Functional consequences of NKCC2 splice isoforms: insights from a *Xenopus* oocyte model

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Lu L, Fraser JA. Functional consequences of NKCC2 splice isoforms: insights from a *Xenopus* oocyte model. *Am J Physiol Renal Physiol* 306: F710–F720, 2014. First published January 29, 2014; doi:10.1152/ajprenal.00369.2013.—The Na$^+\cdot$K$^+\cdot$2Cl$^-$ cotransporter NKCC2 is exclusively expressed in the renal thick ascending limb (TAL), where it exists as three main splice isoforms, NKCC2B, NKCC2A, and NKCC2F, with the latter two predominating. NKCC2A is expressed in both medullary and cortical TAL, but NKCC2F localizes to the medullary TAL. The biochemical characteristics of the isoforms have been extensively studied by ion uptake studies in *Xenopus* oocytes, but the functional consequences of alternative splicing remain unclear. We developed a charge-difference model of an NKCC2-transfected oocyte. The model closely recapitulated existing data from ion-uptake experiments. This allowed the reconciliation of different apparent $K_m$ values reported by various groups, which have hitherto either been attributed to species differences or remained unexplained. Instead, simulations showed that apparent Na$^+$ and Cl$^-$ dependencies are influenced by the ambient K$^+$ or Rb$^+$ bath concentrations, which differed between experimental protocols. At steady state, under bath conditions similar to the outer medulla, NKCC2F mediated greater Na$^+$ reabsorption than NKCC2A. Furthermore, Na$^+$ reabsorption by the NKCC2F-transfected oocyte was more energy efficient, as quantified by $J_{\text{Na}}$/$P_{\text{Na}}$. Both the increased Na$^+$ reabsorption and the increased efficiency were eroded as osmolarity decreased toward levels observed in the cortical TAL. This supports the hypothesis that the NKCC2F is a medullary specialization of NKCC2 and demonstrates the utility of modeling in analyzing the functional implications of ion uptake data at physiologically relevant steady states.

APPROXIMATELY 20–25% OF RENAL SODIUM REABSORPTION OCCURS IN the thick ascending limb (TAL) (5). This is mediated by the apical expression of the renal-specific electroneutral Na$^+\cdot$K$^+\cdot$2Cl$^-$ cotransporter NKCC2 in the TAL epithelium, both directly through transcellular Na$^+$ reabsorption with the Na$^+\cdot$K$^+\cdot$ATPase mediating basolateral Na$^+$ efflux as well as indirectly through the creation of a lumen-positive transtubular potential through apical K$^+$ recycling (17). Consequently, reductions in NKCC2 activity leads to natriuresis; as seen in the salt-wasting characteristic of the loss-of-function mutations in NKCC2 that underpin type I Bartter syndrome (27) as well as the diuresis that accompanies the clinical use of loop diuretics (5).

Na$^+$ reabsorption by NKCC2 fulfills two major physiological functions: salt (and hence volume) conservation and load sensing at the macula densa as a component of tubuloglomerular feedback (TGF) (35). The primary transcript of the NKCC2-coding gene, *Slc12a1*, is subject to alternative splicing, presumably to generate splice isoforms better suited to particular aspects of the aforementioned physiological functions. A key alternative splice locus is exon 4, where the selective inclusion of one of three mutually exclusive exon 4 variants yields three NKCC2 isoforms: NKCC2A, NKCC2B, and NKCC2F (30). As shown by ion uptake studies of NKCC2 isoforms heterologously expressed in *Xenopus* oocytes, the isoforms differ in affinity for cotransported ions (16, 32). Studies have consistently demonstrated an increasing Cl$^-$ affinity from NKCC2F to NKCC2A to NKCC2B (5). The isoforms also differ in axial localization along the TAL. In situ RNA hybridization and RT-PCR studies have revealed that NKCC2A is expressed in the renal cortex and outer medulla outer stripe, NKCC2F in the outer medulla with higher density in the inner stripe, and NKCC2B in the renal cortex. In the macula densa, both NKCC2A and NKCC2B are expressed (5, 16).

The localization of the isoforms provides some clues as to the specific functions of each isoform, i.e., NKCC2A and NKCC2F in Na$^+$ reabsorption for salt conservation and NKCC2A and NKCC2B in Na$^+$ reabsorption for TGF. The mechanisms by which their different biochemical characteristics and the interactions thereof optimize performance of these functions have been investigated experimentally, but remain incompletely understood. The contributions of NKCC2A and NKCC2B toward load sensing at the macula densa were elegantly dissected by Oppermann and colleagues (28, 29) by combining the generation of isoform-specific knockout mice with micropuncture experiments. These studies revealed that the different ion affinities of NKCC2A and NKCC2B enabled load sensing over a wider range of flow rates, with the high-affinity NKCC2B mediating load sensing at low flow rates and the low-affinity NKCC2A at high flow rates.

Similar approaches, however, are ill-suited to the study of Na$^+$ reabsorption for salt conservation. While stop-flow pressure measurements enable assessment of TGF in situ, micropuncture experiments lack sufficient resolution along the nephron to measure Na$^+$ reabsorption specifically along the TAL. Nor have knockout studies provided a full answer. In an NKCC2A knockout mouse, compensatory upregulation of NKCC2B occurred in the renal cortex and outer medulla, and urine osmolality was normal (29). There have been no reports to date of an NKCC2F knockout mice model.
Consequently, current understanding of the functional contribution of NKCC2A and NKCC2F to Na\(^+\) reabsorption remains shaped by inferences made from ion uptake studies in *Xenopus* oocytes. The lower magnitude of uptake in NKCC2F-compared with NKCC2A-expressing oocytes is thought to be explained by the higher \(K_d\) of NKCC2F for cotransported ions and has led to the suggestion that medullary expression of NKCC2F serves to reduce medullary Na\(^+\) reabsorption (32). This is thought to confer the functional advantage of reducing medullary \(O_2\) consumption, since NKCC2 activity is ouabain sensitive and hence a form of secondary active transport (3, 16).

However, experimental support for such a hypothesis is lacking. Microperfusion of isolated cortical (cTAL) and medullary TAL (mTAL) has shown that while the cTAL has higher diluting power as expected from the expression of the higher-affinity NKCC2 isoforms, the mTAL has greater Na\(^+\) reabsorption capacity (3, 34, 36). This was noted by Giménez et al. (16). Taking into account the lower rate of ion uptake by NKCC2F-expressing *Xenopus* oocytes, they suggested that this might be due to higher NKCC2 expression in the medulla. Clearly, such an explanation is incompatible with the suggested functional rationale of NKCC2F expression mediating reduced medullary Na\(^+\) reabsorption.

The underlying problem is the difficulty of assessing transport capacity from ion uptake experiments. To maximize the rate of uptake and hence the signal-to-noise ratio, such experiments are performed under initial rate of reaction type conditions, where the oocyte is depleted of the cotransported ions, for instance, by preincubation in K\(^-\) and Cl\(^-\)-free medium (32). However, physiologically, the ion concentrations of the luminal fluid, renal epithelium, and renal interstitium are all interrelated, as evidenced by the hyperosmolarity of TAL cells compared with plasma revealed by electron microprobe analysis (2). Consequently, the transport capacity which determines Na\(^+\) reabsorption physiologically is not that at initial rate of uptake conditions, but rather that at steady state, with a far reduced overall inward gradient of cotransported ions. Indeed, it is striking that the microperfusion experiments earlier discussed were performed effectively under such steady-state conditions, with the bath and perfusate being of similar composition (3, 36).

We hypothesize that modeling the NKCC2-transfected *Xenopus* oocyte will enable us to characterize the transport capacity of NKCC2 isoforms at physiologically relevant steady states, as determined by experimentally measured biochemical parameters such as ion affinity, thereby extending the utility of available experimental data by extending it into more physiological parameter spaces. This approach is possible because the *Xenopus* oocyte is a well-characterized model system also used to study endogenous ion channels and transporters, enabling development of a model that is closely based on existing experimental work. This approach can be considered as a careful extrapolation from ion uptake data to realize an enhanced value from such experiments. In the present study, we consider the functional consequences of alternative NKCC2 splicing. The same approach could additionally be applied to analyze the numerous other cotransporter studies that have been performed in *Xenopus* oocytes, as well as complement future experimental work in this field.

Therefore, we first developed a charge-difference model (11) of an untransfected denuded *Xenopus* oocyte, such as is routinely used in heterologous expression studies. Using a kinetic model of NKCC2 developed by Weinstein (38), we then developed a model NKCC2-transfected *Xenopus* oocyte and validated it by recapitulating published ion uptake data from different groups. This enabled us to reconcile some apparent discrepancies in \(K_d\) values obtained by different groups (5). Finally, we used this model NKCC2-transfected oocyte to characterize the steady-state transport capacity, and energy efficiency, of NKCC2A and NKCC2F. This reveals that under hyperosmotic conditions similar to in the renal medulla, NKCC2F has a higher transport capacity, and higher energy efficiency of Na\(^+\) reabsorption, compared with NKCC2A. This is eroded as osmolarity decreases, attenuating the functional advantage conferred by NKCC2F, supporting the notion that NKCC2A is the default splice isoform, with NKCC2F being a medullary specialization (14).

**MATHEMATICAL MODEL**

The denuded *Xenopus* oocyte was modeled as a single compartment in the bath medium. The oocyte was described by the following elementary variables: 1) volume, 2) intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\)), 3) intracellular Cl\(^-\) concentration ([Cl\(^-\)]\(_i\)), 4) intracellular K\(^+\) concentration ([K\(^+\)]\(_i\)), and 5) total concentration of membrane-impermeant ions and molecules, [\(X\)]. These membrane-impermeant ions correspond physiologically to complex molecules such as amino acids, cytosolic proteins, and nucleic acids (10). Volume was modeled as a free variable in the NKCC2-transfected oocyte, but not in the untransfected oocyte which was used to solve for unknown parameters as subsequently discussed, because the volume was already known from experimental measurements in the latter case. Surface area was estimated from experimental measurements of volume assuming the oocyte approximated a sphere, and this was invariant during all simulations. Another important fixed parameter was \(z_X\), which referred to the average valency of the membrane-impermeant intracellullar ions.

The bath medium was similarly described by extracellular values [Na\(^+\)]\(_o\), [Cl\(^-\)]\(_o\), [K\(^+\)]\(_o\), and [\(X\)]\(_o\), as well as \(z_X\). \(X\) referring in this case to membrane-impermeant extracellular ions and molecules, such as HEPES. The bath medium was modeled as an infinite medium, i.e., with invariant concentrations, to reflect that in all experimental setups considered here the volume of the oocytes incubated therein, thus making it unlikely for bath ion concentrations to change significantly over the course of the experiments.

When the oocyte was incubated in a given bath medium, changes in its aforementioned elementary variables, and indeed their steady-state values, were determined solely by parameters of the model that determined the movement of water and ions between the compartments. In other words, the steady-state values of model variables were independent of their initializing values, i.e., history independent, as shown in Fig. 1A. Transmembrane fluxes in this model included the osmotic movement of water, the passive movement of Na\(^+\), Cl\(^-\), and K\(^+\) down their electrochemical gradients, and the action of the following pumps and transporters, i.e., Na\(^+\)-K\(^+\)-ATPase, endogenous NKCC1, and heterologous NKCC2 (for transfected oocytes only). Calculations were performed as described below, considering each of the transmembrane fluxes above in succession.

The osmotic movement of water per unit membrane, \(J_{\text{H}_2\text{O}}\), depended on the free water gradient between the two compartments and the transmembrane water permeability, \(P_{\text{H}_2\text{O}}\). This was modeled by the equations

\[
J_{\text{H}_2\text{O}} = P_{\text{H}_2\text{O}}\left(\left[\text{H}_2\text{O}\right]_o - \left[\text{H}_2\text{O}\right]_i\right)
\]
\[ [H_2O] = 55.55 - [Na^+] - [Cl^-] - [K^+] - [X^-] \] (1b)

where 55.55 M refers to the molar concentration of pure water (12). Following transmembrane water movement, the ion concentrations were diluted or concentrated accordingly, and volume was adjusted if it was a free variable.

To determine the electrodialysis of membrane-permeant ions, the membrane potential, \( E_m \), was first calculated. \( E_m \) was calculated using the charge-difference approach (11), using the following equation

\[ E_m = F \left( [Na^+] - [Cl^-] + [K^+] + z_i[X^-] \right) / \text{C}_m \] (2)

where \( F \) is Faraday’s constant, and \( C_m \) is cell membrane capacitance (10). \( E_m \) together with the transmembrane concentration gradient and membrane ionic permeability, \( P \), was used to calculate the transmembrane flux per unit membrane area, \( J \), of the various modeled ions using the Goldman-Hodgkin-Katz flux equation. For the ion \( s \),

\[ J_s = P_s e_m \left( [s^-] e^k - [s^+] e^o \right) \] (3a)

\[ e_m = 2\phi / \left( e^k - e^o \right) \] (3b)

\[ \phi = z_i F E_m / (2RT) \] (3c)

where \( z_i \) is the valency of \( s \), \( R \) is the molar gas constant, and \( T \) is the absolute temperature (22).

\( Na^+-K^+-ATPase \) activity was calculated using the six-stage kinetic model developed by Hernandez et al. (18, 19), for which the cited works provide a complete description. Briefly, pump flux was solely determined by the free energy change of each pump cycle, which is determined by the free energy change of each pump cycle, which in turn depends on \( E_m, [Na^+], [Na^+]_o, [K^+], [K^+]_o, \) [ATP], [ADP], and \( [P_i] \). These variables were used to calculate six forward and six reverse rate constants, which were then used to calculate pump flux per unit membrane, \( J_{pump} \), using the following equation

\[ J_{pump} = N_s (\alpha - \beta) / \Sigma \] (4)

where \( N_s \) is the \( Na^+-K^+-ATPase \) density, \( \alpha \) is the product of the forward rate constants, \( \beta \) is the product of the reverse rate constants, and \( \Sigma \) is a function of all the rate constants and ligand concentrations (19). No further regulation of \( Na^+-K^+-ATPase \) activity was modeled.

Flux mediated by heterologous NKCC2 was calculated using the kinetic model for NKCC2 developed and parameterized by Weinstein (38). This model had a reaction scheme that assumed first-on-first-off symmetry, with binding on the extracellular face being on the order \( Na^+, Cl^-, K^+, \text{and } Cl^- \). Only empty or fully loaded carriers could translocate across the membrane. Separate parameter sets were available for NKCC2A and NKCC2F and were obtained based on experiments performed by Plata and colleagues (32) on mouse NKCC2 heterologously expressed in Xenopus oocytes. Each parameter set comprised seven parameters: the microscopic (as opposed to apparent) affinity constants for \( Na^+, K^+, \text{and } Cl^- \), i.e., \( K_m, K_k, \text{and } K_c \), and the forward and reverse translocation rates of the empty and fully loaded transporter, i.e., \( P_{empty}, P_{full} \), \( P_{full}, P_{empty} \), NKCC2-mediated flux per unit membrane, \( J_{NKCC2} \), was calculated using the following set of equations

\[ J_{NKCC(r)} = J_{NKCC(r)} \] (5a)

\[ J_{NKCC(r)} = \left( [Na^+]_o \beta_0 [K^+]_o / (\sigma_r \beta_r + \sigma_o \beta_o) \right) \] (5b)

\[ \alpha = [Na^+] / K_a \] (5d)

\[ \beta = [K^+] / K_i \] (5e)

\[ \gamma = (Cl^-) / K_c \] (5f)

\[ \sigma_r = 1 \alpha_r + \alpha_r \gamma_r + \alpha_r [K^+] \] (5g)

\[ \sigma_o = 1 \beta_o + \beta_o \gamma_o + \alpha_o [K^+] \] (5h)

\[ J_{NKCCo} = J_{NKCCo} \] (5i)

\[ J_{NKCCo} = P_{empty} + P_{full} \beta_0 [K^+] \] (5j)

where \( J_{NKCC(r)} \) and \( J_{NKCCo} \) refer to microscopic NKCC2-mediated flux in the forward and reverse direction, respectively, and \( N \) denotes NKCC2 density.

Due to the unavailability of a published kinetic model of NKCC1, NKCC1 was modeled as NKCC2F. This simplification is reasonable as, while ion affinities have not been measured for the Xenopus NKCC1, the ion affinities between the closely related human NKCC1 and mouse NKCC2A and NKCC2F are highly similar. No further regulation of NKCC2 activity was modeled.

Finally, the total transmembrane ion flux for each ion was calculated taking into account the passive, pump-, and transporter-mediated transmembrane ion fluxes, and the cellular ion concentrations were adjusted accordingly.

During each iteration, calculations were performed in the order described above. Iterations began with a time step of \( 10^{-10} \) s and this was dynamically adjusted in accordance with the \( E_m \) change in that time step. Preliminary simulations revealed that the optimal dynamic adjustment algorithm to achieve the maximum step size without the model oscillating involved 1) increasing the time step by 0.001% if the change in \( E_m \) was < 2 \times 10^{-7} \) V and 2) decreasing the time step by 10% if the change in \( E_m \) was > 2 \times 10^{-6} \) V.

When the experiments simulated utilized \( Rb^+ \), the variables \( [Rb^+]_o \) and \( [Rb^+]_i \), were introduced into the model, \( Rb^+ \) was treated in exactly the same manner as \( K^+ \), in terms of passive permeabilities as well as transporter and pump ion affinities. When \( ^{86}Rb^+ \) was used in experiments as a radioactive tracer, \( [Rb^+] \) was calculated from the stated activity, assuming an \( ^{86}Rb^+ \)-specific activity of 570 Ci/mol.
RESULTS

Parameterization of the untransfected oocyte. The mathematical model described above was parameterized using the experimental data shown in Table 1, generating a model of an untransfected denuded stage V-VI Xenopus oocyte. The model was used to determine two key parameters not available from the experimental literature: first, NKCC1 density and second, the absolute ionic permeabilities, although relative ionic permeabilities have been measured.

To determine the range of possible parameter values, we fitted the model to four experimental measurements: 1) bumetanide-sensitive K⁺ influx into oocytes (37); 2) [Cl⁻];, 3) [K⁺]i, and 4) Eₘ of oocytes after the application of ouabain (7) (Table 1). In each case, the experimental protocols were simulated precisely, allowing direct comparison of the steady-state values obtained from simulations with experimental results. Ionic permeability ratios were fixed according to available experimental data, while NKCC1 density and the absolute ionic permeability were varied. Simulations revealed the influence of NKCC1 density and absolute ionic permeabilities on the four variables for which experimental data are available, as shown in Fig. 2A. The magnitude of the influence of these parameters was sufficient to allow experimental data to constrain their values. Figure 2B shows the values of these parameters that give steady-state values of the four constraining variables that are within ±1 SE of their mean values. Only a restricted set of values satisfies this requirement for all four variables: an NKCC1 density between 10⁻⁴.5 and 10⁻¹₃.5 mol/cm² and Pₖ between 10⁻⁷.5 and 10⁻⁷ cm/s.

To find a single set of parameter values, this solution set was further explored at a resolution of 0.1 logarithmic units. However, no single set of values could yield an untransfected oocyte that matched the published mean values for [Cl⁻], [K⁺]i, and Eₘ (after ouabain). Given that Eₘ was measured following an acute pharmacological manipulation, we relaxed the Eₘ (after ouabain) constraint to within 2 SE of the experimental mean, yielding four sets of parameter values that satisfy the constraining variables (Fig. 2C). Selecting the parameter set that produced an Eₘ (after ouabain) closest to the experimental mean, a final NKCC1 density and Pₖ of 3.16 × 10⁻¹⁵ mol/cm² and 3.98 × 10⁻⁸ cm/s, respectively was obtained. An oocyte with these parameters was then simulated in oocyte Ringer 2 (OR2) bath medium (84 mM Na⁺, 89.5 mM Cl⁻, 2.5 mM K⁺, 8 mM X, 0.375 zX) (37) until a steady state was reached.

Table 1. Experimental data used in model parameterization, comprising experimentally measured parameters, and experimentally measured variables used for model determination of unknown parameters

<table>
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<th>Parameter</th>
<th>Value</th>
<th>Units</th>
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<td>Volume¹</td>
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<td>Liters</td>
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<tr>
<td>Surface area</td>
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<tr>
<td>Capacitance</td>
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<td>Water and ionic permeabilities</td>
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<td></td>
</tr>
<tr>
<td>PᵣᵣO</td>
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<tr>
<td>Pᵣₖ/Pₖ</td>
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<td>7</td>
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<tr>
<td>Pₚ/Pₖ</td>
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<td></td>
<td>7</td>
</tr>
<tr>
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<td>cm/s</td>
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<td>Pₙₙₑ𝑜ₑ</td>
<td>1.34 × 10⁻⁷</td>
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<td>s⁻¹</td>
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<td>M</td>
<td>38</td>
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</table>

¹When fixed in parameterizing the untransfected denuded oocyte. ²When not assumed to be completely impermeant. See the text for definitions.
was achieved, and this was considered the baseline untransfected denuded oocyte that was used for subsequent simulations.

Development of an NKCC2-transfected oocyte. To model the NKCC2-transfected oocyte, a kinetic model of NKCC2 (38) was incorporated into the untransfected denuded oocyte model. Two separate models simulating an NKCC2A- and an NKCC2F-transfected oocyte were developed. To validate the models, the experiments performed by Plata et al. (32) were simulated in full, including long-term incubation in ND96, preincubation in Cl⁻-free ND96 overnight, and then in K⁺- and Cl⁻-free medium for 30 min, before the actual ion uptake experiments under varying ion concentrations. NKCC2 densities were adjusted to achieve the ion uptake values observed in medium containing maximum concentrations of the cotransported ions, yielding a density of $1.10 \times 10^{-12}$ mol/cm² for NKCC2A and NKCC2F, respectively. Figure 3A reveals that the model is able to recapitulate the ion-dependencies observed by Plata et al. (32). This is not trivial even though the same data set was used by Weinstein (2010) to parameterize the NKCC2 kinetic model, as that parameterization was performed using effectively isolated cotransporters in infinite medium, disregarding the effects on the oocyte of both the extensive preuptake experimental manipulations and the ion uptake itself, which could affect subsequent ion uptake.

To cross-validate the model, experiments performed by Giménez et al. (2002) were simulated in full. The experimental protocol included preincubating the oocytes in furosemide and then in hyposmotic medium, both of which were modelled as a reduction in effective NKCC1 and NKCC2 density in accordance with experimental measurements of the effect of such

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**Fig. 2.** Parameterization of untransfected denuded *Xenopus* oocyte. A: effect of NKCC1 and absolute ionic permeabilities, shown here as $P_K$, on steady-state intracellular K⁺ concentration ([K⁺]ᵢ), intracellular Cl⁻ concentration ([Cl⁻]ᵢ), NKCC1-mediated ion uptake, and $E_m$ after ouabain (clockwise from top left). B: comparison of steady-state [Cl⁻], [K⁺], $E_m$ after ouabain, and NKCC1-mediated ion uptake with measured experimental values delineates (dotted lines) a solution set of NKCC densities and absolute ionic permeabilities that can satisfy all these experimental observations to within 1 SE. Further exploration of the solution set yields combinations of NKCC1 density and absolute permeability values that satisfy experimental observations, but only after the constraint of $E_m$ after ouabain is relaxed to 2 SE from the observed mean. The combination that has $E_m$ after ouabain closest to the experimental mean is circled. Colors represent similar variables as in B.
manipulations on NKCC activity (32, 37). NKCC2A and NKCC2F densities following transfection were respectively estimated as \(1.55 \times 10^{-10}/\text{mol/cm}^2\) and \(1.00 \times 10^{-10}/\text{mol/cm}^2\). As seen in Fig. 3B, simulations are able to recapitulate the observed ion dependencies, thus further validating the fidelity of the NKCC2-transfected oocyte model. Plata et al. (2002) and Giménez et al. (16) both used impermeant ion substitutes to manipulate ion concentrations without changing osmolarity, and the data shown assume zero permeability to these ion substitutes. While some experiments suggest appreciable per-

Fig. 3. NKCC2-transfected oocyte model. A and B: recapitulation of ion dependencies observed by Plata et al. (32; A) and Giménez et al. (16; B) for both NKCC2A and NKCC2F by model simulations. C: validation of Rb\(^+\) uptake as a marker of NKCC2 activity, including NKCC2-mediated ion influx at initial rate of reaction conditions. D: differences in apparent \(K_m\) values measured by Plata et al. (32) and Giménez et al. (16) are abolished when similar external K\(^+\) or Rb\(^+\) concentrations are employed when assessing the Cl\(^-\) (left) and Na\(^+\) (right) dependence of ion uptake for NKCC2F and NKCC2A. E: apparent \(K_{Cl}\) for NKCC2F decreases with increasing ambient bath K\(^+\) or Rb\(^+\) concentration.
meability to these ion substitutes (6), simulations reveal that including these levels of permeability do not alter the ability of the model to recapitulate experimental data.

**Differences in apparent \( K_m \) values.** The ability of the model to recapitulate the data of both Plata et al. (32) and Giménez et al. (16) is striking, because of the differences between the apparent \( K_m \) values reported by both groups as shown in Table 2. Comparison of the experimental protocols used by Plata et al. (32) and Giménez et al. (16) reveals multiple differences, including the length and nature of the preincubation steps, the ambient concentrations of unmanipulated ions during ion uptake, and the duration of ion uptake. In principle, differences in the determined \( K_m \) values could be due to genuine differences in NKCC2-mediated ion influx, or due to \(^{86}\text{Rb}^+\) being unable to accurately mark NKCC2-mediated ion influx. As shown in Fig. 3C, almost all of the oocyte’s 

\[ J^\text{Rb} \]

is quantitatively marked by 

\[ J^\text{Na} + J^\text{Cl} \]

as shown in Fig. 3A, indicating that the NKCC2 activity in the two studies. Further simulations were conducted to determine whether this could have resulted from the different experimental protocols, or to species differences in the NKCC2 isoforms used.

A notable difference between the experimental protocols of Plata et al. (32) and Giménez et al. (16) was that \([K^+ + Rb^+]_o\) was 10 and 2 mM, respectively, when the \([Na^+ + K^+ + Rb^+]_o\) set at 2 mM in each case. This correction led to very similar observed Na\(^+\) and Cl\(^-\) dependencies, whereas when tested at the original values of \([K^+ + Rb^+]_o\) used in the experiments, the Na\(^+\) and Cl\(^-\) dependencies were different for each protocol. This is because these levels of \([K^+ + Rb^+]_o\) are nonsaturating (Fig. 3E), whereby increases in \([K^+ + Rb^+]_o\) continue to increase initial \( J_{\text{NKCC}^\text{F}} \). This means that at higher \([Na^+ + K^+ + Rb^+]_o\), \( V_{\text{max}} \) can be achieved at lower concentrations of \([Na^+ + K^+ + Rb^+]_o\), and \([Cl^- + C1^- + Rb^+]_o\), thus enhancing the apparent Na\(^+\) and Cl\(^-\) dependence of \( J_{\text{NKCC}^\text{F}} \), and shifting apparent \( K_{\text{Na}} \) and \( K_{\text{Cl}} \) to lower values. It thus follows that the apparent \( K_{\text{Na}} \) and \( K_{\text{Cl}} \) in experiments by Plata et al. (32) when 10 mM \([K^+ + Rb^+]_o\) was used were indeed lower than those estimated by Giménez et al. (16), when 2 mM \([K^+ + Rb^+]_o\) was used (Fig. 3D).

**Steady-state transport capacity.** To assess the steady-state transport capacity of NKCC2 isoforms, simulations of oocytes expressing NKCC2A or NKCC2F at a density of \(1.50 \times 10^{-13}\) mol/cm\(^2\) were run to steady state. Bath conditions were chosen to recapitulate conditions in the mTL, cTL, and the end of the thick ascending limb, with respective osmolarities of 600, 300 and 180 mosmol/l. Ion concentrations at 600 mosmol/l comprised 250 mM Na\(^+\), 225 mM Cl\(^-\), 10 mM K\(^+\), and 115 mM of other osmoregulatory ions that are assumed to be impermeant (39), and this was scaled to obtain the ionic composition of the medium of varying osmolarity. To achieve bulk electroneutrality in the bath medium, \( z^X \) was set at \(-0.304\). As seen in Fig. 4A, at steady state, \( J_{\text{NKCC}^\text{F}} \) was 73% higher for NKCC2F than NKCC2A at 600 mosmol/l, but this increased transport capacity of NKCC2F was eroded with decreasing osmolarity, to 20 and 5% greater than NKCC2A fluxes at 300 and 180 mosmol/l respectively. The pattern of these observations was not sensitive to the NKCC2 density used for simulation (Fig. 4A).

As seen in Fig. 4B, at steady state, \( J_{\text{NKCC}^\text{F}} \) is \( \approx 100 \) times less than \( J_{\text{NKCC}^\text{A}} \). This implies that \( J_{\text{NKCC}^\text{F}} \) and \( J_{\text{NKCC}^\text{A}} \) are closely matched, as is indeed the case across a range of bath osmolarities. This is because at steady state, unlike initial rate of reaction conditions, cytosolic ion concentrations are also influenced by bath ion concentrations. Therefore, even though NKCC2A has a higher \( J_{\text{NKCC}^\text{A}} \) than NKCC2F, as predicted by its higher ion affinities, this does not necessarily translate into a higher \( J_{\text{NKCC}^\text{F}} \) than NKCC2F.

Consequently, while \( J_{\text{NKCC}^\text{A}} \) must still depend on the absolute magnitude of fluxes, a physiologically more important determinant of \( J_{\text{NKCC}^\text{F}} \) at steady state is the ratio of forward to reverse fluxes, as can be seen in the formulation: \( J_{\text{NKCC}^\text{F}} = \left( J_{\text{NKCC}^\text{F}} / J_{\text{NKCC}^\text{A}} \right) - 1 \) \( \times J_{\text{NKCC}^\text{A}} \). Indeed, this is the basis for the higher \( J_{\text{NKCC}^\text{F}} \) observed for NKCC2F compared with NKCC2A. At 600 mosmol/l, although \( J_{\text{NKCC}^\text{F}} \) and \( J_{\text{NKCC}^\text{A}} \) are \( \approx 48\% \) greater for NKCC2A compared with NKCC2F, NKCC2A has \( \sim 160\% \) greater \( \left( J_{\text{NKCC}^\text{F}} / J_{\text{NKCC}^\text{A}} \right) - 1 \) than NKCC2F. Therefore, transport asymmetry, as seen in \( J_{\text{NKCC}^\text{F}} / J_{\text{NKCC}^\text{A}} \), is a key determinant of steady-state \( J_{\text{NKCC}^\text{F}} \), and it is the function of the relative enhancement of NKCC2F to NKCC2A that results in similar transport capacities between isoforms with decreasing osmolarity.

The basis of interisoform differences in \( J_{\text{NKCC}^\text{F}} / J_{\text{NKCC}^\text{A}} \), and its modulation by bath osmolarity, can be dissected out by considering a theoretical NKCC2F affinity mutant, which has the translocation properties of NKCC2F but the ion affinities of NKCC2A. Like NKCC2F, this affinity mutant displays consistently enhanced \( J_{\text{NKCC}^\text{F}} / J_{\text{NKCC}^\text{A}} \) compared with NKCC2A at all osmolarities but displays a marked increase in \( J_{\text{NKCC}^\text{F}} / J_{\text{NKCC}^\text{A}} \) with decreasing osmolarity like NKCC2A (Fig. 4B). Therefore, it appears that the enhancement of NKCC2F transport capacity resides in its translocation rates, whereas erosion of this enhancement with decreased osmolarity is a consequence of its ion affinities. Since NKCC2A and NKCC2F have identical loaded transporter forward translocation rates, \( P_{\text{full},r} \), but NKCC2F has smaller loaded transporter reverse translocation rate, \( P_{\text{full},r} \) (Table 1), the key difference leading to enhanced \( J_{\text{NKCC}^\text{F}} \) is likely to be the increased transporter asymmetry, i.e., \( P_{\text{full},r} / P_{\text{full},r} \) displayed by NKCC2F compared with NKCC2A.

**Steady-state transport efficiency.** Energy efficiency is an important issue, particularly in the renal medulla where the supply of respiratory substrates is poor due to low blood flow as well as countercurrent exchange. The energy efficiency of Na\(^+\) reabsorption was quantified by normalizing \( J_{\text{NKCC}^\text{F}} \) by Na\(^+\)-K\(^+\)-ATPase activity, \( J_{\text{Pump}} \). As shown in Fig. 5A, \( J_{\text{NKCC}^\text{F}} / J_{\text{Pump}} \) was consistently higher for NKCC2F than NKCC2A.

Table 2. Comparison of apparent \( K_{\text{Na}} \) and \( K_{\text{Cl}} \) values measured by Giménez et al. (16) and Plata et al. (32) for rabbit and mouse NKCC2, respectively

<table>
<thead>
<tr>
<th>Ion</th>
<th>( K_m ) (Giménez et al.), mM</th>
<th>( K_m ) (Plata et al.), mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+)</td>
<td>16.45 ± 1.9</td>
<td>5.0 ± 3.9</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>44.65 ± 3.87</td>
<td>22.2 ± 4.8</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>66.72 ± 5.8</td>
<td>20.6 ± 7.2</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>111.3 ± 13.4</td>
<td>29.2 ± 2.1</td>
</tr>
</tbody>
</table>

Values are means ± SE.

**References**

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and this efficiency enhancement was likewise eroded with decreasing osmolarity. These observations were not sensitive to the NKCC2 density used for simulations (Fig. 5A).

A closer examination reveals that $J_{\text{Pump}}$ is actually similar at steady state for NKCC2F and NKCC2A oocytes (Fig. 5B). Hence the increase in energy efficiency is actually due to the higher $J_{\text{NKCC}}$ displayed by NKCC2F oocytes earlier described, despite similar $J_{\text{Pump}}$. Since at steady state, when $[\text{Na}^+]_i$ is constant, Na$^+$ influx must be equal to Na$^+$ efflux via the Na$^+$-K$^+$-ATPase, the ability of higher $J_{\text{NKCC}}$ to be supported by a similar $J_{\text{Pump}}$ implies a reduction in other sources of Na$^+$ influx. Indeed, NKCC2F oocytes have reduced Na$^+$ leak into the cell, which actually accounts for the majority of Na$^+$ reabsorption (Fig. 5B).

Since $[\text{Na}^+]_i$ is effectively set by the Na$^+$-K$^+$-ATPase when pump density is adequate (10), $[\text{Na}^+]_i$ is similar in NKCC2F and NKCC2A oocytes at all bath osmolarities studied. Consequently, the reduced Na$^+$ leak in NKCC2F oocytes must be due to a reduced electrical gradient for Na$^+$ entry. Indeed, as shown in Fig. 5C, the changes of $E_m$ with osmolarity closely match the changes in transport efficiency, and the relative depolarization of NKCC2F oocytes compared with NKCC2A oocytes is due to higher steady-state [Cl$^-$]$_i$ and hence Cl$^-$ leak. Since at steady state, Cl$^-$ entry via NKCC must be equal to Cl$^-$ leak out of the cell, and Cl$^-$ leak is driven by the outward Cl$^-$ concentration gradient, the heightened $J_{\text{NKCC}}$ in NKCC2F is responsible for the elevated [Cl$^-$]$_i$ and hence depolarized $E_m$ and increased transport efficiency. Therefore, by coupling Na$^+$ reabsorption with reabsorption efficiency, Na$^+$ reabsorption is effectively decoupled from energy costs.

**DISCUSSION**

**Model development.** In the initial phase of this study, we developed a model untransfected denuded Xenopus oocyte closely based on experimental data. The only parameters which required determination through the model were NKCC1 density and absolute ionic permeability. The available experimental data tightly constrained the parameter set, yielding model-determined values which are plausible compared with experimental data in other preparations. NKCC1 density of $3.16 \times 10^{-15}$ mol/cm$^2$ is similar to the NKCC1 density of $1.0 \times 10^{-15}$ mol/cm$^2$ observed in duck erythrocytes (30). Similarly, the model-determined $P_K$ of $3.98 \times 10^{-8}$ cm/s in the modeled stage V-VI oocytes is similar to the $P_K$ of $9.55 \times 10^{-8}$ cm/s observed in oocytes at earlier stages of development (6).

The subsequent introduction of the NKCC2 kinetic model yielded an NKCC2-transfected oocyte that was able to recapitulate the ion uptake studies performed by Plata et al. (32) and Giménez et al. (16) for both NKCC2A and NKCC2F, despite apparent discrepancies between the results of these studies that have hitherto been explained as species differences (5). Instead, results from the transfected oocyte model suggest that differences in the $K_m$ values obtained in these studies are artifacts of the different experimental protocols. Thus the simulations suggest that much of the variation between the apparent $K_m$ values is due to the ambient [K$^+$+Rb$^+$]$_o$ when $K_{\text{Cl}}$ and $K_{\text{Na}}$ are determined, with increasing [K$^+$+Rb$^+$]$_o$ decreasing apparent $K_m$. This agrees well with experimental data. [K$^+$+Rb$^+$]$_o$ increases from Giménez et al. (16) (2 mM) to Gagnon et al. (13) (5 mM) to Plata et al. (32) (10 mM), and...
hence, for instance, apparent $K_m$ of NKCC2F decreases in that order, i.e., 66.7, 54, and 20.6 mM, respectively.

These findings are supported by the fact that even when cRNA of NKCC2 from the same species is used, different groups still obtain different apparent $K_m$ values (5, 13, 16). Furthermore, alignment of the TM2 and ICL1 regions of NKCC2 (4), where the residues determining differences in ion affinities between isoforms lie (15), reveals that there are only one and two conservative amino acid substitutions between the mouse and rabbit sequences for NKCC2A and NKCC2F respectively, i.e., where isoleucine is substituted with or for valine, making large differences between true $K_m$ values unlikely.

These findings demonstrate the utility of our model in providing an improved quantitative approach to analyzing results from ion uptake experiments. The routine use of the NKCC2 oocyte model to interrogate proposed differences in NKCC2 behavior based on ion uptake studies will complement the use of statistical tests, excluding systematic and random error, respectively. Further work can apply the model to a recent study of human NKCC2 isoforms, where in contrast to all other species studied, NKCC2F has lower apparent $K_m$ than NKCC2A (4).

Steady-state NKCC2-mediated transport. On the basis of higher $K_m$ values for the cotransported ions as well as lower rates of uptake into ion-depleted oocytes in the presence of maximal bath ion concentrations, it is thought that NKCC2F has reduced intrinsic transport capacity compared with NKCC2A, but that this may be attenuated by the higher luminal ion concentrations in the mTAL which are within the range of NKCC2F $K_m$ values (16, 32).
This understanding is challenged by model simulations, which instead suggest that at steady state, under conditions similar to the outer medulla inner stripe where NKCC2F is localized, NKCC2F mediates 73% more Na\(^+\) reabsorption than NKCC2A. This extrapolation of ion uptake data serves to bring findings from heterologous expression studies in line with observations in mammalian kidneys. Not only can this help to explain the aforementioned greater Na\(^+\) reabsorption observed in the mTAL compared with the eTAL (3, 34, 36), it is also consistent with the observation that in NKCC2A\(^{-/-}\) mice, incomplete compensation by NKCC2B upregulation is only unmasked through micropuncture at supranormal flow rates, suggesting that at normal flow rates, there is a functional reserve in NKCC2A-mediated reabsorption, pointing to appreciable, predominantly upstream, NKCC2F-mediated reabsorption (29). The functional advantage of the high NKCC2F-mediated Na\(^+\) reabsorption is obvious, i.e., enhanced salt conservation and greater urinary concentrating capacity, and the fact that this advantage is eroded with decreasing osmolality is in line with the concept of NKCC2A as the default isoform (14), with NKCC2F as a medullary specialization.

The discordance between the inferences drawn about NKCC2 physiology from apparent \(K_m\) values and an extrapolation of ion-uptake data into physiological parameter spaces performed by the NKCC2 oocyte model is striking. It advises against a simplistic interpretation of the results obtained due to experimental necessity under initial rate of reaction type conditions and showcases the utility of the oocyte model in understanding the physiological implications of such data more rigorously.

Therefore, a rigorous examination of ion uptake data should include fitting to a suitable kinetic model to obtain translocation rates and microscopic (as opposed to apparent) ion affinities, and modeling to understand its implications at steady state. For NKCC2 physiology, this can help in understanding the biochemical basis of the effects of certain mutations in Slc2a1 which are not immediately obvious. For example, the P254A mutation in a human NKCC2A background displays normal \(36^\circ\)Rb\(^-\) uptake in the presence of high bath ion concentrations, and indeed enhanced Rb\(^-\) affinity (26), arguing for a predisposition to the development of hypertension, rather than the protection actually observed (25). Other potential applications in NKCC2 physiology include understanding the effect of K\(^+\)-independent NaCl transport observed under hyposmotic conditions (5) on cell homeostasis as well as the magnitude and ion dependency of Na\(^+\) reabsorption (31).

Modeling NKCC2 transport at steady state also allowed us to study the efficiency of Na\(^+\) reabsorption, normalizing \(J_{\text{NKCC}}\) by \(J_{\text{Pump}}\). While the previously hypothesized reduction in NKCC2F-mediated Na\(^+\) reabsorption was suggested to reduce medullary O\(_2\) and nutrient consumption (16), simulations revealed that enhanced Na\(^+\) reabsorption in the NKCC2F oocyte was not accompanied by an increase in \(J_{\text{Pump}}\). Hence the functional advantage of increased steady-state reabsorption that NKCC2F expression might confer does not necessarily come at the price of increased energy expenditure or risk of ischemia. This provides some support for the idea that NKCC2F is indeed a medullary specialization in vivo.

In conclusion, we have developed a high-fidelity model of an untransfected denuded Xenopus oocyte. Employing a kinetic model of NKCC2, we have used the model to success-