Characterization of a Rat Na\textsuperscript{+}-Dicarboxylate Cotransporter*

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The metabolism of Krebs cycle intermediates is of fundamental importance for eukaryotic cells. In the kidney, these intermediates are transported actively into epithelial cells. Because citrate is a potent inhibitor for calcium stone formation, excessive uptake results in nephrolithiasis due to hypocitraturia. We report the cloning and characterization of a rat kidney dicarboxylate transporter (SDCT1). In situ hybridization revealed that SDCT1 mRNA is localized in S3 segments of kidney proximal tubules and in enterocytes lining the intestinal villi. Signals were also detected in lung bronchioli, the epididymis, and liver. When expressed in Xenopus oocytes, SDCT1 mediated electrogenic, sodium-dependent transport of most Krebs cycle intermediates ($K_m = 20-60 \mu M$), including citrate, succinate, α-ketoglutarate, and oxaloacetate. Of note, the acidic amino acids L- and D-glutamate and aspartate were also transported, although with lower affinity ($K_m = 2-18 \mu M$). Transport of citrate was pH-sensitive. At pH 7.5, the $K_m$ for citrate was high (0.64 μM), whereas at pH 5.5, the $K_m$ was low (57 μM). This is consistent with the concept that the –2 form of citrate is the transported species. In addition, maximal currents at pH 5.5 were 70% higher than those at pH 7.5, and our data show that the –3 form acts as a competitive inhibitor. Simultaneous measurements of substrate-evoked currents and tracer uptakes under voltage-clamp condition, as well as a thermodynamic approach, gave a Na\textsuperscript{+} to citrate or a Na\textsuperscript{+} to succinate stoichiometry of 3 to 1. SDCT1-mediated currents were inhibited by phloretin. This plant glycoside also inhibited the SDCT1-specific sodium leak in the absence of substrate, indicating that at least one Na\textsuperscript{+} binds to the transporter before the substrate. The data presented provide new insights into the biophysical characteristics and physiological implications of a cloned dicarboxylate transporter.

In kidney proximal tubules, reabsorption of Krebs cycle intermediates such as citrate, succinate, α-ketoglutarate, malate, and fumarate has been shown to be accomplished by Na\textsuperscript{+}-coupled transporters (1–9). Numerous studies have been performed in intact proximal tubules (10), isolated brush border membrane vesicles (BBMV)\textsuperscript{1} (1–5, 11, 12), and basolateral membrane vesicles (BLMV) (12–14), mostly using citrate or succinate as substrates.

In BLMV, succinate uptake was found to be mediated with low affinity ($K_m = 1 \text{ mM}$) (5, 8, 12, 15). Studies on the pH dependence suggested that citrate is transported in its protonated divalent form (Cit\textsuperscript{2+}) (1, 2, 12), whereas succinate is transported either in its deprotonated (–2) or protonated (–1) form (11). In addition, it was shown that the –3 form of citrate (trivalent form, Cit\textsuperscript{3–}) inhibits transport of Cit\textsuperscript{2+} (11). Radio-tracer studies revealed that the cotransport process exhibits a stoichiometry of 2–3 sodium ions/dicarboxylate molecule (2, 8, 16). On the other hand, experiments with a voltage-sensitive dye showed that the cotransport was electroneutral (14, 17), which favors a 3:1 stoichiometry.

In BLMV, succinate transport was pH-sensitive and with high affinity ($K_m = 10 \mu M$; Refs. 12 and 13), and citrate uptake was hardly pH-sensitive (12), suggesting that both divalent and trivalent citrate can be transported. The functional differences between BBMV and BLMV transport suggest that there exist different transporter isoforms on the apical and basolateral sides. Also, a possible presence of a trivalent citrate transport system in the basolateral membrane of the proximal tubule cell line from opossum kidney was proposed (9).

Citrates transport in the proximal tubule is of considerable interest because it has several implications for the kidney function. Citrate is metabolized by the kidney via the intramitochondrial tricarboxylic acid cycle, and this process provides up to 10–15% of renal oxidative metabolism (18, 19). Urinary citrate is a potent inhibitor of calcium stone formation by chelating calcium and inhibiting precipitation of calcium and crystalization of calcium-oxalate crystals (20). Hypocitraturia is found in about half of patients with renal stone diseases (21). Low urinary citrate levels are found in many conditions associated with decreases either in intraluminal or intracellular pH in the proximal tubules (i.e. systemic acidosis) or with potassium depletion. These conditions are known to increase citrate reabsorption (6, 22, 23). Interestingly, hypocitraturia may be found without apparent cause (idiopathic), but the underlying mechanism is still undetermined (24).

cDNAs of Na\textsuperscript{+}-dicarboxylate cotransporters have recently been isolated from rabbit kidney (NaDC-1) (25), human kidney (hNaDC-1) (26), rat intestine (27), and rat kidney (28). Both the rabbit and human transporters can transport tricarboxylic acid cycle metabolites with low affinities (29, 30). The expression of these cloned cotransporters in Xenopus oocytes allowed kinetic analyses under steady-state and presteady-state conditions and, in contrast to vesicle studies, with excellent control of membrane potential, external milieu, and in some cases internal milieu. In the present paper, we report the characterization, using the two-microelectrode voltage-clamp technique, and the tissue distribution of a rat dicarboxylate transporter (SDCT1) that has been recently cloned in our laboratory by homology screening.
Characterization of SDCT1

Isolation of the Rat SDCT1 Clone—Sprague-Dawley rat kidney cortex mRNA was reverse transcribed and used for polymerase chain reaction with a set of degenerative primers corresponding to the amino acids 35–40 and 142–137 of rabbit NaDC-1 (25). Polymerase chain reaction products were used to screen the cDNA library of a rat kidney cortex in the vector λgt10 at high stringency. A positive clone 2.4 kilobases in size was subcloned into the EcoRI site of the pBluescript vector and sequenced.

Oocyte Preparation—Stage VI and VII oocytes were extracted from female Xenopus laevis frogs and prepared as described previously (31). Capped cDNA of rat SDCT1 was synthesized in vitro transcription from cDNAs in pBluescript SK−. Defolliculated oocytes were injected with 25–50 ng of cRNA or water at the same day or the following day after defolliculation and maintained in Barth’s solution (88 mM NaCl, 1.8 mM CaCl2, 1 mM MgCl2, 0–5 mM citrate, pH 5.5–9.5 by Tris-base or 10 mM HEPES, 1.8 mM MgCl2, 1 mM KCl, 0.82 mM CaCl2, 0.82 mM choline-chloride, 10 mM HEPES, 1.8 mM MgCl2, 0.1 mM succinate). Because cold and hot substrates were premixed, the ratio of specific current to the radiolabeled uptake will not be affected by spatial and temporal variations in substrate concentration within the chamber.

Thermodynamic Determination of Stoichiometry—This procedure consists of measuring the reversal potential (Vr), i.e. the membrane potential where the inhibitor-sensitive current is zero. In the present study, succinate was used as the substrate at 20 and 200 μM, and phlorizin at 0.5 mM was used as the inhibitor. Because at pH 7.5 succinate is predominantly in its divalent (−2) form and assuming that n sodium ions are coupled to one succinate molecule, the relationship between Vr and succinate concentration ([Succinate]) at 22 °C is (33) as follows.

\[ V_r = \frac{58.5}{n - 2} \log \frac{[Na^+][Succinate]}{[Na^+][Succinate]} + C \]  

where \( C \) is a constant if it is assumed that bilateral Na+ and intracellular substrate concentrations remained unchanged during measurements. C can be eliminated when the \( V_r \) shift is determined by changing succinate from 20 to 200 μM.

RESULTS

Sequence Homology and in Situ Hybridization—The SDCT1 clone encoded a 587-amino acid-residue protein that has 75% identity to the rabbit NaDC-1 (25) and 77% to the human kidney hNaDC-1 (26). During this study, rat intestinal and renal Na+−dicarboxylate cotransporters were also identified (27, 28) and showed 98% amino acid identity to SDCT1 respec-
tively. However, functional characterization of these proteins has not been reported. High stringency in situ hybridization experiments demonstrated that SDCT1 is predominantly localized in S3 segments of proximal tubules in the outer stripe of outer medulla (Fig. 1a) and in a small subset of tubular cells in the outer part of inner medulla (not shown). In duodenum, SDCT1 is strongly expressed by enterocytes lining the intestinal villi (Fig. 1b). In liver, SDCT1 is relatively strongly expressed by a small subset of cells (Fig. 1c). In lung, SDCT1 is expressed in cells of the bronchiole epithelium (Fig. 1f) and in cells of the alveolar epithelium (Fig. 1h). In epididymis, SDCT1 is strongly expressed in cells in the initial segment of the tubular epithelium (Fig. 1j). In the epididymis head, more proximal segments express moderate SDCT1 levels, whereas more distal segments express very low levels (Fig. 1l).
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rate-evoked inward currents were pH-sensitive and stimulated by increasing the proton concentration in the solution (Fig. 2c). In contrast, succinate-evoked cotransport was not pH-sensitive between pH 5.5 and pH 7.5, although it was slightly reduced at pH 8.5 (Fig. 2d). Because succinate has a pK value (pK2) of 5.6, these data suggest that it can be transported either in its −1 or −2 form. When chloride was replaced by gluconate, no remarkable difference in current was observed at various potentials (Fig. 2e), demonstrating that SDCT1-mediated transport is chloride-independent.

Ion and Substrate Specificity—In addition to sodium, potassium can also drive substrate transport. At −50 mV, 50 mM K+ and pH 7.5, 1 mM citrate stimulated currents averaging 20 ± 3% (n = 5) of those evoked in the presence of 100 mM Na+, under the same conditions. In contrast, when 1 mM succinate was used in place of 1 mM citrate, the K+-coupled current was only 10% of the Na+-coupled current, and no detectable currents were observed with 50 μM succinate. These data indicate that K+ couples to substrates with a lower efficiency than Na+.

When 1 mM citrate or 50 μM succinate was added to solution containing 100 mM Li+ in place of Na+, no detectable currents were stimulated, indicating that lithium cannot drive SDCT1-mediated cotransport. In fact, lithium had a significant inhibitory effect. In the presence of 50 μM succinate, when 3 mM Li+ was added to solutions containing 100 and 20 mM Na+, the Na+-coupled currents were reduced to 50 ± 2 and 28 ± 1% (n = 3), respectively. Because higher Li+ inhibition was observed at lower Na+ concentration, these data suggest that Li+ can compete with Na+ for binding but is not itself translocated.

Currents evoked by citrate addition were voltage-dependent (Figs. 3a and 4a). At −50 mV and pH 7.5, the apparent affinity for citrate was 0.64 ± 0.01 mM (n = 5) (Fig. 3b). Currents elicited by application of substrates (1 mM) to the same oocytes ranked in the following order: fumarate > l-malate > succinate > α-ketoglutarate > oxaloacetate > citrate > l-aspartate > l-glutamate > d-aspartate > β-glutamate (Fig. 3d). On the other hand, neutral and positively charged amino acids, maleic acid, amiloride, dimethylsulfoxide, and monoxycarboxylates (l-lactate, pyruvate, nicotinate, acetate, and γ-hydroxybutyrate), all at 1 mM levels, did not evoke detectable currents (Vh = −50 mV). Likewise, these substances had no inhibitory effects on 1 mM citrate-evoked currents, which is similar to results obtained from tracer measurements for human NaDC-1 (29). These results indicate that monocarboxylates are not transported by SDCT1. The Krebs cycle metabolites succinate, α-ketoglutarate, and oxaloacetate stimulated SDCT1-mediated currents with high affinities and high efficiencies, whereas both L-/D-glutamate and aspartate generated currents with low affinities and low efficiencies (Table I).

The apparent affinity constant for sodium was 19.5 ± 0.7 mM, and the Hill coefficient (nH) was 2.07 ± 0.09 (n = 6) at 2 mM citrate, Vh = −50 mV, and pH 7.5 (Fig. 4b). This suggests that at least 2 sodium ions are coupled to each citrate molecule. In 4 oocytes from different batches, the maximal current for sodium (I max(Na)) increased about 3-fold upon hyperpolarization from 0 to −160 mV, and the apparent affinity constant for sodium (K Na(Na)) decreased 50% from −40 to −160 mV (Fig. 4c).

Proton Dependence of Citrate and Glutamate Transport—At −50 mV, the apparent affinity for citrate increased about 10-fold to 57 ± 8 μM (n = 4) when pH decreased from 7.5 to 5.5 (Fig. 3, b and c). These observations are consistent with previous findings on rabbit and human NaDC-1 (29) and renal membrane vesicles (2, 11). In contrast, succinate-evoked currents were pH-independent (Fig. 2d). The proton affinity constant (K H(Na)) was determined at [Cit] = 1 mM (Fig. 5a) and averaged 62 ± 14 nM (corresponding to pH 7.2 ± 0.1) from 3

tocytes, was strongly labeled for SDCT1 message (Fig. 1d). These SDCT1-positive cells did not form a particular pattern and were scattered throughout the liver. In lung, SDCT1 message was expressed by cells in the bronchiole epithelium (Fig. 1f) and by cells in the alveolar epithelium (Fig. 1h). Finally, SDCT1 mRNA was expressed in the tubular epithelium of epidiymis: high levels of SDCT1 mRNA were present in epithelial cells in the initial segment and more moderate levels in proximal segments of the epidiymis head (Fig. 1f). In more distal segments of epidiymis head (Fig. 1f) or in segments of epidiymis tail (not shown), SDCT1 labeling was negative.

Expression in Xenopus Oocytes—When mRNA of the SDCT1 was injected into Xenopus oocytes, 25- and 50-fold increases in [14C]citrate and succinate uptakes were obtained, respectively, with H2O-injected oocytes (Fig. 2a). Using the two-microelectrode voltage-clamp technique, SDCT1-mediated transport was shown to be electrogenic and sodium-dependent, as no significant currents were observed upon citrate addition when NaCl was substituted by choline-chloride (Fig. 2b). Citrate-evoked inward currents were pH-sensitive and stimulated by increasing the proton concentration in the solution (Fig. 2c). In contrast, succinate-evoked cotransport was not pH-sensitive between pH 5.5 and pH 7.5, although it was slightly reduced at pH 8.5 (Fig. 2d). Because succinate has a pK value (pK2) of 5.6, these data suggest that it can be transported either in its −1 or −2 form. When chloride was replaced by gluconate, no remarkable difference in current was observed at various potentials (Fig. 2e), demonstrating that SDCT1-mediated transport is chloride-independent.

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**FIG. 3.** I-V curves of citrate-evoked currents and pH dependence of the apparent affinity constants and maximal currents for citrate. a, currents due to addition of citrate ranging from 0.02 to 0.5 mM at pH 5.5 were plotted against the membrane potential. b, concentration dependence of SDCT1-mediated currents at V_m = -50 mV and pH 7.5. For these representative data, a Michaelis-Menten fit (Equation 4) gave K_m = 0.70 ± 0.10 mM and I_max = -58.6 ± 3.4 nA. The average K_m was 0.64 ± 0.01 (n = 5). c, currents recorded at pH 5.5 yielded K_m = 49 ± 4 μM and I_max = -90.6 ± 2.4 nA. d, to obtain the substrate specificity, currents due to application of various substrates at 1 mM were compared in the same oocytes (n = 3–8).

![Image](image.png)

oocytes. The pH dependence of the [14C]citrate uptake was also determined and exhibited a Michaelis-Menten relationship with a similar K_m value (pH 7.2 ± 0.3, see Fig. 5b). The uptake vanished at high pH, indicating that trivalent citrate was not remarkably transported. Because proton translocation is not associated with transport of succinate (and other Krebs cycle intermediates, e.g. α-ketoglutarate) (data not shown), protons are unlikely to be coupling ions for citrate uptake. We propose that protons serve to protonate the trivalent form of citrate and that the divergent form is the predominant form transported.

For pH ≥ 5.5, citrate is both in the Cit^-2 and Cit^-3 form, and their relative proportion in solution is described by the equilibrium constant pK_a (≈ 6.4). If Cit^-3 had no effect on Cit^-2 transport, then we would expect to have the same maximal currents for citrate (I_max) at different pH. However, measurements performed in the same oocytes revealed that I_max at pH 5.5 was 70% higher than I_max at pH 7.5 (see example in Fig. 3), indicating that Cit^-3 inhibits Cit^-2 transport. If inhibition was noncompetitive, currents at high substrate or proton concentrations would be expected to decrease (34). If inhibition was uncompetitive, the current as a function of [H^-] would be expected to be sigmoidal and to drop at high substrate concentrations. Because both of these predictions are not supported by our data, it is reasonable to assume competitive inhibition of Cit^-2 transport by Cit^-3. Under this assumption and using Equation 5, we found that K^2 = 1.0 ± 0.1 μM, which corresponds to a pK value of 6.0 ± 0.1, K^3 = 33 ± 4 μM and K^3' = 1.5 ± 0.2 μM (n = 3). The obtained pK value is close to pK_a of citrate, indicating that citrate protonation is likely to be determined by bulk solution. This result shows that the apparent affinity constant for divalent citrate is in fact high and close to K_m for (total) citrate at pH 5.5 and that the trivalent citrate is a relatively low efficiency inhibitor. Because divalent citrate is equivalent to a dicarboxylate in terms of the number of negative charges, our data show that SDCT1 transports only dicarboxylates.

![Image](image.png)

**TABLE I**

| Substrate       | K_m (μM) | I_max/K_m (nA/μM) |
|-----------------|----------|-------------------|
| Succinate       | 24 ± 2   | 4410 ± 1147       |
| α-Ketoglutarate | 45 ± 6   | 1893 ± 376        |
| Oxaloacetate    | 53 ± 11  | 1508 ± 241        |
| Citrate at pH 5.5 | 57 ± 8 | 1299 ± 210        |
| Citrate         | 0.64 ± 0.01 | 105 ± 17          |
| L-Aspartate     | 1.9 ± 0.4 | 77 ± 15           |
| L-Glutamate     | 5.5 ± 0.6 | 21 ± 3            |
| D-Aspartate     | 18 ± 5    | 18 ± 5            |
| D-Glutamate     | 11 ± 3    | 12 ± 7            |

**FIG. 4.** Sodium dependence of SDCT1-mediated currents at pH 7.5. a, I-V curves at various [Na^+] were obtained as the difference between currents before and after application of 2 mM citrate. b, at -50 mV, the sigmoidal relationship of the oocyte transport versus [Na^+] was fitted to the following Hill equation:

\[
I = \frac{I_{\text{max}} [S]^n}{K_{\text{Na}}^n + [S]^n}
\]

yielding K_{Na} = 17.6 ± 0.7 mM, n_H = 1.9 ± 0.1, and I_{\text{max}} = 202 ± 4 nA. The averaged K_{Na} and n_H from 3–6 oocytes were 19.5 ± 0.7 mM and 2.07 ± 0.09, respectively. c, voltage dependence of K_{Na}, n_H and I_{\text{max}} was obtained by fitting the data shown in panel a to the Hill equation.
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Fig. 5. \( H^+ \) sensitivities obtained from current and/or tracer measurements. a, under voltage-clamp conditions \((V_m = -100 \text{ mV})\), average currents (for pH from 5.5 to 8.5 with increment of 0.5) obtained from 3 oocytes were fitted to the Hill equation with \( K_{\text{H\text{max}}} = 59.7 \pm 16.7 \text{ nM} \) (corresponding to pH 7.3 \pm 0.3), \( n_H = 0.67 \pm 0.08 \), and \( I_{\text{H\text{max}}} = 392 \pm 65 \text{ nA} \). b, \(^{14}C\)citrate uptakes at 1 mm level were obtained and averaged from 8–10 oocytes at each pH value (from 5.5 to 9.5 with increment of 1.0). From the Michaelis-Menten fit, \( K_{\text{Cit\text{H}}} = 62 \pm 20 \text{ nM} \) (or pH 7.2 \pm 0.3) and \( I_{\text{Cit\text{H\max}}} = 653 \pm 46 \text{ pmol/10 min/oocyte} \). c, currents evoked by 2 mm l-glutamate at pH 5.5, 6.5, 7.5, and 8.5 are illustrated. The solid line represents the Michaelis-Menten fit plus a constant c with \( K_{\text{Glu\text{H}}} = 8.1 \pm 2.4 \text{ nM} \) (or pH 8.1 \pm 0.3), \( I_{\text{Glu\text{H\max}}} = 233 \pm 20 \text{ nA} \), and \( c = -9.0 \pm 4.6 \text{ nA} \). The dashed line is the best fit assuming that only the \(-2\) form of glutamate is transported \((I = I_{\text{Glu\text{H\max}}} K_{\text{Glu\text{H}}} / (K_{\text{Glu\text{H}}} + [\text{Glu}])\), with \( K_{\text{Glu\text{H}}} = 10.8 \pm 2.7 \text{ nM} \) (or pH 8.0 \pm 0.2) and \( I_{\text{Glu\text{H\max}}} = 228 \pm 18 \text{ nA} \).

Interestingly, glutamate transport exhibited a pH dependence opposite to that of citrate transport. Currents resulting from addition of 2 mm l-glutamate at pH 7.5, 6.5, and 5.5 represented 31.8, 9.6, and 4.4% of that at pH 8.5 (Fig. 5c). Because glutamate has a pK2 of 9.67 for the amino group, this result indicates that glutamate is largely transported in its \(-2\) form (Glu\(^{-2}\)) and that the affinity for Glu\(^{-2}\) is in fact high. However, if only the \(-2\) form was transported, we would predict, based on the Michaelis-Menten fit (dashed curve in Fig. 5c), a much lower current at pH 6.5 and no current at pH 5.5, contrary to the observed data (solid circles in Fig. 5c). This suggests that the predominant \(-1\) form is also transported, although at a much lower transport rate. The pH dependence of glutamate and citrate transport supports our concept that protons affect SDCT1-mediated transport through substrate protonation or deprotonation. Selective transport of either Cit\(^{-2}\) or Glu\(^{-2}\) would result in pH changes on both trans and cis sides of the membrane.

Voltage-dependent Steps—To gain insight into the voltage dependence of the Na\(^{+}\),H\(^{+}\) and substrate concentration, the binding processes are fast enough so that translocation of the loaded transporter or relocation of the free transporter across the membrane becomes rate-limiting and determines \( I_{\text{max}} \). We determined the voltage dependence of \( I_{\text{max}} \) for citrate, sodium, and proton (Fig. 6a), all of which exhibited a 3-fold increase upon hyperpolarization of \( V_m \) from \(-20\) to \(-160\) mV. This indicates that translocation of either the loaded or the free transporter or both are voltage-dependent.

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To gain insight into the voltage dependence of the Na\(^{+}\),H\(^{+}\) and substrate binding processes, we plotted the apparent affinity constants \( K_m \) as a function of \( V_m \) (Fig. 6b). The citrate affinity decreased with hyperpolarization, which is consistent with unfavorable binding of negative charge at hyperpolarized \( V_m \). The sodium affinity increased with hyperpolarization, consistent with preferred binding of positive charge at negative
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Under voltage-clamp conditions (measurements of substrate-evoked currents and tracer uptakes), the ratio averaged 1.04 when the incubation time was 4 min. When both data are plotted together, the charge moved was calculated by integrating the succinate-evoked currents at positive potentials. For experiments using succinate (open circles), the slope of the linear fit, which is equal to the charge:uptake ratio, is the same over the uptake period.

V_m Interestingly, despite the positive charge of H^+, its affinity had the same V_m dependence as that of citrate. This paradoxical V_m dependence can be explained if H^+ and Cit^{-2} react to form Cit^{-2} before being transported, resulting in a proton-biding affinity that is characterized by binding of the negatively charged Cit^{-2}. Thus H^+, unlike Na^+, is likely not a coupling ion but serves to protonate citrate before binding to the transporter.

Charge:Uptake Ratio—We measured the citrate or succinate-evoked current under voltage-clamp conditions and, at the same time, [14C]citrate or [14C]succinate uptake (see “Experimental Procedures”). The charge moved during uptake is equal to the time integral of the current (Fig. 7b). At pH 7.5, succinate is mainly in its -2 form, and our data presented above showed that citrate is also transported in its -2 form. It follows that both the sodium:citrate and the sodium:succinate stoichiometries are 3:1 for SDCT1.

Inhibition of SDCT1 Transport and Reversal Potentials—In oocytes expressing SDCT1 but not in H_2O-injected oocytes, external Na^+ in the absence of external substrate inhibited outward currents at positive potentials (Fig. 8, a, b, and e). These SDCT1-specific outward currents are likely to correspond to the reversed transport generated by intracellular dicarboxylates. In contrast, succinate (200 μM) or citrate (2 mM) did not significantly inhibit outward currents in the absence of extracellular Na^+ (see curve corresponding to 0 Na^+ in Fig. 4a). Phloretin (0.5 mM) inhibited both inward and outward currents obtained in the presence of 20 mM external Na^+ and 200 μM succinate (Fig. 8, c, d, and e) with an estimated inhibition constant (K_i) of 40 μM at -50 mV. No significant phloretin-inhibitable currents were detected in H_2O-injected oocytes. In the absence of external substrate but in the presence of sodium, a small phloretin-inhibitable inward current (at V_m < -90 mV, Fig. 9) along with a phloretin-inhibitable outward current (at V_m > -90 mV) was observed. This indicates that an SDCT1-mediated uncoupled sodium leak exists and that at least one sodium ion binds first to the transporter. Thus, the small currents at V_m < -100 mV in Fig. 8c (open circles) arise from
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**DISCUSSION**

**Localization**—In the present study, we described a high affinity Na\(^+\)-coupled dicarboxylate transporter, SDCT1, from rat kidney, its localization, and a number of biophysical characteristics. Our in situ hybridization studies demonstrated that SDCT1 is localized in the late portion of proximal tubules (S3 segments). Previous studies using membrane vesicles prepared from renal cortex revealed low affinity transport in BBMV (K\(_{\text{m}}\) for succinate = ~1 mM) and high affinity transport in BLMV (K\(_{\text{m}}\) for succinate = ~10 mM). In BBMV, citrate but not succinate transport was highly dependent on extracellular pH. In BLMV, extracellular pH had a remarkable effect on succinate and methylsuccinate uptake but little effect on citrate uptake (12, 13). In the present study, using oocytes expressing SDCT1, succinate transport was with high affinity and pH-independent (Fig. 2d), and citrate transport increased about 4-fold when pH was decreased from 8.5 to 5.5 (Fig. 5). Thus, the pH dependence of SDCT1-mediated transport suggests that SDCT1 is an apical transporter. It is possible that the previously studied low affinity system in BBMV has a high capacity and masked the high affinity transport of SDCT1 in S3 segments. In fact, tubule perfusion studies in rabbit kidney indicated that dicarboxylate reabsorption occurs in S3 segments with low capacity compared with the early part (i.e. S1 segments) (10, 38). It is likely that high affinity low capacity SDCT1 participates in final reabsorption of dicarboxylates that escape the early part of proximal tubules where the low affinity transporters rabbit and human NaDC-1 are expected to be expressed (25, 26). Similar situations have been demonstrated for the reabsorption of other solutes such as glucose.

**Stoichiometry**—Previous vesicle studies indicated a sodium:substrate stoichiometry of 2:1 to 3:1. For SDCT1, a 3:1 stoichiometry was obtained using both the voltage-clamp tracer method and the thermodynamic method. The voltage-clamp condition was critical in these stoichiometry studies to accurately determine specific charge accumulation in oocytes. Currents were continuously recorded during the entire radioisotope uptake. This is important as currents remarkably change during uptake (Fig. 7a). The 3:1 stoichiometrical ratio has physiological implications. Firstly, it can create higher dicar-
boxylate gradients across the cell membrane than a 2:1 coupling mechanism. Secondly, the 3:1 stoichiometry of dicarboxylate transport results in an electronegenic transport that can utilize the existing membrane potential as a driving force for substrate accumulation. The high stoichiometry and high affinity ensure efficient reabsorption of trace amounts of dicarboxylates that escaped the early proximal kidney tubules.

**Inhibition of SDCT1-mediated Currents**—In oocytes expressing SDCT1, addition of 100 mM Na\(^+\) without external substrate evoked a large inward current (−120 nA; Fig. 2b). This current is SDCT1-specific because H\(_2\)O-injected oocytes only showed small currents (less than 20 nA). The current is not a sodium leak because 1) the total conductance of the oocyte at 100 mM Na\(^+\) is lower than that in the absence of Na\(^+\) and 2) the current amplitude decreased with hyperpolarization (Fig. 8, a, b, and e). This indicates that addition of 100 mM Na\(^+\) reduces outward currents at positive membrane potentials (trans-inhibition). Trans-inhibition by Na\(^+\) was also observed for sodium-dependent succinate transport in renal BBMV (4). The small currents at \(V_m < -120 \text{ mV}\) may be attributed to the SDCT1-mediated sodium leak and SDCT1-independent sodium fluxes. Phloretin (as well as Li\(^+\)) were found to inhibit both inward and outward currents mediated by SDCT1 (Fig. 8, c and d). At −50 mV and without external substrate, the phloretin-sensitive SDCT1 currents were generally outwardly directed with an \(V_i\) average of −85.3 mV. This observation validates the concept that Na\(^+\) inhibits SDCT1-specific outward currents.

**Endogenous SDCT1 Substrates in Xenopus Oocytes**—The \(V_i\) values can be used to estimate the concentration of intracellular substrate for SDCT1 in oocytes. Assuming that [Na\(^+\)]\(_i\) is 10 mM (44) and using Equation 1, the intracellular substrate concentration is equivalent to −100 \(\mu\)M succinate. If all of the intracellular substrate was citrate, this would be equivalent to an endogenous citrate concentration in the mM range, the same order of magnitude as in the renal tissue (45).

**Substrate Binding Order and Kinetic Model**—Although it is well established now that 3 sodium ions are stoichiometrically coupled to one substrate molecule, the order by which these 3 sodium ions and the substrate bind to SDCT1 remains to be resolved. Assuming that Na\(^+\) and substrate bind to the protein in an orderly fashion, there are four possibilities: SNNN, NSNN, NNSN, and NNNS, where N = Na\(^+\) and S = substrate. The presence of a phloretin-inhibitable sodium leak indicates that sodium ions bind before the substrate, eliminating SNNN. On the other hand, because external Na\(^+\) alone but not external substrate alone trans-inhibits outward currents, this suggests that Na\(^+\) and not substrate binds last to SDCT1, as can be explained by the King and Altman algorithm: in the absence of external Na\(^+\), NNSN (but not NNNS or NSNN) predicts a term containing [S]\(_i\) in the denominator of the expression for the outward current \(I_o\) (34, 46). When [S]\(_i\) is high, \(I_o\) will decrease (i.e. \(I_o\) is trans-inhibited by [S]\(_i\)), which contradicts our experimental observation. In contrast, using the above algorithm, both NNNS and NSNN predict a trans-inhibition by [Na\(^+\)]\(_i\), in the absence of external substrate, as observed, because [Na\(^+\)]\(_i\) appears in the denominator of the expression for \(I_o\). On the other hand, if the substrate was the last to bind, then the electroneutral exchange between external tracer substrate and internal cold substrate would be expected to be remarkable, resulting in an underestimation of charge to substrate uptake ratio, even under voltage-clamp conditions. However, the stoichiometry obtained by the tracer method was the same as that obtained by the thermodynamic method. Thus, our data are consistent with the models where the binding order for SDCT1 is NNSN or NSNN (Fig. 10).

**Pathophysiological Implications**—The importance of dicarboxylate reabsorption in the proximal tubules has been emphasized as the major determinant of urinary excretion of citrate, the potent inhibitor of calcium salt crystallization (20, 38). Hypocitraturia is therefore an important risk factor for kidney stone formation. Among many factors modulating renal citrate excretion, the most important is systemic acid-base status and K\(^+\) depletion (6). In metabolic alkalosis, proximal tubular citrate reabsorption is decreased, whereas it is increased in metabolic acidosis and chronic K\(^+\) depletion, the conditions associated with intracellular acidosis in the proximal tubular cells. Reduction of intracellular pH results in decreased citrate levels in the cytoplasm by increasing citrate entry into the mitochondria via proton-coupled tricarboxylate transport, followed by oxidative phosphorylation (6), and possibly by increasing cytosolic citrate utilization through ATP citrate lyase (47). This change stimulates citrate uptake into the cells, and citrate clearance decreases. Although it has been inferred that the key determinant of hypocitraturia is intracellular acidosis and changes in citrate metabolism, the significance of extracellular (luminal) pH in the alteration of citrate reabsorption was also emphasized (38). Our studies clearly confirm the concept that the pH sensitivity of citrate transport mediated by SDCT1 is due to changes in the proportion between the transported form (Cit\(^-\)) and the inhibitory form (Cit\(^2-\)).

There is evidence that apical citrate uptake is regulated by chronic adaptations. Brush border membrane vesicles from chronically K\(^+\)-depleted rats demonstrate increases in the maximal rate of the Na\(^+\)-coupled citrate transport without changes in the affinities for sodium or citrate (48). Chronic metabolic acidosis in rats also resulted in enhanced citrate transport in brush border membrane vesicles when compared with control rats (23). Future experiments will be needed to determine the regulation of SDCT1 in chronic adaptations.

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