Characterization of the thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter: a new model for ions and diuretics interaction

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The thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter (TSC) is the major pathway for salt reabsorption in the apical membrane of the mammalian distal convoluted tubule. When expressed in *Xenopus laevis* oocytes, rat TSC exhibits high affinity for both cotransported ions, with the Michaelis-Menten constant for Na\(^+\) of 8.1 ± 1.6 mM and for Cl\(^-\) of 6.3 ± 1.1 mM, and Hill coefficients for Na\(^+\) and Cl\(^-\) consistent with electroneutrality. The affinities of both Na\(^+\) and Cl\(^-\) were increased by increasing concentration of the counterion. The IC50 values for thiazides were affected by both extracellular Na\(^+\) and Cl\(^-\). The higher the Na\(^+\) or Cl\(^-\) concentration, the lower the inhibitory effect of thiazides. Finally, rTSC function is affected by extracellular osmolarity. We propose a transport model featuring a random order of binding in which the binding of each ion facilitates the binding of the counterion. Both ion binding sites alter thiazide-mediated inhibition of transport, indicating that the thiazide-binding site is either shared or modified by both Na\(^+\) and Cl\(^-\).

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The functional characteristics of TSC from the winter flounder urinary bladder have been studied in some detail (15), revealing that teleost TSC exhibits Km values for Na\(^+\) and Cl\(^-\) of 25.0 ± 0.4 and 13.5 ± 0.2 mM, respectively. Hill coefficients for both ions were close to unity, consistent with electroneutral cotransport. In addition, thiazide inhibition of flounder TSC function revealed a potency profile that was similar to...
that previously shown for inhibition of Cl\(^-\)-dependent Na\(^+\) absorption in the flounder urinary bladder [assessed as the short-circuit current (22)] and for thiazide competition for the high-affinity \(^{3}H\)metolazone binding site on rat kidney cortical membranes (2). In this study we present a functional characterization of the mammalian Na\(^+\)-Cl\(^-\) cotransporter, rTSC, as expressed in Xenopus laevis oocytes.

**METHODS**

**X. laevis oocytes preparation.** Adult female X. laevis frogs were purchased from two different vendors: Nasco (Fort Atkinson, WI) and Carolina Biological Supply (Burlington, NC). Oocytes were surgically harvested from anesthetized frogs under 0.17% tricaine and incubated during 1 h under vigorous shaking in ND-96 [(in mM) 96 NaCl, 2 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), and 5 HEPES/Tris, pH 7.4] in the presence of 2 mg/ml of collagenase B after which oocytes were washed four times in ND-96 and manually defolliculated. Oocytes were incubated overnight in ND-96 at 18°C supplemented with 2.5 mM sodium pyruvate and 5 mg/100 ml of gentamicin. Stage V-VI oocytes (8) were then injected with 50 nl of rTSC cRNA at a concentration of 0.5 cin. After injection, oocytes were incubated 3–4 days in ND-96 with sodium pyruvate and gentamicin. Stage V-VI oocytes (8) were then injected with 50 nl of water or rTSC cRNA at a concentration of 0.5 μg/μl or 25 ng cRNA per oocyte. After injection, oocytes were incubated 3–4 days in ND-96 with sodium pyruvate and gentamicin. The incubation medium was changed every 24 h. The day before the uptake experiments were performed, oocytes were switched to a Cl\(^-\)-free ND-96 [(in mM) 96 Na\(^+\)-isethionate, 2 K\(^+\)-gluconate, 1.8 Ca\(^{2+}\)-gluconate, 1.0 Mg\(^{2+}\)-gluconate, 5 HEPES, 2.5 sodium pyruvate, 5 mg% gentamicin, pH 7.4] for at least 12 h (15).

**In vitro rTSC cRNA translation.** To prepare the rTSC cRNA, rTSC cDNA in pSPORT1 (14) was linearized at the 3’ end by using Not I from Boehringer (Mannheim, Germany) and cRNA was transcribed in vitro, by using the T7 RNA polymerase mMESSAGE kit (Ambion). Transcription product integrity was confirmed on agarose gels, and concentration was determined by absorbance reading at 260 nm (DU 640, Beckman, Fullerton, CA). cRNA was stored frozen in aliquots at –80°C until used.

**Assessment of the Na\(^+\)-Cl\(^-\) cotransporter function.** Functional analysis of the Na\(^+\)-Cl\(^-\) cotransporter was assessed by measuring tracer \(^{22}\)Na\(^+\) uptake (New England Nuclear) in groups of at least 15 oocytes. \(^{22}\)Na\(^+\) uptake was measured by using the following protocol: a 30-min incubation period in an isotonic K\(^+\)- and Cl\(^-\)-free medium [(in mM) 96 Na\(^+\)-gluconate, 6.0 Ca\(^{2+}\)-gluconate, 1.0 Mg\(^{2+}\)-gluconate, 5 HEPES/Tris, pH 7.4] with 1 mM ouabain, 100 μM bumetanide, and 100 μM amiloride, followed by 60-min uptake period in a K\(^+\)-free isotonnic medium. For most experiments the isotonic medium contained (mM) 40 NaCl, 56 Na\(^+\)-isethionate, 5.6 Ca\(^{2+}\)-gluconate, 1.0 Mg\(^{2+}\)-gluconate, 6.0 HEPES, 5 Tris, pH 7.4), with 1 mM ouabain, 100 μM bumetanide, and 100 μM amiloride, and 2.5 μCi of \(^{22}\)Na\(^+\). Ouabain was added to prevent sodium exit via the Na\(^+\)-K\(^+\)-ATPase, bumetanide to inhibit the oocyte Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, and amiloride to block other Na\(^+\) pathways in the oocytes, such as the Na\(^+\)-H\(^+\) antiporter and Na\(^+\) channels.

To determine the ion transport kinetics and the order of ion binding to the Na\(^+\)-Cl\(^-\) cotransporter, we performed experiments using varying concentrations of Na\(^+\) and Cl\(^-\). To maintain osmolality and ionic strength, gluconate was used as a Cl\(^-\) substitute and NMDG as a Na\(^+\) substitute. The sensitivity and kinetics for thiazide-type diuretics were assessed by exposing groups of rTSC cRNA-injected oocytes to each diuretic at concentrations varying from 10\(^{-8}\) to 10\(^{-4}\) M. For these experiments, the desired concentration of the diuretic was present in both the incubation and uptake periods. In addition, concentration-dependent effect of thiazides on rTSC function was assessed by using uptake solutions containing different concentrations of extracellular Na\(^+\) or Cl\(^-\). Finally, uptake experiments were also performed by using three different osmolality conditions for the oocytes: hypotonic (110 mosmol/kgH\(_2\)O), isotonicity (210 mosmol/kgH\(_2\)O), and hypertonicity (305 mosmol/kgH\(_2\)O). All solutions for these experiments contained 40 mM NaCl, which resulted in an osmolarity of ~110. For the isotonic and hypertonic solutions, osmolarity was adjusted by adding 90 mM or 190 mM sucrose, respectively.

All uptakes were performed at 30°C. At the end of the uptake period, oocytes were washed 5 times in ice-cold up-

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Fig. 1. Functional expression of rat thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter (rTSC) in Xenopus laevis oocytes that were injected with water or with rTSC cRNA (c). Each circle represents the mean ± SE of 15 oocytes. Uptake at 30 min in rTSC-injected oocytes was thiazide sensitive (data not shown).

Fig. 2. Time course over 30 min of Na\(^+\) uptake in X. laevis oocytes injected with water (●) or with rTSC cRNA (○). Each circle represents the mean ± SE of 15 oocytes. Uptake at 30 min in rTSC-injected oocytes was thiazide sensitive (data not shown).
Expression of rTSC in *X. laevis* oocytes. We have previously shown that *X. laevis* oocytes do not exhibit endogenous expression of the thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter (14, 15). Figure 1 shows a summary from six experiments of rTSC expression in oocytes. Na\(^+\) uptake increased from a level of 157 ± 10 pmol oocyte\(^{-1}\) h\(^{-1}\) in water-injected oocytes to a value of 7,555 ± 228 pmol oocyte\(^{-1}\) h\(^{-1}\) in rTSC cRNA-injected oocytes. Thus injection of *X. laevis* oocytes with rTSC cRNA resulted in an average 48-fold increase in Na\(^+\) uptake (range 40 to 150-fold in different experiments). The increased Na\(^+\) uptake was chloride dependent and thiazide sensitive, with uptake in the absence of extracellular Cl\(^-\) being 709 ± 114 pmol oocyte\(^{-1}\) h\(^{-1}\) and in the presence of 10\(^{-4}\) M metolazone being 1,039 ± 118 pmol oocyte\(^{-1}\) h\(^{-1}\). Control uptake in water-injected oocytes collected from different frogs varied from 70 ± 24 to 450 ± 34 pmol oocyte\(^{-1}\) h\(^{-1}\) but was always insensitive to thiazides.

Figure 2 shows that Na\(^+\) uptake in water-injected oocytes was small and linear during 30 min of uptake. In rTSC cRNA-injected oocytes, the uptake increased rapidly and was also linear during the first 30 min. Thus we used a 60-min uptake period for all experiments, except when evaluating ion kinetic analyses where 15-min uptakes were performed.

Kinetics of ion binding in rTSC. Figure 3 shows the Na\(^+\) dependency (Fig. 3A) and Cl\(^-\) dependency (Fig. 3B) of Na\(^+\) uptake in rTSC cRNA-injected oocytes. Uptakes were performed with a fixed concentration of Na\(^+\) or Cl\(^-\) at 40 mM, with changing concentrations of counterion from 0 to 40 mM. Uptakes were also measured in water-injected oocytes (data not shown), and mean values for water groups were subtracted in corresponding rTSC groups to analyze only Na\(^+\) uptake due to rTSC. As shown in Fig. 1, Na\(^+\) uptake in H\(_2\)O-injected oocytes is low, making this latter correction small. Lines were fit using Michaelis-Menten equation. The Hill coefficient for Na\(^+\) and Cl\(^-\) was close to unity: 1.04 ± 0.16 and 1.07 ± 0.14 for Na\(^+\) and Cl\(^-\), respectively. [Na\(^+\)]\(_e\) and [Cl\(^-\)]\(_e\), extracellular Na\(^+\) and Cl\(^-\) concentrations, respectively.

Table 1. *rTSC* Cl\(^-\) apparent *K*\(_m\) and *V*\(_{max}\) in oocytes exposed to increased extracellular Na\(^+\) concentration

<table>
<thead>
<tr>
<th>Extracellular Na(^+) Concentration, mM</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K</em>(_m), mM</td>
<td>21.21 ± 0.4</td>
<td>17.05 ± 4.8</td>
<td>18.68 ± 3.5</td>
<td>8.46 ± 1.7</td>
<td>6.46 ± 1.7</td>
</tr>
<tr>
<td><em>V</em>(_{max}), pmol oocyte(^{-1}) h(^{-1})</td>
<td>1,220 ± 188</td>
<td>2,125 ± 288</td>
<td>3,206 ± 293</td>
<td>2,868 ± 215</td>
<td>3,539 ± 317</td>
</tr>
</tbody>
</table>

Values are means ± SE. rTSC, rat thiazide-sensitive Na\(^+\)-Cl\(^-\) cotransporter; *K*\(_m\), Michaelis-Menten constant; *V*\(_{max}\), maximal velocity.
1.54 mM and 3,542 ± 286 pmol·oocyte⁻¹·h⁻¹, respectively. The Hill coefficient for both ions remained close to unity: 1.04 ± 0.17 and 1.07 ± 0.14 for Na⁺ and Cl⁻, respectively.

To test whether extracellular Na⁺ and Cl⁻ concentrations influence the binding of the counterion, we examined the counterion concentration dependency of Na⁺ uptakes at varying fixed concentrations of extracellular Na⁺ or Cl⁻. Tables 1 and 2 show the results of these experiments as the kinetic parameters (Kₘ and Vₘₐₓ) for Cl⁻ or Na⁺, respectively. Table 1 shows that the apparent Kₘ for Cl⁻ was significantly affected by extracellular Na⁺ concentration. The apparent Kₘ for Cl⁻ increased from 6.46 ± 1.7 to 21.2 ± 0.4 mM (P < 0.01) when extracellular Na⁺ decreased from 40 to 2 mM. Thus the higher the sodium concentration, the higher the affinity of rTSC for extracellular Cl⁻. Similarly, the Kₘ for Na⁺ also was affected by extracellular Cl⁻. As Table 2 shows, Kₘ for extracellular Na⁺ varied from 7.26 ± 2.4 to 41.9 ± 6.9 mM (P < 0.01), when extracellular Cl⁻ concentration varied from 40 to 2 mM. Thus the higher the chloride concentration, the higher the affinity of rTSC for Na⁺.

**Kinetics of thiazide inhibition of rTSC.** Thiazide-induced inhibition of rTSC has been considered for years as the hallmark of the Na⁺-Cl⁻ cotransporter that is expressed in the apical membrane of the mammalian DCT and the teleost urinary bladder. Thus we analyzed the inhibitory kinetics of several thiazide-type diuretics on rTSC cRNA-injected-oocytes. The results of this series of experiments are shown in Fig. 4. The rank of order for rTSC inhibition was polythiazide > metolazone = bendroflumethiazide > trichlormethiazide > hydrochlorothiazide > chlorothalidone (chlorthalidone not shown). rTSC function was not affected by the addition of a non-diuretic thiazide derivative such as diazoxide, tested in concentrations from 10⁻¹⁴ to 10⁻⁴ M in the uptake medium (data not shown). In addition, rTSC function was not inhibited by furosemide or acetazolamide (data not shown).

**Effect of pH on rTSC function and thiazide inhibition.** Table 3 shows that there is no effect of extracellular pH in the range of 6.0 to 8.0 on both rTSC function and thiazide sensitivity. Uptakes were performed in solutions containing 40 mM NaCl, with pH of 6.0, 6.5, 7.0, 7.5, and 8.0. As Table 3 shows, all groups exhibited similar uptakes. In addition, we tested two different thiazides: metolazone at a concentration of 5 × 10⁻⁶ M that is just above IC₅₀ and bendroflumethiazide at 5 × 10⁻⁷ M that is just below IC₅₀. In these experiments, pH had no effect on metolazone or bendroflumethiazide rTSC inhibition of Na⁺ uptake (not significant by using one-way ANOVA).

**Effects of extracellular ions on thiazide inhibition of rTSC.** Tran et al. (30) observed that binding of tracer [³H]metolazone to its putative receptor was inhibited by increased Cl⁻ and stimulated by increased Na⁺ concentrations. They proposed that thiazides and Cl⁻ competed for the same site or at least for part of the same binding site on the protein. To examine this issue at a functional level, we evaluated the effect of extracellular Cl⁻ on the kinetics of inhibition of several thiazides. To this end, we assessed kinetics of rTSC inhibition of five different thiazide-type diuretics, in the presence of 2 or 100 mM extracellular Cl⁻. We used 2 mM Cl⁻ because this concentration is clearly below

<table>
<thead>
<tr>
<th>Extracellular Cl⁻ Concentration, mM</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>10</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Kₘ, mM]</td>
<td>41.9 ± 6.9</td>
<td>50.88 ± 15.7</td>
<td>21.26 ± 8.4</td>
<td>12.11 ± 1.4</td>
<td>7.46 ± 1.0</td>
<td>7.26 ± 2.4</td>
</tr>
<tr>
<td>[Vₘₐₓ, pmol·oocyte⁻¹·h⁻¹]</td>
<td>1,736 ± 175</td>
<td>2,867 ± 570</td>
<td>3,253 ± 629</td>
<td>2,693 ± 131</td>
<td>2,862 ± 135</td>
<td>3,570 ± 400</td>
</tr>
</tbody>
</table>

Values are means ± SE.

Fig. 4. Kinetic analyses of inhibition of rTSC function by thiazide-type diuretics. All Na⁺ uptakes were preformed during 60 min with thiazides tested at concentrations from 10⁻⁴ to 10⁻¹ M. The profile of inhibition was polythiazide (□), > metolazone (●) = bendroflumethiazide (○) > trichlormethiazide (□) > hydrochlorothiazide (●). Uptakes were performed during 60 min in uptake solution containing 40 mM Na⁺ and 96 mM Cl⁻.

Table 3. **Effect of pH on the thiazide sensitivity of rTSC**

<table>
<thead>
<tr>
<th>pH</th>
<th>rTSC Function [% of Inhibition by metolazone (5 × 10⁻⁶ M)]</th>
<th>[rTSC Function [% of Inhibition by metolazone (5 × 10⁻⁶ M)]</th>
<th>[rTSC Function [% of Inhibition by metolazone (5 × 10⁻⁶ M)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>5,599 ± 293</td>
<td>71.36 ± 3.04</td>
<td>32.46 ± 6.11</td>
</tr>
<tr>
<td>6.5</td>
<td>5,348 ± 357</td>
<td>57.07 ± 7.71</td>
<td>33.10 ± 6.86</td>
</tr>
<tr>
<td>7.0</td>
<td>6,318 ± 573</td>
<td>79.15 ± 1.38</td>
<td>30.59 ± 5.51</td>
</tr>
<tr>
<td>7.5</td>
<td>5,376 ± 500</td>
<td>66.79 ± 5.00</td>
<td>27.42 ± 4.99</td>
</tr>
<tr>
<td>8.0</td>
<td>6,422 ± 225</td>
<td>74.07 ± 6.22</td>
<td>21.48 ± 3.54</td>
</tr>
</tbody>
</table>

Values are means ± SE.
the apparent $K_m$ for Cl$^-$ (Figs. 3 and 4). The results of these series of experiments are shown in Fig. 5 and Table 4. It is clear that the affinity of rTSC for each thiazide is shifted to the left in the presence of a lower extracellular Cl$^-$ concentration, indicating that Cl$^-$ affects the binding of thiazide diuretics to rTSC. We also assessed the effect of extracellular Na$^+$ on inhibition of rTSC by metolazone. As illustrated in Fig. 6, the IC$_{50}$ was shifted to the left as Na$^+$ concentration decreased in the uptake medium. When Na$^+$ concentration was 2 mM, the IC$_{50}$ was $3 \times 10^{-7}$ M, whereas in the presence of 100 mM Na$^+$, the IC$_{50}$ was $2 \times 10^{-6}$ M. Thus the concentration of Na$^+$ in extracellular fluid also influences the inhibition of rTSC function by metolazone.

To further examine the effect of ions on thiazide inhibition of rTSC function, we assessed the effect of increased concentrations of extracellular Na$^+$ or Cl$^-$ on the inhibitory effect of metolazone at a concentration of $5 \times 10^{-7}$ M, the IC$_{50}$ of this diuretic. For these experiments, all solutions had the same osmolarity (~210 mosmol/kgH$_2$O), as well as ionic strength. As Fig. 7 shows, there is a significant negative correlation between both extracellular Na$^+$ ($r^2 = 0.82, P < 0.0001$) or Cl$^-$ concentration and rTSC function.

Table 4. IC$_{50}$ of each thiazide at 2 or 100 mM Cl$^-$ concentration.

<table>
<thead>
<tr>
<th>Thiazide</th>
<th>Cl$^-$ 2 mM</th>
<th>Cl$^-$ 100 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metolazone</td>
<td>$3.5 \times 10^{-7}$ M</td>
<td>$2.5 \times 10^{-6}$ M</td>
</tr>
<tr>
<td>Bendroflumethiazide</td>
<td>$5.0 \times 10^{-7}$ M</td>
<td>$2.5 \times 10^{-6}$ M</td>
</tr>
<tr>
<td>Trichloromethiazide</td>
<td>$5.0 \times 10^{-7}$ M</td>
<td>$5.0 \times 10^{-6}$ M</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>$7.0 \times 10^{-8}$ M</td>
<td>$7.0 \times 10^{-7}$ M</td>
</tr>
<tr>
<td>Polythiazide</td>
<td>$2.0 \times 10^{-8}$ M</td>
<td>$3.5 \times 10^{-7}$ M</td>
</tr>
</tbody>
</table>
Cl\(^{-}\) \((r^2 = 0.80, P < 0.001)\) and the percentage of rTSC inhibition by metolazone.

**Regulation of rTSC by osmolarity.** The Na-Cl cotransporter, rTSC, is highly expressed in the apical membranes of the DCT (24, 26). In this nephron segment, the tubular fluid arriving from the medullary thick ascending limb can vary in osmolarity from hypotonic to isotonic. Accordingly, we studied the effect of osmolarity on the transport function of rTSC. The Cl\(^{-}\)-dependent fraction and the thiazide-sensitive fraction of Na\(^{+}\) uptake was assessed in rTSC-injected oocytes that were exposed to an uptake medium containing 40 mM NaCl at three different osmolarities: hypotonic (\(<110\) mosmol/kgH\(_2\)O), the osmolarity obtained by the 40 mM NaCl concentration in the uptake medium; and isotonic (\(<205\) mosmol/kgH\(_2\)O) or hyper-tonic (\(<310\) mosmol/kgH\(_2\)O) by adding sucrose to the 40 mM NaCl uptake medium. Thus uptakes were performed in different osmolar conditions, without changing the extracellular NaCl concentration or ionic strength. Figure 8 shows a representative experiment. Compared with the amount of Cl\(^{-}\)-dependent or thiazide-sensitive Na\(^{+}\) uptake that was observed in hypotonicity, rTSC function was increased by isotonicity. No further activation was seen with the hypertonic uptake medium.

**DISCUSSION**

In the present study we have functionally characterized the rat thiazide-sensitive Na\(^{+}\)-Cl\(^{-}\) cotransporter, rTSC. As shown previously (14), rTSC gives rise to thiazide-sensitive Na\(^{+}\)-Cl\(^{-}\) cotransport when expressed in *X. laevis* oocytes. The kinetic analyses for both ions reveal that rTSC exhibits very high affinities for Na\(^{+}\) and Cl\(^{-}\). Hill coefficients for each ion were unity, consistent with a stoichiometry of 1Na:1Cl and the electroneutral nature of the cotransport process. The *K*\(_{m}\) values for both ions were < 8 mM. These ion affinities are somewhat higher than those obtained for the Na-Cl cotransporter from winter flounder urinary bladder [*K*\(_{m}\) for Na\(^{+}\) and Cl\(^{-}\) were \(=25\) and 13 mM, respectively (15)]. The large central hydrophobic domain containing the 12 transmembrane segments of the related Na-K-2Cl cotransporter has been shown to determine the diuretic [in this case bumetanide] and ion binding (20). Because rat and flounder TSC exhibit \(~80\%\) amino acid identity in this central domain, it seems likely that small changes in TSC sequences in this region account for the kinetic differences between these two TSC proteins.

In the DCT, urinary fluid arrives from the loop of Henle with NaCl concentrations and osmolarities that are usually significantly lower than in plasma, due to the intense reabsorption of ions, without water, in the thick ascending limb. Thus, to maintain an appropriate rate of salt reabsorption, the apically expressed Na-Cl cotransporter in the DCT must have very high affinities for both cotransported ions. The high Na\(^{+}\) and Cl\(^{-}\) affinities of \(~8\) mM for rTSC are consistent with this model and with the previous observations by Velázquez and coworkers (31) using
in vivo microperfusion experiments in rat DCT in which they found half-maximal stimulation of salt transport at Na\(^+\) or Cl\(^-\) concentrations of \(\sim 10 \text{ mM}\). In addition, any reduction of ion reabsorption in the thick ascending limb or increased flow out of the loop of Henle will result in increased salt (and osmolarity) delivery to DCT. In this latter circumstance, the rate of NaCl transport has been shown to be directly related to the rate of NaCl delivery to the DCT. In this regard, we found in the present study that NaCl transport by TSC was regulated by osmolarity, independently of changes in NaCl concentration or ionic strength, at least in oocytes. Thus it is possible that osmotic-induced activation of TSC could be one of the mechanisms that account for the known immediate increase in NaCl reabsorption rate in DCT when a loop diuretic is administered (4).

Our functional study confirms the predictions made by Tran et al. (30) using \[^{3}H\]metolazone binding analysis to renal cortical membranes and by Chang and Fujita (5) using a recently developed computer-based program that TSC possesses two binding sites: one selective for Na\(^+\) and another for Cl\(^-\). However, in contrast to their findings, we observed that the affinity of the cotransporter for Na\(^+\) or Cl\(^-\) is clearly affected by the concentration of the counterion in the uptake medium. The \(K_m\) for Na\(^+\) is affected by Cl\(^-\) and the apparent \(K_m\) for Cl\(^-\) is affected by extracellular Na\(^+\). The higher the counterion concentration, the higher the Na\(^+\) or Cl\(^-\) affinity of the cotransporter. We suggest that these results indicate that the order of binding for Na\(^+\) and Cl\(^-\) to the cotransporter is random. As shown in the appendix, to analyze the order of binding of both ions to the cotransporter we followed the “rapid equilibrium” approach suggested by Segel (28).

According to this model, in random bireactant systems, when \(\alpha = 1\), one ion has no effect on the binding of the other and the apparent \(K_m\) (\(K_{app}\)) is held constant as the counterion concentration increases; when \(\alpha > 1\) the binding of one ion decreases the affinity for the second ion and the \(K_{app}\) increases as the concentration of counterion increases, and when \(\alpha < 1\) the binding of one ion increases the affinity of the cotransporter for the counterion, and the \(K_{app}\) for the varied ion decreases as the concentration of the fixed ion increases. Thus the observed mutual effects of Na\(^+\) and Cl\(^-\) concentration on \(K_{app}\) and \(V_{max}\) in our results are characteristic for random binding of these ions with an \(\alpha < 1\). If the ion binding were ordered, with Na\(^+\) binding first, then we would expect that the \(V_{max}\) for chloride remains unaffected. As Table 1 and 2 show, the \(V_{max}\) for both ions was affected by the concentration of the counterion.

The binding of the thiazide-like compound \[^{3}H\]metolazone exclusively to cellular membranes from renal cortex has been used for years as a surrogate to study changes in cotransporter expression with modulation of physiological conditions and during different pathophysiological states (1, 3, 6, 11–13). Our results show that TSC function is inhibited by several different thiazides with an inhibitory profile similar to their effect in clinical medicine, as well as their potency to block the \[^{3}H\]metolazone binding to renal cortical membranes (2). In addition, the benzothiadiazine derivative and vasodilator drug diazoxide, which causes vasodilation but does not cause diuresis, possesses no inhibitory effect on TSC function.

Our data show that the concentration of Na\(^+\) as well as Cl\(^-\) in the extracellular medium affects the affinity of TSC for thiazides. The higher the concentration of both ions, the lower the thiazide-induced inhibition of TSC function. For example, the IC\(_{50}\) for metolazone inhibition of TSC was shifted by one order of magnitude to the left when either Cl\(^-\) (Fig. 5) or Na\(^+\) (Fig. 6) in the extracellular uptake medium was decreased from 100 to 2 mM. As shown in Fig. 7, the higher the Na\(^+\) or Cl\(^-\) concentration, the lower the TSC inhibition by thiazides. On the one hand, our findings are in agreement with the observations of Tran and co-workers (30), who found an inhibitory effect of Cl\(^-\) on thiazide binding to renal cortical membranes, but otherwise diverge from their finding of a stimulatory effect of Na\(^+\) on thiazide binding. We show that both Na\(^+\) and Cl\(^-\) decrease the inhibitory potency of thiazides on TSC, whereas Tran and co-workers found that raising the Na\(^+\) concentration increases the binding of thiazides to plasma membranes from renal cortex (5, 30). Thus, regarding the Na\(^+\) and thiazide interactions, our result appears to be inconsistent with those of Tran et al. (30).

Differences between predictions based on tracer diuretic binding to plasma membranes and analysis of the cotransporter function have been shown to occur. It was predicted by studies using \[^{3}H\]bumetanide binding to renal outer medulla membranes that, in the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, bumetanide binds to the second Cl\(^-\) site (17). However, recent studies of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporters using chimeras and point mutations demonstrate that altering the second transmembrane domain affects Na\(^+\), K\(^+\), and bumetanide kinetics, but not Cl\(^-\) kinetics (18–20). If bumetanide and Cl\(^-\) bind to the same site on the protein, the kinetics of both should be affected by the same alterations in amino acid sequence. Thus bumetanide binding to the Na-K-2Cl cotransporter appears to be distinct from the Cl\(^-\)-binding site.

Finally, on the basis of our data, we propose a modification of the presently accepted TSC model for NaCl transport and inhibition by thiazides. In this revised Na-Cl cotransporter model, either ion can first (and presumably randomly) bind to the transporter, but the binding of this first ion affects the binding affinity of the second ion (or counterion). In other words, the occupancy of either ion binding site increases the probability for occupancy of the other one. Moreover, both ion binding sites alter thiazide-mediated inhibition of transport, indicating that the thiazide binding site is either shared or modified by both Na\(^+\) and Cl\(^-\).
APPENDIX

To determine the order of ion binding to the thiazide-sensitive sodium-chloride cotransporter we used the rapid equilibrium approach by Segel (28). For a random binding assumption we have

\[
\begin{align*}
T + Na^+ & \xrightleftharpoons{Kv Na^+} \text{TNa}^+ \\
\text{Cl}^- & \xrightleftharpoons{Kv Cl^-} \text{Cl}^-
\end{align*}
\]

where \( T \) is transporter, \( v \) is the velocity, \( K_{Na^+} \) and \( K_{Cl^-} \) are the dissociation constants for \( Na^+ \) and \( Cl^- \), respectively, and \( \alpha \) is the factor by which the dissociation constant of one ion is modified by the binding of the other ion to the transporter. \( V_{max} \) is defined as the product of the rate constant, \( K_v \), and the sum of all states of the transport molecule.

If \( \alpha < 1 \) (the binding of one ion increases the affinity of the cotransporter for the counterion), the \( V_{max} \) for the varied ion decreases as the concentration of the fixed ion increases. If \( \alpha = 1 \) (one ion has no effect on the binding of the other), the \( V_{max} \) is equal to the \( K \) for the ion and there are not changes with the variation of the counterion.

If \( \alpha > 1 \) (the binding of one ion decreases the affinity for the second ion), in this case the apparent \( K \) value for the varied ion increases as the concentration of fixed ion increases.

The rapid equilibrium approach for an ordered binding if \( Na^+ \) binds first yields

\[
\begin{align*}
T + Na^+ & \xrightleftharpoons{Kv Na^+} \text{TNa}^+ \\
\text{Cl}^- & \xrightleftharpoons{Kv Cl^-} \text{Cl}^- \\
\text{TNa}^+ Cl^- & \xrightleftharpoons{K_v} T + Na^+ + Cl^- \\
K_{Cl^- app} & = K_{Cl^-} \left( 1 + \frac{K_{Na^+}}{[Na^+]} \right) \\
V_{max app} & = \frac{V_{max}}{1 + \frac{K_{Na^+}}{[Na^+]} + [Cl^-]}
\end{align*}
\]

where \( T \) and \( v \) are the transporter and velocity, \( K_{Na^+} \) and \( K_{Cl^-} \) are the dissociation constants for \( Na^+ \) and \( Cl^- \), respectively, and \( \alpha \) is the factor by which the dissociation constant of one ion is modified by the binding of the other ion to the transporter. \( V_{max} \) is defined as the product of the rate constant, \( K_v \), and the sum of all states of the transport molecule.

The apparent \( K \) for \( Cl^- \) varies with varying concentrations of \( Na^+ \) and the \( V_{max} \) remains unaffected by the sodium concentration.

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