The transport properties of the human renal Na\(^+\)-dicarboxylate cotransporter under voltage-clamp conditions

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Yao, Xiaozhou, and Ana M. Pajor. The transport properties of the human renal Na\(^+\)-dicarboxylate cotransporter under voltage-clamp conditions. Am J Physiol Renal Physiol 279: F54–F64, 2000.—The transport properties of the human Na\(^+\)-dicarboxylate cotransporter, (hNaDC-1), expressed in Xenopus laevis oocytes were characterized using the two-electrode voltage clamp technique. Steady-state succinate-evoked inward currents in hNaDC-1 were dependent on the concentrations of succinate and sodium, and on the membrane potential. At −50 mV, the half-saturation constant for succinate (\(K_{0.5}^{\text{succinate}}\)) was 1.1 mM and the half-saturation constant for sodium (\(K_{0.5}^{\text{sodium}}\)) was 65 mM. The Hill coefficient was 2.3, which is consistent with a transport stoichiometry of 3 Na\(^+\):1 divalent anion substrate. The hNaDC-1 exhibits a high-cation selectivity. Sodium is the preferred cation and other cations, such as lithium, were not able to support transport of succinate. The preferred substrates of hNaDC-1 are fumarate (\(K_{0.5}^{\text{fumarate}}\) 0.5 2.8 mM), citrate (\(K_{0.5}^{\text{citrate}}\) 0.5 1.8 mM) and succinate, followed by methysuccinate (\(K_{0.5}^{\text{methylsuccinate}}\) 0.5 2.8 mM), citrate (\(K_{0.5}^{\text{citrate}}\) 0.5 6.8 mM) and \(\alpha\)-keto-glutarate (\(K_{0.5}^{\text{\(\alpha\)-keto-glutarate}}\) 16 mM). The hNaDC-1 may also transport sodium ions through an uncoupled leak pathway, which is sensitive to phloretin inhibition. We propose a transport model for hNaDC-1 in which the binding of three sodium ions is followed by substrate binding.

sodium; succinate; Xenopus laevis oocytes; electrogentic cotransport

The epithelial cells of the renal proximal tubule reabsorb filtered Krebs cycle intermediates, such as succinate, \(\alpha\)-ketoglutarate, and citrate, on a low-affinity Na\(^+\)-dicarboxylate cotransporter, NaDC-1, found on the apical membrane (16). A high-affinity Na\(^+\)-dicarboxylate cotransporter is found on the basolateral membrane of proximal tubule cells (16). The low-affinity NaDC-1 plays an important role in regulating the concentration of urinary citrate, which acts as a chelator of calcium. Hypocitraturia is a risk factor for kidney stone formation (19). NaDC-1 belongs to a distinct gene family of sodium-coupled anion transporters that includes the Na\(^+\)-sulfate transporter, NaSi-1, and is not related to any other known families of transport proteins (16). NaDC-1 orthologs, corresponding to low-affinity NaDC-1, have been isolated from rabbit, human, and rat (4, 14, 22). Other members of this family include a Na\(^+\) or Li\(^+\)-dependent dicarboxylate transporter from Xenopus laevis intestine, NaDC-2 (1), and the high affinity Na\(^+\)-dicarboxylate cotransporters from rat, NaDC-3 (SDCT2), and flounder, flNaDC-3 (3, 11, 23).

The human Na\(^+\)-dicarboxylate cotransporter, hNaDC-1, is 78% identical in sequence to the transporter from rabbit, rbNaDC-1 (15). In transport assays using radiotracer substrates, both transporters have low affinities for succinate and both exhibit a stimulation of citrate transport at acidic pH. However, the two transporters differ in their affinities for citrate, in cation binding, and in sensitivity to inhibitors (18). For example, hNaDC-1 has a much lower affinity for citrate and for sodium than rbNaDC-1. Furthermore, unlike rbNaDC-1, hNaDC-1 is relatively insensitive to inhibition by lithium (18). Because the rabbit and human Na\(^+\)-dicarboxylate cotransporters differ in their transport properties, it is likely that their electrophysiological characteristics are also somewhat different.

In the present study, we characterized the transport properties of hNaDC-1 expressed in X. laevis oocytes using the two-electrode voltage-clamp technique. Many of the effects of voltage on hNaDC-1 are similar to the effects of voltage on rbNaDC-1 (17). For example, the half-saturation constant for sodium (\(K_{0.5}^{\text{sodium}}\)) in hNaDC-1 is very sensitive to membrane potential whereas the half-saturation constant for succinate (\(K_{0.5}^{\text{succinate}}\)) is relatively voltage independent. However, unlike rbNaDC-1, the substrate-dependent currents in hNaDC-1 were very dependent on sodium, and no currents were seen in the presence of lithium. Interestingly, hNaDC-1 also exhibited a phloretin-sensitive leak pathway for the transport of sodium uncoupled to the movement of substrate, suggesting that hNaDC-1 could also act as a sodium transport pathway. In conclusion, this study provides new insights into the functional properties of the Na\(^+\)-dicarboxylate cotransporter from human kidney.
METHODS

cRNA transcription. The hNaDC-1 cDNA in pSPORT1 plasmid was linearized with XbaI and in vitro cRNA transcription was done using the T7 mMessage mMachine Kit (Ambion) (15).

Oocyte preparation. Stage V and VI oocytes were obtained from female X. laevis frogs (Xenopus I) and collagenase treated as described previously (14, 18). The oocytes were injected with ~46 nl of hNaDC-1 cRNA (0.5 μg/μl) 1 day after isolation and cultured at 18°C in modified Barth’s medium. Culture dishes and medium were changed daily.

Transport solutions. Sodium buffer consisted of (in mM) 100 NaCl, 2 KCl, 1 MgCl2, 1 CaCl2, and 10 HEPES, buffered to pH 7.5 using Tris base. Choline buffer, lithium buffer, and cesium buffer were prepared by replacing NaCl with 100 mM cholineCl, LiCl, or CsCl, respectively. For transport solutions containing different sodium concentrations, the NaCl was replaced by cholineCl. Stock solutions of inhibitors were prepared as follows: 100 mM phloretin in ethanol; 50 mM tetrodotoxin (TTX), citrate-free (Calbiochem) in 100 mM acetic acid; 50 mM niflumic acid in ethanol; 500 mM amiloride in DMSO; 500 mM DIDS in DMSO, gadolinium chloride (GdCl3), and 100 mM tetraethylammonium (TEA) in water. The inhibitors were diluted to their final concentration in sodium buffer just before use. Control solutions received vehicle alone (ethanol, DMSO, or acetic acid).

Electrophysiology. Experiments were performed 3–5 days after cRNA injection using the two-electrode voltage clamp method with a Geneclamp 500 amplifier (Axon Instruments), as described (17). The microelectrodes were filled with 3 M KCl and had resistances between 0.4 and 0.8 MΩ. Test voltage pulses were applied for 100 ms between +50 and −150 mV (in 20-mV decrements) at a holding potential of −50 mV, and membrane currents were recorded. The voltage pulses were controlled with the pCLAMP version 6.0 program suite (Axon Instruments). For most experiments, the substrate-dependent currents were determined from the difference between currents measured in sodium buffer in the absence and presence of substrate. The substrate was washed away by superfusion with choline buffer, and experiments were continued only when the oocytes returned to control condition, usually after 5–10 min. The sodium leak currents were determined from the difference between currents measured in choline buffer (0 sodium) and sodium buffer (100 mM sodium).

Data analysis. Steady-state substrate-dependent currents were fitted to the Hill/Michaelis-Menten equations using SigmaPlot software (Jandel Scientific); \( I = I_{\text{max}}S^n/(K_{0.5} + S)^n \), where \( I \) is the substrate-induced current, \( I_{\text{max}} \) is the maximum current observed at saturating substrate concentrations, \( K_{0.5} \) is the substrate concentration at half-maximal current, and \( n \) is the Hill coefficient. For the Michaelis-Menten equation, \( n = 1 \). The error bars for figures of kinetic data represent errors of the fit. Other experimental results are expressed as mean ± SE (\( N = \) no. of experiments using different donor frogs). Unless otherwise noted, all experiments were repeated with oocytes from at least three different frogs.

RESULTS

Steady-state inward currents activated by succinate. The presence of substrate and sodium produced inward currents in oocytes expressing hNaDC-1. Figure 1A

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**Fig. 1.** Current traces were obtained from an oocyte expressing human Na^-dicarboxylate cotransporter (hNaDC-1) before (A) and after (B) addition of 1 mM succinate to bathing medium. Experiments were performed on the 4th day after cRNA injection. The oocyte membrane was held at a holding potential of −50 mV. Pulse protocol is shown. In C is shown steady-state currents recorded in the absence (●) and presence (○) of 1 mM succinate from the experimental data shown in A and B, respectively. In D the current-voltage relationship (I-V) curve was obtained as the difference between currents before and after application of 1 mM succinate.
shows typical current tracings from an oocyte superfused with sodium buffer and subjected to the voltage-pulse protocol, from a holding potential of −250 mV. In the presence of sodium and succinate, inward currents were produced (Fig. 1B). Pre-steady-state charge movements were not evident (Fig. 1A and B). Figure 1C shows the steady-state currents measured at each voltage in the presence and absence of succinate. The difference between the currents measured with and without substrate is the substrate-dependent current (Fig. 1D). Control, uninjected, or water-injected oocytes had very small currents in the presence of sodium, as seen previously (17). No inward currents were detected in control oocytes when succinate was added to the medium (results not shown).

The steady-state inward currents produced by succinate in hNaDC-1 were concentration and voltage dependent. The currents increased with increasing concentrations of succinate and also with hyperpolarizing potentials (Fig. 2A). For each voltage, the currents were plotted against succinate concentration and fit to the Michaelis-Menten equation. The maximal succinate-induced current ($I_{\text{max}}^{\text{succinate}}$) as a function of membrane potential.

Figure 3 shows the effects of sodium on the kinetics of succinate-induced currents in hNaDC-1. Decreasing the sodium concentration resulted in a large decrease in succinate-induced currents, consistent with our previous study showing that hNaDC-1 has a very low cation affinity, with an apparent $K_m$ for sodium of ~78 mM (15). The $I_{\text{max}}^{\text{succinate}}$ appeared to be unaffected by sodium concentrations between 25 and 100 mM, although there were large errors in the data fits at lower sodium concentrations (Fig. 3B). The $I_{\text{max}}^{\text{succinate}}$ increased with more negative voltages at all sodium concentrations (Fig. 3B). In contrast, the $K_{0.5}^{\text{succinate}}$ increased considerably as the sodium concentration was reduced. The $K_{0.5}^{\text{succinate}}$ at −50 mV was 1.3 mM at 100 mM sodium, 4.2 mM at 75 mM sodium, and 11.4 mM at 25 mM sodium (Fig. 3C). The differences in $K_{0.5}^{\text{succinate}}$ at different sodium concentrations were more pronounced at more positive membrane potentials whereas no differences in $K_{0.5}^{\text{succinate}}$ were evident at very negative membrane potentials (Fig. 3D).

**Substrate specificity.** To examine substrate specificity, oocytes expressing hNaDC-1 were superfused with 10 mM concentrations of various substrates and steady-state currents were recorded (Fig. 4). The currents were expressed as a percentage of the succinate-induced current. Similar to the substrate specificity of rNaDC-1 (17), the currents recorded in the presence of fumarate in hNaDC-1 were larger than those measured in succinate (Fig. 4). Methylsuccinate, dimethylsuccinate, citrate, and α-ketoglutarate induced <50%...
of the succinate-induced currents in the human NaDC-1 (Fig. 4). In contrast, the largest currents in rbNaDC-1 were seen with methylsuccinate, citrate, and tricarballylate (17). In hNaDC-1, small currents of 20% of the succinate-induced currents were observed with tricarballylate and glutarate, whereas sulfate, lactate, and L-glutamate produced currents that were 5% of control. Pyruvate did not induce any measurable inward current. None of the substrates induced inward currents in water-injected control oocytes (data not shown).

To determine whether the differences in substrate-induced currents were due to differences in $I_{\text{max}}$ or $K_{0.5}$, kinetic measurements were made. Although the $K_{0.5}$ is lowest for succinate (1.1 mM), the transport capacity ($I_{\text{max}}$) for fumarate is greater than for succinate (Table 1). The $I_{\text{max}} / K_{0.5}$ ratio for fumarate is more than twofold greater than that for succinate, indicating that it is transported more efficiently. The rank order of substrate preference in hNaDC-1 is fumarate, followed by succinate, methylsuccinate, citrate, and $\alpha$-ketoglutarate (Table 1). However, it should be noted that the preferred species of citrate transported by hNaDC-1 is citrate$^{2-}$, which accounts for only $\sim$1.3% of the total citrate at pH 7.5 (pK$_a$ 5.62) (2, 18). Therefore, the $K_{0.5}$ of citrate$^{2-}$ in hNaDC-1 is $\sim$88 $\mu$M, although the possible inhibition of transport by citrate$^{3-}$ (18) could affect this value.
The kinetic measurements for citrate shown in Table 1 were made in the presence of 100 mM niflumic acid to inhibit currents through an endogenous hemi gap-junction channel that is seen in some batches of uninjected oocytes. As reported previously, the currents through this hemi gap-junction channel are very large (0.5–1 mA), outwardly directed, and they are activated by the removal of calcium (28). We have observed these currents in some batches of uninjected oocytes in the presence of large concentrations of citrate or in the presence of 5 mM EDTA, suggesting that the effect of citrate is the chelation of calcium (results not shown). Niflumic acid does not affect succinate or citrate transport or currents in hNaDC-1 (results not shown).

**Sodium effects on succinate-dependent inward currents.** The dependence of succinate-induced steady-state currents on external sodium concentration in oocytes expressing hNaDC-1 is illustrated in Fig. 5. There was no measurable current at 5 mM sodium, but above this concentration the succinate-induced currents increased with increasing concentrations of sodium (Fig. 5A). The succinate-induced currents were sigmoidal functions of sodium concentration (Fig. 5B) and could be fit by the Hill equation. The maximum succinate-induced current at saturating sodium concentrations, $I_{\text{max}}$, increased with hyperpolarizing potentials, ~1.8-fold between −50 and −150 mV (Fig. 5C). The apparent affinity constant for sodium, $K_{0.5}$, was very sensitive to voltage, decreasing from 68 mM at −50 mV to 45 mM at −150 mV (Fig. 5D). In three separate experiments, the mean $K_{0.5}$ measured at presence of large concentrations of citrate or in the presence of 5 mM EDTA, suggesting that the effect of citrate is the chelation of calcium (results not shown). Niflumic acid does not affect succinate or citrate transport or currents in hNaDC-1 (results not shown).

![Fig. 5](http://example.com/fig5.jpg)

**Fig. 5.** Effect of sodium concentration on succinate-induced steady-state inward currents. A: typical $I$-$V$ curves obtained from an oocyte expressing hNaDC-1 at different sodium concentrations. B: steady-state currents obtained from an hNaDC-1-expressing oocyte at −10, −50, −90, −130, and −150 mV plotted as a function of sodium concentration. Data were fit to the Hill equation. C: dependence of maximal sodium-induced current ($I_{\text{max}}$) on membrane potential. D: dependence of $K_{0.5}$ on membrane potential. E: the apparent Hill coefficients were plotted against membrane potential. Error bars represent SE of fit. Concentration of succinate was 10 mM in these experiments. It was not possible to obtain a reliable fit of data measured at +50 mV.
of the current seen in sodium (17). A previous study of rbNaDC-1 found that lithium could cause inward currents in the absence of substrate, the current being more negative than those seen in choline at pH 5.5 but only at membrane potentials less than −50 mV (results not shown). Small succinate-dependent inward currents were also seen in hNaDC-1 when sodium was replaced by lithium, choline (pH 7.5), or cesium. In a single experiment, no currents were observed in potassium and there was also no chloride dependence (results not shown). Small succinate-dependent inward currents, ~8% of those seen in sodium, were observed in choline at pH 5.5 but only at membrane potentials more negative than −70 mV (Fig. 7). In contrast, our previous study of rbNaDC-1 found that lithium could substitute for sodium, and lithium produced much larger currents than sodium in hNaDC-1. The sodium-dependent leak current, which represented ~20% of the total maximal current measured in saturating concentrations of succinate (Fig. 8A). The mean leak current was 17 ± 1% (mean ± SE, N = 15 oocytes, 8 frogs). The sodium-dependent leak current was a linear function of the amount of expression of hNaDC-1 whereas uninjected or water-injected oocytes had low-sodium currents of approximately −7 nA at −50 mV (Fig. 8B). The current-voltage relationship of the substrate-independent sodium currents is shown in Fig. 8C. The currents were measured at sodium concentrations between 5 and 100 mM. The currents were inwardly directed at all voltages tested, and there was a steep response to voltage between −50 and −150 mV. In addition, the sodium-dependent leak currents in hNaDC-1 were saturable with increasing concentrations of sodium. The currents at −50 mV from Fig. 8C were replotted as a function of sodium concentration. In the experiment shown in Fig. 8D, the half-saturation constant for leak (K_{leak}) was 186 mM. In three separate experiments, the K_{leak} was 193 ± 11 mM (mean ± SE). The substrate-independent sodium currents were also seen in rbNaDC-1 and represented ~13% of the maximal substrate-induced currents (results not shown).

The sodium-dependent leak current in oocytes expressing hNaDC-1 was sensitive to inhibition by 0.5 mM phloretin, which was more pronounced at hyperpolarized membrane potentials (Fig. 9A). The effect of phloretin was completely reversible after a 15-min washout with choline buffer. However, phloretin had no effect on substrate-dependent currents in hNaDC-1, either at 10 mM succinate (Fig. 9B) or at concentrations of succinate as low as 50 μM (results not shown). Phloretin also had no effect on currents in water-injected oocytes (data not shown). The concentration dependence of phloretin inhibition of the sodium current in hNaDC-1 at −50 mV is shown in Fig. 9C. The IC_{50} in this experiment was 0.2 mM, and the maximal inhibition was 59%. In three experiments, the IC_{50} for concentration of succinate was 10 mM.

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**Fig. 6. Sodium activation of succinate-dependent currents in an oocyte expressing hNaDC-1.** Inward currents at increasing sodium concentrations are plotted for succinate concentrations of 0.5, 1, and 5 mM. Holding potential was −50 mV. Kinetic constants are K_{Na} = 54 ± 11 mM (5 mM succinate); 59 ± 19 mM (1 mM succinate); f_{max} = 160 ± 32 nA (5 mM succinate); −72 ± 19 nA (1 mM succinate). Hill coefficient, n, was 2.4 ± 0.7 (5 mM succinate); 1.9 ± 0.5 (1 mM succinate).

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**Fig. 7. Cation specificity of hNaDC-1.** Substrate-dependent steady-state inward currents were recorded in choline (pH 7.5), choline (pH 5.5), cesium, lithium, and sodium buffer in an oocyte expressing hNaDC-1. Currents were plotted as a function of membrane potential. Concentration of succinate was 10 mM.
phloretin was 0.2 ± 0.05 mM, and the maximal inhibition was 58 ± 9% (mean ± SE).

To test whether activation of endogenous sodium channels in the oocyte membranes could potentially account for the sodium-dependent leak currents in oocytes expressing hNaDC-1, the sensitivity of the currents to inhibitors was measured. There have been reports that heterologous expression of channels or transporters in X. laevis oocytes can induce the expression of a hyperpolarization-activated cation-selective current that is blocked by DIDS and TEA (24). However, the sodium-dependent leak pathway in oocytes expressing hNaDC-1 was insensitive to 1 mM DIDS or 100 μM TEA (data not shown). X. laevis oocytes also express nonselective mechanosensitive channels that are inhibited by amiloride or Gd3+ (25). However, neither 500 μM amiloride nor 100 μM Gd3+ had any effect on the sodium-dependent leak pathway in our experiments (data not shown). Niflumic acid was tested as a potential inhibitor of the hemi-gap junction channel (28), but it also had no effect on the sodium-dependent leak pathway in oocytes expressing hNaDC-1 (data not shown). Finally, we tested TTX because the endogenous sodium channels of X. laevis oocytes are voltage-gated and sensitive to micromolar TTX (12). However, 10−4 M TTX did not affect the sodium-dependent leak current in hNaDC-1 (data not shown). Furthermore, none of these agents affected the substrate-dependent currents in hNaDC-1 (data not shown).

DISCUSSION

The coupled transport of succinate and sodium by hNaDC-1 is electrogenic and produces an inwardly directed current. The voltage-sensitive steps in transport by hNaDC-1 appear to be sodium binding and substrate turnover, whereas the binding of substrate appears to be relatively independent of voltage. The response to voltage in hNaDC-1 is similar to that of many sodium-dependent transporters, including rb-NaDC-1 (17), the Na+-glucose cotransporter, SGLT1 (20), the Na+-iodide symporter, NIS (5), and the Na+-phosphate cotransporter, NaPi-5 (7). Therefore, despite differences in structure between different families of sodium-coupled transporters, many of these transporters share similarities in mechanism.

Substrate kinetics. The kinetic constants for hNaDC-1 from the two-electrode voltage clamp studies agree quite well with previous data from radiotracer uptake experiments. The $K_{\text{0.5}}$ was 1.1 mM, similar to the $K_m$ for succinate of 0.8 mM (18), verifying that hNaDC-1 has a relatively low affinity for substrates. In transport experiments, the $K_{\text{0.5}}$ was 185 ± 85 mM and $I_{\text{max}}$ was −74 ± 24 nA. Hill coefficient was 1.01 ± 0.27 (mean ± SE of the fit).
dicarboxylate cotransporters rNaDC-1 (SDCT1) had an increase in the $K_{0.5}^{citrate}$ with more negative membrane potentials (4).

The Na$^+$-dicarboxylate cotransporters have broad, overlapping substrate specificities for 4-carbon, terminal dicarboxylic acids in the trans-configuration, including many Krebs cycle intermediates, such as succinate, α-ketoglutarate, and citrate (27). However, there are species differences in preferred substrates among the NaDC-1 orthologs. For example, the largest currents in hNaDC-1 were induced by fumarate and succinate, whereas the currents induced by α-ketoglutarate and citrate were only ~50% of the succinate-induced currents. The rabbit NaDC-1 differs from hNaDC-1 primarily in having large citrate-induced currents, likely because rbNaDC-1 has a higher affinity for citrate (17, 18). In contrast, the rNaDC-1 has small citrate-induced currents like hNaDC-1 (4, 22). However, the rNaDC-1 substrate selectivity differs from that of hNaDC-1 mainly in the α-ketoglutarate-induced currents, which were almost as large as the currents produced by succinate (4, 22). Therefore, it is likely that these closely related transporters contain subtle differences in the structures of their substrate binding sites that distinguish between similar substrate structures. In addition, there are differences in relative $I_{max}$ between substrates, suggesting differences in translocation or intracellular release of the substrates.

Sodium. hNaDC-1 has a relatively low affinity for sodium, with a $K_{0.5}^{sodium}$ of 68 mM, which agrees with the value of 78–150 mM from radiotracer uptake studies (10, 18). The $K_{0.5}^{sodium}$ in hNaDC-1 decreased with membrane hyperpolarization, indicating that the binding of sodium is likely to be voltage dependent. Sodium activation experiments with hNaDC-1 under voltage-clamp conditions resulted in an apparent Hill coefficient of 2.3, compared with 2.1–2.5 in radiotracer uptake experiments (10, 18). The Hill coefficient in hNaDC-1 is consistent with a coupling stoichiometry of 3 sodium ions:1 divalent anion substrate.

hNaDC-1 has a very strong preference for sodium as the coupled cation. Lithium was not able to substitute for sodium, consistent with our previous studies that showed that hNaDC-1 is insensitive to inhibition by lithium (18). Interestingly, there appear to be species and isoform differences in lithium handling in the family related to NaDC-1. rbNaDC-1 is very sensitive to inhibition by millimolar concentrations of lithium, which competes with sodium at one of the three cation binding sites (17). At higher concentrations, lithium can drive transport in rbNaDC-1, although the $K_{0.5}^{sodium}$ is very large, ~30 mM (17). In contrast, lithium inhibition but not substitution is seen in rNaDC-1 (SDCT1) and in the high-affinity Na$^+$-dicarboxylate cotransporter, rNaDC-3 (SDCT2) (3, 4, 11). The X. laevis intestine Na$^+$-dicarboxylate cotransporter, NaDC-2, is driven equally well by either lithium or...
sodium, and lithium does not inhibit transport in the presence of sodium (1). The differences in lithium handling suggest that there are differences in the structures of the cation binding sites among the members of this family.

Leak currents. The results presented in this study suggest that hNaDC-1 may be able to transport sodium by an uncoupled substrate-independent mechanism. In the absence of substrate, a sodium-dependent inward current that was sensitive to inhibition by phloretin was observed in the hNaDC-1-injected oocytes, but not in control un.injected or water-injected oocytes. Phloretin did not affect the substrate-dependent current in hNaDC-1. The magnitude of the sodium leak current in hNaDC-1 was proportional to the amount of expression of hNaDC-1. The leak current in hNaDC-1 was also saturable with increasing concentrations of sodium, indicating a low-affinity carrier-mediated pathway ($K_{\text{leak}}$ 191 mM at $-50 \text{ mV}$). It should be noted, however, that the hyperbolic kinetics of the leak pathway do not necessarily rule out channel-like activity. For example, sodium currents through the acetylcholine channel also exhibit saturation kinetics that can be modeled by the Michaelis-Menten equation, with an apparent $K_m$ for sodium of 102 mM (9). However, the leak current in oocytes expressing hNaDC-1 was insensitive to inhibitors of ion channels that have been observed previously in X. laevis oocytes, including amiloride, tetrodotoxin, Gd$^{3+}$, tetraethylammonium, niflumic acid, and DIDS (12, 24, 25, 28). Therefore, the substrate-independent sodium current is likely to be a property of hNaDC-1 rather than the result of activation of an endogenous channel in the oocytes.

There is evidence that some neurotransmitter transporters, such as the GABA transporter, exhibit significant efflux activity, which would be particularly important in neurons during an action potential (13). Therefore, an alternate hypothesis to account for the sodium currents seen in the absence of substrate in hNaDC-1 would be the inhibition of outward currents by addition of extracellular sodium. In this hypothesis, hNaDC-1 would operate in efflux mode when the cells were bathed in choline, and the addition of extracellular sodium would cause a trans-inhibition of the efflux, which would appear as an inward current relative to the current measured in choline. However, this hypothesis is not adequately supported by our experimental results with hNaDC-1. For example, the substrate-independent sodium current in hNaDC-1 exhibits hyperbolic kinetics with low affinity ($K_{\text{leak}}$ 191 mM), and saturation is not reached at the highest sodium concentration used in these experiments, 100 mM. This result is not consistent with a simple inhibition of efflux, which should saturate at a much lower sodium concentration. In experiments with rabbit renal brush-border membrane vesicles, trans-sodium concentrations inhibited Na$^+$-succinate uptake with sigmoid kinetics (Hill coefficient of 1.75) and a $K_{0.5}$ of 23 mM (26). Second, the size of the substrate-independent sodium current is too large to be only efflux, considering that the sodium concentration inside the oocytes is likely to be 6–10 mM (4, 28). In experiments measuring uptake of radiotracer substrates or inward substrate-dependent currents in hNaDC-1, there is almost no uptake at sodium concentrations below 25 mM, even with substrate concentrations as high as 10 mM (18). Therefore, the simplest explanation for the substrate-independent sodium current in hNaDC-1 is a sodium leak current (or slippage), and this is our present working hypothesis. However, further experiments with hNaDC-1 in which the intracellular and extracellular sodium and substrate concentrations can be controlled are clearly needed.

Sodium-dependent leak currents in the absence of substrate have also been reported for other transporters including SGLT1 (20), NIS (5), and NaPi-2 (6), and the magnitude of the leak current relative to the substrate-induced current depends on the transporter. For example, the leak current in SGLT1 is ~15% of the substrate-induced current (20), whereas NIS exhibits a leak current of ~35% of the substrate-induced current (5). rNaDC-1, or SDCT1, also exhibits a small sodium leak, ~4% of the maximal succinate-induced current and both the succinate-dependent and -independent currents are inhibited by phloretin (4).

Transport model. Figure 10 shows an ordered binding model of hNaDC-1 function, based on the results of this study, which is very similar to a previous model proposed for the Na$^+$-dicarboxylate cotransporter in rabbit renal brush-border membrane vesicles (26). The stoichiometry of Na$^+$-dicarboxylate cotransport in hNaDC-1 is three sodium ions for each divalent anion substrate molecule. The presence of the substrate-independent sodium leak indicates that at least one of the sodium ions binds before the substrate. The Hill

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**Fig. 10.** Model of Na$^+$-dicarboxylate cotransport by hNaDC-1. **C** represents carrier and **S** represents substrate, ′ refers to outside of cell, and ″ refers to inside of cell. In this model, Na$^+$ binds to transporter before substrate. In the absence of substrate, the transporter carries a single sodium ion as a facilitated diffusion carrier in a leak or slippage pathway. However, in the presence of substrate, three sodium ions bind before substrate binds. The fully-loaded carrier reorients so that the binding sites are exposed to the inside of cell, and the substrate and sodium are then released on the inside. Another conformational change occurs which reorients empty substrate and cation binding sites to outside.
coefficient of one for the sodium leak current indicates that the leak or slippage occurs after a single sodium ion has bound to the transporter. However, the $K_{0.5}^{\text{sodium}}$ was the same at different sodium concentrations (Fig. 3B), providing evidence for an ordered binding model in which succinate binds last because increasing concentrations of succinate can overcome the effect of decreased sodium concentration. Therefore, in the presence of substrate, the first step in the model proposed for hNaDC-1 function is the cooperative binding of three sodium ions, which increases the affinity of the transporter for substrate. The substrate then binds to the transporter, and the fully-loaded transporter undergoes a conformational change that exposes the substrate and cation binding sites to the inside of the cell. The substrate and cations are released on the inside of the cell after which the empty carrier reorients its binding sites to face the outside of the cell. This model is very similar to the models proposed for SGLT1 (21) and NIS (5). However, it differs from the model describing NaPi-2 function, in which two sodium ions bind before the substrate and one sodium ion binds last (6).

The $K_{0.5}^{\text{sodium}}$ in hNaDC-1 is very sensitive to voltage. In contrast, the $K_{0.5}^{\text{succinate}}$ is relatively insensitive to voltage changes at negative membrane potentials. However, at low-sodium concentrations, the $K_{0.5}^{\text{succinate}}$ is affected by voltage (Fig. 3D). One explanation for the results is that the binding of sodium is voltage sensitive whereas the binding of substrate is voltage independent. At low-sodium concentrations, the transporter does not have the optimal configuration for substrate binding (as shown by the larger $K_{0.5}^{\text{succinate}}$, Fig. 3C). However, changes in membrane potential at low-sodium concentrations could affect substrate binding indirectly by affecting sodium binding. The effects of low sodium can be overcome at high enough substrate concentrations.

The direction of transport by hNaDC-1, as in other sodium-coupled transporters, depends on the direction of the electrochemical gradients of sodium and succinate. It has been estimated that intracellular concentrations of sodium in X. laevis oocytes are between 6 and 10 mM, and intracellular succinate is $\sim$100 $\mu$M (4, 28). Therefore, a reversal of the substrate-dependent current in hNaDC-1 would be expected under the appropriate conditions, for example, if the extracellular concentrations of succinate and sodium were low enough or at positive membrane potentials. However, we do not see significant outward currents by hNaDC-1, suggesting that the rates of the outward fluxes may be slower than the rates of the inward fluxes. This observation is supported by previous kinetic experiments with rabbit renal brush-border membrane vesicles that showed that the $V_{\text{max}}$ for succinate efflux is much lower than the $V_{\text{max}}$ for influx (8). Furthermore, the rate of transport by hNaDC-1 is very low at the sodium concentrations (6–10 mM) that would be found inside the oocytes (Fig. 5, Ref. 18). It is likely that, in addition to allowing transport against a larger electrochemical gradient, the coupling of three sodium ions in hNaDC-1 also prevents significant efflux of substrate from the renal proximal tubule cells during substrate accumulation.

**Conclusion.** In conclusion, the succinate-dependent steady-state inward currents associated with hNaDC-1 expressed in X. laevis oocytes were analyzed by using the two electrode voltage-clamp technique. The substrate-induced currents in hNaDC-1 are dependent on membrane potential and on the concentrations of succinate and sodium. The $K_{0.5}^{\text{sodium}}$ decreases with hyperpolarizing potentials, whereas the $K_{0.5}^{\text{succinate}}$ is relatively insensitive to membrane potential. The only cation that is able to support succinate transport in hNaDC-1 is sodium. Finally, a phloretin-sensitive leak pathway for sodium was observed in the oocytes expressing hNaDC-1. We propose an ordered binding model in which three sodium ions bind before the substrate. The results provide new information for clarifying the functional role of the Na$^+$-dicarboxylate cotransporter in the human kidney.

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