Functional differences between flounder and rat thiazide-sensitive Na-Cl cotransporter

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Vázquez, Norma, Adriana Monroy, Elisa Dorantes, Rosario A. Muñoz-Clares, and Gerardo Gamba. Functional differences between flounder and rat thiazide-sensitive Na-Cl cotransporter. Am J Physiol Renal Physiol 282: F599–F607, 2002.—The purpose of the present study was to determine the major functional, pharmacological, and regulatory properties of the flounder thiazide-sensitive Na-Cl cotransporter (fTSC) to make a direct comparison with our recent characterization of the rat TSC (rTSC; Monroy A, Plata C, Hebert SC, and Gamba G. Am J Physiol Renal Physiol 279: F161–F169, 2000). When expressed in Xenopus laevis oocytes, fTSC exhibits lower affinity for Na+ than for Cl−, with apparent Michaelis-Menten constant (Km) values of 58.2 ± 7.1 and 22.1 ± 4.2 mM, respectively. These Km values are significantly higher than those observed in rTSC. The Na+ and Cl− affinities decreased when the concentration of the counterion was lowered, suggesting that the binding of one ion increases the affinity of the transporter for the other. The effect of several thiazides on fTSC function was biphasic. Low concentrations of thiazides (10−9 to 10−7 M) resulted in activation of the cotransporter, whereas higher concentrations (10−6 to 10−4 M) were inhibitory. In rTSC, this biphasic effect was observed only with chlorthalidone. The affinity for thiazides in fTSC was lower than in rTSC, but the affinity in fTSC was not affected by the Na+ or the Cl− concentration in the uptake medium. In addition to thiazides, fTSC and rTSC were inhibited by Hg2+, with an apparent higher affinity for rTSC. Finally, fTSC function was decreased by activation of protein kinase C with phorbol esters and by hypertonicity. In summary, we have found significant regulatory, kinetic, and pharmacological differences between fTSC and rTSC orthologues.

distal tubule; osmolarity; salt reabsorption; thiazide-sensitive sodium-chloride cotransporter

EARLY WORK IN THE MARINE TELOSTS

The existence of an active and interdependent pathway for Na+ and Cl− absorption in the apical membrane (36, 37), together with localization of the Na+–K+–ATPase in the basolateral membrane (38). Thus the model suggested for salt reabsorption in the bladder included an apical Na-Cl cotransporter. Years later, Stokes et al. (41) reported clear evidence that the Na-Cl cotransporter in the apical membrane of the winter flounder urinary bladder was inhibited by the thiazide-type diuretics hydrochlorothiazide and metolazone, and Ziyadeh et al. (47) observed that inhibition of this cotransporter in the urinary bladder with thiazides was followed by an increase in Ca2+ absorption. Therefore, ion reabsorption mechanisms in the teleost urinary bladder and mammalian distal convoluted tubule (DCT) are similar. We thus took advantage of this similarity to clone a cDNA that encodes the flounder thiazide-sensitive Na-Cl cotransporter (fTSC), using a functional expression strategy in Xenopus laevis oocytes (11), followed by the identification of cDNA in the mammalian TSC (rTSC) with a homology-based approach (10, 20, 40).

TSC belongs to the superfamily of electroneutral cation-coupled Cl− cotransporters from which eight genes have been identified: two that encode the bumetanide-sensitive Na-K-2Cl cotransporter (10, 45); one that encodes the thiazide-sensitive Na-Cl cotransporter (10); four genes that encode the K-Cl cotransporters (13, 29, 32); and one gene that encodes a membrane protein with a function that is still unknown (1). In humans, the gene of the thiazide-sensitive Na-Cl cotransporter is localized in chromosome 16. Several mutations in TSC have been associated with an autosomal recessive disease featuring hypokalemic metabolic alkalosis, chronic arterial hypotension, hypomagnesemia, and hypocalciuria, known as Gitel-
man’s syndrome (40), and some of these mutations have been shown to preclude the protein to be functional due to disruption of the adequate intracellular processing of the transporter (20).

Little is known about the structure-function relationships of the thiazide-sensitive cotransporter. The identity between the human and shark basolateral Na-K-2Cl cotransporters is ~74%, and the ion and bumetanide inhibition kinetics are very different. For instance, the affinity for the three cotransported ions and for bumetanide of the shark’s Na-K-2Cl cotransporter is remarkably lower than the affinity of the human cotransporter. The similar structure but different functional properties between species allowed Isenring et al. (16, 17) to construct and guide experiments using chimeric clones between the human and shark proteins that have been useful in finding out the membrane-spanning domains that determine the ions and bumetanide affinities. Similarly, rTSC and fTSC also show a high degree of homology. The identity is 62% at the amino acid level, plus 10% of conservative changes. Moreover, comparison of our recent analysis of the functional and pharmacological properties of rTSC protein as expressed in *X. laevis* oocytes (28) with the initial characterization of fTSC (11) suggested that important differences in TSC functional properties could occur among species. Thus the major goal of the present study was to determine the major functional properties of fTSC, also expressed in *X. laevis* oocytes, to make a direct comparison with rTSC functional properties. In addition, in the present study we extended our previous characterization of rTSC. As a result, here we show important functional, pharmacological, and regulatory differences between rTSC and fTSC, which, together with our present knowledge of the primary structure of both proteins, can be used to begin to explore the structure-function relationships of this cotransporter.

**METHODS**

*X. laevis* oocyte preparation. Oocytes surgically harvested from adult female *X. laevis* frogs (Nasco, Fort Atkinson, MI) anesthetized by immersion in 0.17% tricaine were incubated for 1 h under vigorous shaking in frog Ringer-ND-96 [(in mM) 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPES/Tris, pH 7.4] in the presence of 2 mg/ml of collagenase. Then, oocytes were washed four times in ND-96, manually defolliculated, and incubated at 18°C overnight in ND-96 with 2.5 mM sodium pyruvate and 5 mg/100 ml of gentamicin. The next day, the mature oocytes (stages V-VI (7)) were injected with 50 nl of a 1 mM Tris solution containing either fTSC or rTSC cRNA at a concentration of 0.5 μg/μl. Control oocytes were injected with the same solution without cRNA. Oocytes were incubated for 3–4 days in ND-96 with sodium pyruvate and gentamicin. The incubation medium was changed every 24 h. The night before uptake experiments, oocytes were incubated in Cl⁻-free ND-96 [(in mM) 96 Na⁺-isethionate, 2 K⁺-gluconate, 1.8 Ca²⁺-gluconate, 1.0 Mg²⁺-gluconate, 5 mM HEPES, and 2.5 sodium pyruvate, plus 5 mg% gentamicin, pH 7.4] (10).

In vitro fTSC or rTSC cRNA translation. fTSC or rTSC cDNAs were linearized at the 3’-end using NotI (Life Technologies, Bethesda, MD), and cRNA was transcribed in vitro, using a T7 RNA polymerase mMESSAGE kit (Ambion). Transcription product integrity was confirmed on agarose gels, and concentration was determined by an absorbance reading at 260 nm (DU 640, Beckman, Fullerton, CA). cRNA was stored frozen in aliquots at ~80°C until use.

**Assessment of the Na-Cl cotransporter function.** Functional analysis of the Na-Cl cotransporter was assessed by measuring the uptake of tracer 22Na⁺ (New England Nuclear) in groups of at least 15 oocytes, using our previously published protocol (28). In brief, oocytes were exposed to a 30-min incubation period in an isotonic K⁺-and Cl⁻-free medium [(in mM) 96 Na⁺-gluconate, 6.0 Ca²⁺-gluconate, 1.0 Mg²⁺-gluconate, 5 HEPES/Tris, pH 7.4] with 1 mM ouabain, 100 μM bumetanide, and 100 μM amiloride, followed by a 60-min uptake period in a K⁺-free isotonic medium. For most experiments, the isotonic medium contained (in mM) 96 NaCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPES, pH 7.4, supplemented with 1 mM ouabain, 100 μM bumetanide, and 100 μM amiloride, and 2.0 μCi of 22Na⁺. All uptakes were performed at 30°C. At the end of the uptake period, oocytes were washed five times in ice-cold uptake solution without isotopes to remove extracellular fluid tracer. After the oocytes were dissolved in 10% sodium dodecyl sulfate, tracer activity was determined for each oocyte by β-scintillation counting.

To determine ion transport kinetics, uptake experiments were performed with varying concentrations of Na⁺ and Cl⁻. N-methyl-D-glucamine was used as a Na⁺ substitute and gluconate as a Cl⁻ substitute to maintain osmolality and ionic strength. The sensitivity and effect of thiazide-type diuretics were assessed by exposing groups of rTSC or fTSC cRNA-injected oocytes to each diuretic at concentrations varying from 10⁻³ to 10⁻⁴ M. The diuretic was present in both the incubation and the uptake periods. In addition, the concentration-dependent effect of thiazides on fTSC function was assessed using uptake solutions containing different concentrations of extracellular Na⁺ or Cl⁻. The effect of second messengers was also tested by adding the phorbol O-tetradecanoylphorbol 13-acetate (TPA), dibutyryl-cGMP, dibutyryl-cAMP, or IBMX only during the incubation period, after which oocytes were washed in uptake medium and exposed to Na⁺ uptake under conditions identical to those of the control group. Finally, uptake experiments were also performed using three different osmolality conditions for the oocytes: hypotonicity (110 mosmol/kgH₂O), isosmotic (210 mosmol/kgH₂O), and hypertonicity (310 mosmol/kgH₂O). All solutions for these experiments contained 40 mM NaCl or 40 mM Na₂-gluconate, which resulted in an osmolality of ~110 mosmol/kgH₂O. For the isotonic and hypertonic solutions, osmolality was adjusted by adding 90 or 190 mM sucrose, respectively.

**Statistical analysis.** Kinetic parameters were estimated by nonlinear regression fit of the uptake rate data to the Michaelis-Menten (Kₘ) equation. Statistical significance was defined as two-tailed *P* < 0.05, and the results are presented as means ± SE. The significance of the differences between groups was tested by one-way ANOVA with multiple comparison using Bonferroni correction or by Kruskal-Wallis one-way ANOVA on ranks with Dunn’s method for multiple comparison procedures, as needed.

**RESULTS**

Figure 1 depicts a representative experiment showing that water-injected *X. laevis* oocytes do not express a thiazide-sensitive Na-Cl pathway (10, 28), whereas oocytes injected with fTSC cRNA exhibited an increased 22Na⁺ uptake that was sensitive to the thia-
zide-type diuretic metolazone and was completely abolished in the absence of extracellular Cl\(^-\). As shown in the inset in Fig. 1, the increased \(^{22}\text{Na}\) uptake in ftTSC cRNA-injected oocytes was linear, at least during the first 15 min of exposure to the tracer Na\(^+\).

Figure 2 shows the \(^{22}\text{Na}\) uptake in ftTSC-injected oocytes exposed to different extracellular pH values, in a range from 6.0 to 8.0. For this experiment, oocytes were exposed to the tracer Na\(^+\) in uptake media containing 80 mM NaCl, titrated to pH of 6.0, 6.5, 7.0, 7.5, and 8.0, with HCl or NaOH, respectively. The uptake rate, the degree of Cl\(^-\) dependency, and the sensitivity to the thiazide-type diuretic metolazone were similar in all groups (not significant using 1-way ANOVA), suggesting that extracellular pH had no effect on ftTSC function or on metolazone-induced inhibition of the cotransporter.

Kinetic properties of ftTSC. To determine the kinetic properties of ftTSC, we assessed \(^{22}\text{Na}\) uptake in ftTSC cRNA-injected oocytes as a function of the concentration of each transported ion. The results of these experiments are shown in Fig. 3, A and B. Uptake experiments were performed with Na\(^+\) or Cl\(^-\) fixed at 96 mM and changing concentrations of the counterion from 0 to 80 or 96 mM. The small mean values of uptake for water-injected oocytes (data not shown) were subtracted from those corresponding to ftTSC cRNA-injected groups. \(^{22}\text{Na}\) influx in ftTSC showed saturation kinetics, with estimated \(K_m\) and maximal velocity (\(V_{max}\)) values for extracellular Na\(^+\) of 58.2 ± 7.1 mM and 33,670 ± 2,239 pmol-oocyte\(^{-1}\)·h\(^{-1}\), respectively (Fig. 3A), and for extracellular Cl\(^-\) of 22.1 ± 4.2 mM and 25,820 ± 2,022 pmol-oocyte\(^{-1}\)·h\(^{-1}\), respectively (Fig. 3B).

To determine whether extracellular Na\(^+\) and/or Cl\(^-\) concentrations influence the binding of the counterion, we again evaluated the kinetics parameters for Na\(^+\) and Cl\(^-\), but in the presence of a very low concentration of the counterion. Figure 3, C and D, shows the results of these experiments for Na\(^+\) and Cl\(^-\), respectively. The \(K_m\) and \(V_{max}\) values for Na\(^+\) in the presence of 10 mM extracellular Cl\(^-\) were 118.3 ± 74.1 mM and 12,630 ± 4,948 pmol-oocyte\(^{-1}\)·h\(^{-1}\), respectively, and those for Cl\(^-\) in the presence of 20 mM Na\(^+\) were 46.2 ± 15.1 mM and 8,439 ± 1,208 pmol-oocyte\(^{-1}\)·h\(^{-1}\), respectively. Therefore, when the counterion concentration was fixed at a value that is lower than its own \(K_m\) (10 mM Cl\(^-\) or 20 mM Na\(^+\)), the \(K_m\) values for Na\(^+\) and Cl\(^-\) increased and the \(V_{max}\) values decreased. These results suggest that reduction of the extracellular Na\(^+\) or Cl\(^-\) concentration decreased the ftTSC affinity for the counterion.

Effect of thiazides and Hg\(^{2+}\) on ftTSC. The inhibition of the Na-Cl cotransporter by thiazide-like diuretics is one of the distinctive features of this cotransporter pathway in the teleost urinary bladder and the apical membrane of the mammalian DCT. For this reason, we analyzed the effects of increasing concentrations of several thiazide-type diuretics on the functional expression of the cotransporter on ftTSC cRNA-injected oocytes. Figure 4 shows the results of these series of experiments, and Table 1 shows the percentage of Na\(^+\) uptake by ftTSC in the presence of the different concentrations of each of the thiazide-type diuretics. Interestingly, the effect of the thiazide-type diuretics on ftTSC function was biphasic. At low concentrations (10\(^{-6}\) to 10\(^{-5}\) M), thiazides induced an increase in Na\(^+\) uptake that was statistically significant compared with the uptake in the absence of thiazide. At higher concentrations, 10\(^{-5}\) to 10\(^{-4}\) M, all thiazides reduced the
Na⁺ uptake through the Na-Cl cotransporter. The inhibitory profile for ftTSC inhibition was metolazone > polythiazide > bendroflumethiazide > trichloroethiazide > chlorthalidone. This profile was similar to the profile observed for rTSC (28) but with lower sensitivity. In fact, at a 10⁻⁴ M concentration, trichloroethiazide and chlorthalidone reduced the function of ftTSC by only 68 and 46%, respectively, whereas in rTSC the same concentration of all thiazides inhibited the function of the cotransporter by >95% (28). Thus ftTSC exhibits lower affinity for thiazides than does rTSC. The biphasic effect was not observed for most thiazides in rTSC (28), with the exception of chlorthalidone, which is the thiazide with the lowest inhibitory potency. The effect of increased concentrations of chlorthalidone on rTSC function is shown in Fig. 5. Between 10⁻⁹ and 10⁻⁷ M concentration, the function of rTSC was significantly increased. The increased Na⁺ uptake with the low concentration of thiazides was Cl⁻ dependent and sensitive to 10⁻⁴ M polythiazide (data not shown), indicating that it was secondary to stimulation of the Na-Cl cotransporter function. At 10⁻⁶ M chlorthalidone, rTSC function was similar to that in the absence of thiazide, and higher concentrations inhibited the function of the rat cotransporter.

We previously observed that the kinetics for thiazide inhibition of rTSC was affected by the extracellular Na⁺ and Cl⁻ concentrations (28). Thus we evaluated the effect of low Na⁺ (10 mM) or low Cl⁻ (5 mM) on the inhibitory kinetics of metolazone on ftTSC. As shown in Fig. 6, the concentration of extracellular Na⁺ or Cl⁻ has no effect on the IC₅₀ for metolazone inhibition of ftTSC.

Many ion transporters are dramatically affected by exposure to Hg²⁺ (18, 25, 43), which at some point in the last century was used as a diuretic agent. In the electroneutral cotransporter family, Jacoby et al. (18) showed that the basolateral isofrom of the Na-K-2Cl cotransporter exhibits variable inhibition by Hg²⁺, whereas Mercado et al. (26) have shown that the endogenously expressed K-Cl cotransporter in X. laevis oocytes is activated by Hg²⁺. We thus analyzed the effect of increased concentration of Hg²⁺ on the ⁴²Na⁺ uptake induced by rTSC or ftTSC in X. laevis oocytes. As Fig. 7 shows, the exposure of oocytes to Hg²⁺ resulted in significant inhibition of cotransporter function in both rTSC and ftTSC, the former being more susceptible than the latter.
Regulation of fTSC. The function of all members of the electroneutral cotransporter family is regulated by cell volume. For example, BSC2 is activated by hypertonicity and inhibited by hypotonicity (6). BSC1 expresses a long COOH-terminal isoform that is activated by hypertonicity and partially inhibited by hypotonicity (10), and a short COOH-terminal isoform that is activated by hypotonicity (34). The four KCC isotypes are activated by cell swelling (27, 29). In addition, we have recently shown that rTSC is also inhibited by cell swelling (28). Thus we analyzed the effect of exposing oocytes to hypotonic or hypertonic media on the function of fTSC. For these experiments, oocytes were exposed to an uptake medium containing 40 mM NaCl or Na-glucuronate at three different osmolarities ([hypotonic (210 mosmol/kgH2O), isotonic (310 mosmol/kgH2O), or hypertonic (310 mosmol/kgH2O), by adding sucrose to the 40 mM NaCl uptake medium]). Therefore, uptake rates were determined in different osmolar conditions, without a change in the extracellular NaCl concentration or ionic strength. As shown in Fig. 8, when oocytes were incubated in hypotonic and in isotonic media, the function of fTSC was similar. However, when oocytes were exposed to hypertonicity, the Na+ uptake rate by fTSC was significantly reduced.

Finally, we analyzed the effect of second messengers on the Na+ uptake induced by fTSC in X. laevis oocytes. As shown in Fig. 9, the addition of the phorbol ester TPA in the preuptake period resulted in a significant reduction of cotransporter function. This effect is similar to the one we have reported for rTSC (15) and the endogenously expressed Na-K-2Cl cotransporter in oocytes (35). In contrast, the addition of cAMP, cGMP, or the phosphodiesterase inhibitor IBMX had no effect on cotransporter function.

DISCUSSION

In the present study, we determined the major functional, pharmacological, and regulatory properties of the electroneutral Na-Cl cotransporter that is expressed in the apical membrane of the winter flounder urinary bladder. As shown before (11), microinjections of X. laevis oocytes with fTSC cRNA induce the appearance of a Na+ uptake mechanism that is Cl-dependent and sensitive to thiazide-type diuretics. The increased Na+ uptake is not sensitive to inhibitors of other electroneutral cotransporter systems such as furosemide or acetazolamide (11). In the present study, we observed a number of interesting similarities and differences between rTSC (28) and fTSC.

Tran et al. (42) observed that external pH modulated the [3H]metolazone binding to rat renal cortical membranes. Maximum [3H]metolazone binding was observed at extracellular pH of 5.5, followed by a slight decrease, as the pH was increased up to 8.0, suggesting that external pH, within the range of changes that can be observed along the DCT, could affect binding of the thiazide-type diuretics to the cotransporter. However, our results in fTSC in the present study, comparable to those observed in rTSC (28), revealed that extracellular pH within the range of 6.0 to 8.0 has no effect on either cotransporter function, Cl- dependency, or thiazide sensitivity. Similar to our observations in fTSC and rTSC, the function of another member of the electroneutral cation chloride cotransporter, the K-Cl isoform KCC1, is not affected by external pH (24).

Analysis of fTSC ion transport kinetics revealed Km values for extracellular Na+ and Cl- at physiological concentrations of the counterion of ~58 and ~22 mM, respectively. Similar values were obtained when uptake rates were measured using 90- or 15-min incubations. These results are significantly higher than those

![Fig. 5. Biphasic effect of chlorthalidone on the function of rat (r)TSC. Na+ uptake was assessed in rTSC cRNA-injected oocytes in the absence or presence of chlorthalidone at concentrations from 10^-9 to 10^-4 M. Each point represents the mean ± SE of 40 oocytes from 2 different experiments. Data were normalized to the rTSC function, with the amount of Na+ uptake in the absence of chlorthalidone in each experiment taken as 100% function. *P < 0.01 vs. control in the absence of chlorthalidone.](http://ajprenal.physiology.org/)

Table 1. fTSC percentage of function in the presence of increasing concentrations of several thiazide-type diuretics

<table>
<thead>
<tr>
<th>Thiazide Concentration, -log M</th>
<th>10^-9</th>
<th>10^-8</th>
<th>10^-7</th>
<th>10^-6</th>
<th>10^-5</th>
<th>10^-4</th>
</tr>
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<tbody>
<tr>
<td>Chlorthalidone</td>
<td>137.8 ± 24.9</td>
<td>99 ± 10.4</td>
<td>111.2 ± 11.8</td>
<td>131.5 ± 17.9</td>
<td>92.4 ± 17.6</td>
<td>64.4 ± 11.0</td>
</tr>
<tr>
<td>Trichloromethiazide</td>
<td>118.5 ± 15.6</td>
<td>134.6 ± 23.1</td>
<td>108.8 ± 15.4</td>
<td>95.9 ± 13.6</td>
<td>81.7 ± 13.1</td>
<td>32.8 ± 5.1</td>
</tr>
<tr>
<td>Bendroflumethiazide</td>
<td>113.6 ± 13.0</td>
<td>145.1 ± 16.5</td>
<td>101.1 ± 10.4</td>
<td>83.3 ± 12.2</td>
<td>61.2 ± 7.6</td>
<td>5.6 ± 1.1</td>
</tr>
<tr>
<td>Metolazone</td>
<td>200.0 ± 31.7</td>
<td>132.2 ± 17.0</td>
<td>170.0 ± 20.8</td>
<td>54.6 ± 9.18</td>
<td>28.1 ± 5.3</td>
<td>6.8 ± 2.9</td>
</tr>
<tr>
<td>Polythiazide</td>
<td>163.5 ± 19.5</td>
<td>134.0 ± 19.9</td>
<td>205.3 ± 28.8</td>
<td>125.1 ± 16.2</td>
<td>27.4 ± 4.6</td>
<td>3.1 ± 1.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. fTSC, flounder thiazide-sensitive Na-Cl cotransporter. *P < 0.05 vs. uptake with no thiazide.
osmolarity and ionic strength of all solutions were similar.

%control 22Na HgCl2.

oocytes. *P/H11006 uptake. Each bar represents the mean features observed in media was lowered. Although more concentrations concentration of the counterion used in the uptake well as the apparent $V_{\text{max}}$ values are dependent on the concentration of the counterion kept constant during the experiment. That is, the apparent $V_{\text{max}}$ should decrease as the fixed counterion does. Therefore, we believe that our data are consistent with a rapid-equilibrium, random addition of the ions to the cotransporter. The other mechanism that could explain our data is an ordered one in the steady state. However, given that the cotransport of ions across a membrane most likely does not take place under steady-state conditions, because the rate of movement of ions is much slower than the rate of the association and dissociation steps, we favor a rapid-equilibrium, random mechanism over an ordered steady-state mechanism. Interestingly, however, although Na+ and Cl− interaction in fTSC is similar to that in rTSC (28), we observed no interaction between ions and metolazone in fTSC. The inhibitory kinetics of metolazone in fTSC were similar when the concentration of both ions was 96 mM and when either Na+ or Cl− was used at a very low concentration (Fig. 6). In contrast, we observed in rTSC that lowering the extracellular Na+ or Cl− concentration increased the apparent affinity of the cotransporter for metolazone, suggesting that in rTSC both ions compete or interact with the diuretic (28). Therefore, our present data suggest that this interaction is not present in fTSC.

fTSC is specifically inhibited by thiazide-type diuretics, but the affinity of the cotransporter for these compounds is lower than the affinity shown by rTSC (28). In fact, even at $10^{-4}$ M concentration of some thiazides like chlorthalidone or trichloromethiazide, the inhibition of fTSC was not complete, whereas in rTSC this concentration of any thiazide inhibited the cotransporter by >95% (28). In addition, a biphasic response to thiazides was observed in fTSC. Lower concentrations were often associated with an increase in the Na+ uptake, whereas higher concentration reduced Na+ uptake. This behavior was observed in rTSC only for chlorthalidone, the thiazide with the lower potency. We believe that the increase in Na+ uptake when oocytes were exposed to low concentrations of thiazides was due to stimulation of the cotransporter function because the increased uptake was Cl−.
dependent and completely sensitive to a $10^{-4}$ M concentration of polythiazide.

It has been suggested by several authors that inhibition of the cotransporter by thiazide is due to the direct interaction of the drug with the cotransporter, presumably due to competition of the diuretic with Cl$^-$ for the same site on the transporter (42), or with both Na$^+$ and Cl$^-$ (28). The increase in cotransporter function, however, is unlikely to result from such a direct interaction. Instead, it is possible that thiazides activate a second messenger pathway within the oocytes that probably results in activation of the cotransporter. This effect is lost with the higher concentrations of the thiazide because, regardless of the activation of any intracellular pathway, at high concentrations the thiazide blocks the function of the cotransporter itself. It has been suggested for years that thiazides reduce blood pressure not only because of their diuretic and saluretic action but also by reducing the peripheral resistances due to a direct vasodilatory action (4, 5).

Some thiazide derivatives, like indapamine or diazoxide, are vasodilators (39) and not diuretics, and several authors have shown direct effects of hydrochlorothiazide on blood vessels (2). Thiazides activate ion channels in vascular smooth muscle cells (33), and a recent study shows that the mechanism by which thiazides induce vasodilatation requires endothelium and is inhibited by the nitric oxide synthesis inhibitor $N^\text{ω}$-nitro-L-arginine, suggesting that the nitric oxide pathway could be implicated (3). Moreover, in the central nervous system it has been shown that cyclothiazide modulates the desensitization of the AMPA and kainate receptors by a mechanism that includes the nitric oxide/cGMP pathway (9). This effect of cyclothiazide on glutamate and kainate receptors has been shown to be present when $X$. laevis oocytes were used as the expression system (31), suggesting that the required intracellular pathway is present in oocytes. Thus it is possible that thiazides activate a second messenger pathway within oocytes that, in turn, results in TSC activation. However, we observed no effect of cAMP, cGMP, or IBMX on flTSC function, whereas PKC activation with a phorbol ester resulted in significant reduction of the cotransporter function. This inhibitory effect of protein kinase C activation was also observed in rTSC (15). Further studies will be necessary to elucidate the intracellular pathway involved.

Hg$^{2+}$ was used in the first half of the twentieth century as the first potent diuretic agent available in clinical medicine (8). The prescription of Hg$^{2+}$ was then discontinued due to the toxicity and tendency toward tachyphylaxis, together with the development of better diuretics such as thiazides and loop diuretics. The site of action in the nephron was localized at the

![Fig. 8. Effect of extracellular osmolarity in flTSC functional expression. flTSC cRNA-injected oocytes were exposed to uptake media with osmolarities of 110, 210, or 310 mosmol/kgH$_2$O as stated. In the 3 osmolar conditions, uptake was performed in control solutions (open bars) or in the absence of extracellular Cl$^-$ (solid bars). Uptake was performed in all groups for 60 min. Each bar represents the mean ± SE of 25–30 oocytes from 2 different experiments. *P < 0.05 vs. uptake in control solution with NaCl. **P < 0.05 vs. uptake in isotonicity.](http://ajprenal.physiology.org/)

![Fig. 9. Regulation of flTSC in $X$. laevis oocytes. Before the uptake period, flTSC cRNA-injected oocytes were exposed to metolazone (100 nM), O-tetradecanoylphorbol 13-acetate (TPA; 100 nM), dibutyryl-cAMP (1 mM), dibutyryl-cGMP (1 mM), or IBMX (1 mM) as stated. Solid bar, uptake in the control group (water-injected oocytes). Each bar represents the mean ± SE of 20 oocytes. *P < 0.05 vs. uptake in flTSC control group.](http://ajprenal.physiology.org/)

![Fig. 10. Structural differences between rTSC and the flTSC. The figure depicts the proposed topology of the cotransporter to highlight the identities and differences between the 2 cotransporters. PKC and PKA: protein kinase C and protein kinase A, respectively.](http://ajprenal.physiology.org/)
thick ascending limb and distal nephron, regions in which Hg$^{2+}$-inhibited net Cl$^{-}$ reabsorption (46). However, the precise mechanism of action was never determined. We show here that Hg$^{2+}$ reduces the function of rTSC and fTSC (Fig. 7). The inhibition of fTSC by Hg$^{2+}$ was observed by Wilkinson et al. (44) using isolated sheets of the flounder urinary bladder and also by Jacoby et al. (18) on the basolateral isoform of the Na-K-2Cl cotransporter. In addition, Hg$^{2+}$ also inhibits the function of the apical renal-specific isoform of the Na-K-2Cl cotransporter (Plata C and Gamba G, unpublished observations). Thus it is possible that the diuretic effect of Hg$^{2+}$ was due to direct inhibition of the Na-K-2Cl and the Na-Cl cotransporters located at the apical membrane of the thick ascending loop of Henle and the distal tubule, respectively.

The function of all members of the electroneutral cotransporter family is regulated by cell volume. For example, the Na-K-2Cl cotransporter is activated by cell shrinkage (45), whereas the K-Cl cotransporters are activated by cell swelling (27). In any case, the increase or decrease in the activity of the cotransporter is related to phosphorylation and dephosphorylation processes. TSC was less known as a cell volume-regulated cotransporter. However, when expressed in X. laevis oocytes, rTSC function, compared with isotonicity, is partially reduced in the presence of a hypotonic medium, whereas hypertonicity has no further effect on the cotransporter function (28). Interestingly, fTSC exhibits the opposite behavior. When expressed in X. laevis oocytes, the function of fTSC was similar in hypotonicity and isotonicity, whereas exposure of oocytes to hypertonicity resulted in a significant reduction of cotransporter function.

The urinary bladder in teleosts is produced by the fusion and expansion of the archinephric ducts and thus constitutes an epithelium that is embryologically similar to the mammalian DCT (21). The urinary bladder and the DCT provide a mechanism to reduce amounts of salt and water and concentrate divalent cations in the urine, in an environment in which tubular fluid is often more diluted than plasma (19). Thus we believe that differences in TSC properties in the flounder and rat are more related to their primary structure than to the intraluminal milieu. Figure 10 depicts the proposed topology for TSC. There is a central hydrophobic segment containing 12 putative membrane-spanning domains that are flanked by a short NH$_2$- and a long COOH-terminal domain. The hydrophilic segment that connects membrane-spanning domains 7 and 8 is glycosylated and thus located outside the cell (30). Although this topology has not been experimentally confirmed for TSC, it has been shown to be the topology of the basolateral isoform of the Na-K-2Cl cotransporter (12). Also shown in Fig. 10 are the differences between the flounder and the rat cotransporters. fTSC exhibits three putative N-glycosylation sites, whereas rTSC has only two of them. The highest degree of identity (∼80%) is present along the membrane-spanning domains. However, in this segment there are more charged amino acid residues in rTSC than in fTSC. The structural requirements for ion specificity or affinity in TSC are completely unknown. However, it has been shown in the basolateral isoform of the Na-K-2Cl cotransporter that the affinity for the cotransported ions and the diuretic is determined by the central segment containing the membrane-spanning domains, and not by the NH$_2$- or COOH-terminal domains (16). Thus it is possible that the greater number of charged residues in rTSC could be the reason for the higher affinity for both ions in the rat cotransporter. The degree of identity is lower outside the membrane-spanning domains. At the COOH-terminal domain, the identity is ∼55% and, at the NH$_2$-terminal domain, ∼20%. In addition, although there are several putative protein phosphorylation sites shared by both cotransporters in these domains, as Fig. 10 shows, there are some sites that are present in only one or the other. The different NH$_2$- and COOH-terminal domains between rTSC and fTSC could be the reason for differences in regulation between these two isoforms, such as the different response to extracellular osmolarity. In conclusion, we have found significant regulatory, kinetic, and pharmacological differences between the flounder and rat isoforms of the TSC.

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REFERENCES

FUNCTIONAL CHARACTERIZATION OF FLOUNDER TSC


