $^{86}\text{Rb}$ influx (nmol-mg protein$^{-1}$·min$^{-1}$) of HEK cells expressing wild-type and mutant proteins determined after preincubation in media containing various Cl concentrations. Furosemide was used in some preincubation conditions to block transporter-mediated Cl movements. $^{86}\text{Rb}$ influx for wild-type and mutant proteins under basal preincubation conditions (142 mM Cl$^+$-furosemide) are the same values as the data presented in Fig. 3B.

and TM6, may play a role in transporter activity through interaction with cytoplasmic-regulatory domains.

Kinetic analysis of hNKCC2A mutants in stable HEK-293 cell lines: does altered affinity for cotransported ions underlie impaired transporter function? To determine transport behavior of wild-type hNKCC2A and the hNKCC2A variants, we measured the dependence of $^{86}\text{Rb}$ influx on Na, Rb, and Cl concentration in the flux medium. As summarized in Table 1, the $K_m$ values of wild-type hNKCC2A for the three transported ions are consistent with recently reported values for the human ortholog expressed in Xenopus oocytes (3).

Examining the variant transporters, we found P569H and P254A to be significantly different from wild-type hNKCC2A, with all other variants being identical to wild-type within experimental error. Importantly, the affinity for Na is 1.5-fold lower in the P569H mutant compared with wild-type hNKCC2A (Table 1; Fig. 6, A and B). The lower Na affinity would translate into a decreased flux rate from dilute tubular fluid, an effect that would be in addition to the decreased function of P569H observed at 140 mM Na in HEK-293 cells (Fig. 3B). We also found a significant increase in the affinity of the P254A mutant for Rb compared with the wild-type transporter (Table 1; Fig. 6, C and D); while this would predict improved flux behavior at very low tubular K concentrations, it seems unlikely to be of much consequence at normal physiological K levels (10).

DISCUSSION

The present work describes the functional analysis of nine rare independent missense mutations in hNKCC2 that have been linked to altered blood pressure homeostasis and protection from arterial hypertension in a human population (14). Using heterologous expression in X. laevis oocytes and HEK-293 cells, we examined the function, expression, localization, regulation, and ion transport kinetics of wild-type and mutant hNKCC2A proteins. We determined that six of the nine mutations result in hNKCC2A proteins with impaired transport activity and that the mechanisms underlying impaired function reflect defects in protein processing, transport turnover rate, regulation, and ion affinity.

Among the possible mechanisms underlying alterations in the functional activity of mutant transport proteins, decreased cell surface expression is probably the most common. In this study, we found that loss of activity in the R302W, L505V, and T235M mutants was accompanied by decreased production of mature complex-glycosylated cotransporter and the reduction of protein at the plasma membrane. This resulted in the most extreme loss of any of the mutations: for R302W and L505V there was >45% loss of functional expression in X. laevis oocytes and >70% loss for these mutants in HEK cells. Related to this, a point mutation in NKCC2 (R302Q) has been linked to antenatal Bartter’s syndrome in a homozygous patient (27), indicating that this residue is critical to protein function and that mutation at this residue can have a deleterious effect on renal salt handling. Our evidence is that the R302W and L505V variants are synthesized normally but the mutant protein is retained in the endoplasmic reticulum because of folding defects and is targeted for early degradation. This type of mutation has been previously characterized as a processing or “trafficking” mutation and is the basis for many genetic diseases including cystic fibrosis (CFTR), Bartter’s syndrome, and Gitelman’s syndrome (15, 16, 29). Furthermore, we provide evidence that as incubation temperature is lowered the processing of these mutants shows modest recovery, similar to the behavior of other misfolded proteins such as CFTR-ΔF508 (6).

A number of the mutations studied here provide useful insight into structure-function relationships of NKCC2. Mutations P254A and P569H resulted in differences in apparent ion affinity for Rb and Na, respectively. The fact that individual $K_m$ values are affected suggests that these are specific effects

<table>
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<th>Mutant</th>
<th>$K_m$(Na), mM</th>
<th>$K_m$(Rb), mM</th>
<th>$K_m$(Cl), mM</th>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>0.81±0.11</td>
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<td>P1083A</td>
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<td>0.90±0.11</td>
<td>36.8±2.48</td>
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Values are means ± SE ($n = 4–7$ experiments). Shown is a summary of Michaelis constants ($K_m$) for wild-type and mutated human Na-K-Cl cotransporter (hNKCC2A) proteins. Apparent affinities discussed in the text are the reciprocals of these $K_m$ values. Values for R302W and L505V were not determined (ND) as $^{86}\text{Rb}$ influx was uncomfortably close to background HEK-cell levels in these mutants. One-way ANOVA determined a significant difference ($P < 0.01$) among mutants for all 3 ions. *Significant difference from wild-type hNKCC2A ($P < 0.05$, Dunnnett’s post hoc test).
on ion binding sites rather than changes in other transport rate constants. Homology modeling (Fig. 1) places these residues in transmembrane domains 3 and 10, two helices that form part of the translocation pathway in other APC superfamily transporters (8, 24).

P254A and N399S were found to affect the regulatory behavior of the transporter. Regulation of NKCC must involve some mechanism by which phosphorylation in the N terminus determines whether the transmembrane domains are able to undergo their transport conformational changes. Residues P254 and N399 are near the intracellular ends of TM3 and TM6, where they would be in a good position to interact with cytoplasmic domains and form part of this regulatory mechanism.

This report is the first in which the human ortholog of NKCC2 has been successfully studied in a mammalian cell expression system. We find that unlike in attempts with other vertebrate NKCC2s, the FLAG-YFP-tagged hNKCC2A expresses well in HEK-293 cells, with bumetanide-sensitive \(^{86}\)Rb influx an order of magnitude greater than that of the endogenous HEK cell NKCC1 flux. Our experience here is that the mammalian cell expression system is also a more sensitive test-bed for human NKCC2 compared with \textit{Xenopus} oocytes. Comparing the results shown in Figs. 2 and 3, the amount of impairment in many of the mutants is significantly greater in the HEK cell system than in the oocyte system. Quite possibly, the wide tolerance often cited as an advantage for expression of foreign proteins in \textit{Xenopus} oocytes works against the system as a sensitive indicator of expression differences.

We have found that many missense variants of hNKCC2A exhibit significantly decreased transport in a mammalian cell expression system, but of course it is not possible to directly extrapolate to functional consequences in the human kidney.

On the one hand, one might expect the effects to be less marked in the physiological system because these exist as heterozygous mutations. On the other hand, since NKCCs and KCCs are clearly expressed as dimers, dominant negative behavior of poorly processed mutants is both expected and observed (4, 5, 12, 22, 26). Furthermore, just as the mammalian expression system appears to be more sensitive with regard to protein processing compared with \textit{Xenopus} oocytes, it is possible that epithelial cell expression is even more stringently selective.

Overall, our results are supportive of the approach of Ji et al. (14) to use high sequence conservation as a predictor of functional defects, although our finding of six defects in nine variants indicates somewhat less specificity than predicted by their analysis of Barter’s and Gutelman’s mutations (90%). Because of the substantial variation in blood pressure in the human population and the multifactorial nature of the process, individual differences due to partially defective NKCC2 variants may be hidden by other population variables. The results presented here, however, are supportive of the finding that the rare mutations are correlated with reduced blood pressure, since on average our variants have \(>50\%\) reduced activity in HEK cells compared with wild-type hNKCC2, and in general the corresponding blood pressures are below average (14). Interestingly, a recent study which examined the functional consequences of the ROMK mutations identified by Ji et al. came to a similar conclusion by determining that polymorphisms in ROMK inhibit channel function by altering protein processing and channel-gating properties (7).

In conclusion, we have addressed the consequences of a number of heterozygous mutations in NKCC2 on function, expression, localization, regulation, and ion transport kinetics using heterologous expression in \textit{X. laevis} oocytes and HEK-
293 cells. We have determined that six of nine of these mutations lead to impaired transport activity, providing support for the argument that sequence conservation predicts functional importance, and thus for the conclusion of Ji et al. (14) that rare heterozygous mutations in NKCC2 contribute to blood pressure variation in the general population through their effects on renal salt reabsorption. Our results also demonstrate that diverse mechanisms including alteration in protein processing, transport turnover rate, regulatory behavior, and ion affinity underlie the reduced function of hNKCC2A within this set of mutations.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES