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Expression Cloning and Characterization of a Novel Sodium-Dicarboxylate Cotransporter from Winter Flounder Kidney*

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A cDNA coding for a Na^+-dicarboxylate cotransporter, fNaDC-3, from winter flounder (*Pseudopleuronectes americanus*) kidney was isolated by functional expression in *Xenopus laevis* oocytes. The fNaDC-3 cDNA is 2384 nucleotides long and encodes a protein of 601 amino acids with a calculated molecular mass of 66.4 kDa. Secondary structure analysis predicts at least eight membrane-spanning domains. Transport of succinate by fNaDC-3 was sodium-dependent, could be inhibited by lithium, and evoked an inward current. The apparent affinity constant (K_m) of fNaDC-3 for succinate of 30 μM resembles that of Na^+-dicarboxylate transport in the basolateral membrane of mammalian renal proximal tubules. The substrates specific for the basolateral transporter, 2,3-dimethylsuccinate and *cis*-aconitate, not only inhibited succinate uptake but also evoked inward currents, proving that they are transported by fNaDC-3. Succinate transport via fNaDC-3 decreased by lowering pH, as did citrate transport, although much more moderately. These characteristics suggest that fNaDC-3 is a new type of Na^+-dicarboxylate transporter that most likely corresponds to the Na^+-dicarboxylate cotransporter in the basolateral membrane of mammalian renal proximal tubules.

Krebs cycle intermediates represent important fuels for renal proximal tubule cells (1). Di- and tricarboxylates are taken up from the glomerular filtrate via the brush-border (luminal) membrane as well as from the blood across the basolateral membrane by sodium-coupled dicarboxylate transporters (2). The transporter located in the basolateral membrane may be directly involved in the secretion of some anionic drugs (3). In addition, this transporter maintains an outwardly directed dicarboxylate gradient, which drives organic anion excretion via the so-called *p*-aminohippurate (PAH)[1] transporter (4). By this mechanism, a great variety of endogenous and exogenous organic anions, including drugs and environmental chemicals, can be secreted (5).

Experiments with rabbit and rat renal membrane vesicles demonstrated that the dicarboxylate transporters in the brush-border (luminal) and basolateral membranes differ with respect to their substrate affinity (6, 7) and specificity (8). For the luminal transporter a K_m for succinate of 0.61 mM was determined, whereas the basolateral transporter exhibited a much higher affinity for succinate with a K_m of about 12 μM (6). Both 2,3-dimethylsuccinate and *cis*-aconitate inhibited the basolateral transporter, but not the luminal transporter (8, 9). Succinate uptake by the basolateral transporter was decreased by lowering the pH, whereas luminal succinate transport was independent of pH (6, 10, 11). Uptake of citrate by the basolateral transporter was independent of pH (6) or slightly increased by raising pH from 5.5 to 7.0 (12), whereas citrate transport by the luminal transporter was much higher at lower pH than at neutral pH (6).

In recent years, the luminal dicarboxylate transporters from rabbit, NaDC-1 (13), human, hNaDC-1 (14), and rat kidney, rNaDC-1 (15) or SDCT1 (16), as well as homologous transporters from rat, Ri-19 (17), and *Xenopus laevis* intestine, NaDC-2 (18), have been cloned. In contrast, the basolateral transporter has not yet been characterized on the molecular level.

Experiments with renal tubules from flounder have shown that low concentrations of glutarate in sodium-containing bathing medium stimulate basolateral PAH uptake via PAH/dicarboxylate exchange (19). This argues for the existence of a basolateral sodium-dicarboxylate cotransporter in flounder kidney. Since the winter flounder (*Pseudopleuronectes americanus*) kidney consists almost exclusively of proximal tubules (20) resulting in an enrichment of the mRNA of interest, and has been successfully used for cloning of the PAH/dicarboxylate exchanger fROAT (21), we screened a flounder kidney cDNA library for expression of Na^+-dependent dicarboxylate uptake. A Na^+-dicarboxylate cotransporter, named fNaDC-3, was cloned. The functional characteristics of fNaDC-3 suggest that it represents the winter flounder homologue to the mammalian renal basolateral dicarboxylate carrier.

EXPERIMENTAL PROCEDURES

Construction and Screening of a cDNA Library, cDNA Sequencing, and Analysis—To clone the flounder dicarboxylate transporter, we screened a unidirectional cDNA library, which was constructed from a 2–4-kilobase size fraction of mRNA from winter flounder (*P. americanus*) kidney (22), as described previously (21).

Both strands of the isolated clone, fNaDC-3, were sequenced by dye terminator cycle sequencing (Applied Biosystems), starting with T7 and pUC sequencing primers (NAPS) and then proceeding through the sequence with fNaDC-3-specific primers (automatic sequencer: ABI Prism, Applied Biosystems). The sequence was assembled and analyzed with the Genetics Computer Group (GCG) software package, unless otherwise stated. Sequence homology searches were performed at the National Center for Biotechnology Information using the BLAST network service.

Oocytes, Injection, and Uptake Experiments—Stage V and VI oocytes (23) were prepared from *X. laevis* (Nasco) ovaries by treatment with

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF102261.

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[1] The abbreviation used is: PAH, *p*-aminohippurate.

collagenase (Type CLSII, Biochrom) and subsequent washing in Ca^{2+}-free oocyte Ringer's solution (see below) as described earlier (24). Oocytes were injected with 23 or 46 nl of cRNA (0.5–1 $\mu g/\mu l$) using a micropump (Drummond). During the screening process, injected oocytes were maintained for 2 or 3 days at 18 °C in Barth's medium (in mM: 90 NaCl, 2.4 $NaHCO_3$, 1 K_2SO_4, 0.8 $MgSO_4$, 0.3 $Ca(NO_3)_2$, 0.4 $CaCl_2$, 5 HEPES, pH 7.5), containing 12 mg/liter gentamycin (Refobacin^R, Merck). The medium was changed daily, and damaged oocytes were discarded. For uptake experiments, 5–10 oocytes were incubated for the indicated time in a modified Ringer's solution (in mM: 110 NaCl, 3 KCl, 2 $CaCl_2$, and 5 HEPES, pH 7.5) usually containing the radioactively labeled substrate [1,4-^{14}C]succinate (15–60 mCi/mmol, NEN Life Science Products) at 1 μCi/ml incubation solution. To determine the sodium-dependent succinate uptake, sodium was replaced by tetramethylammonium. All experiments were performed at room temperature.

Electrophysiological Measurements—The two-electrode voltage clamp technique was employed either in the current clamp or in the voltage clamp mode using a commercial amplifier (OC 725 A, Warner). After 10 min of membrane potential stabilization following microelectrode impalements, the response to succinate was measured, to verify that the oocytes had expressed fNaDC-3. Afterward, the membrane potential was clamped to a holding potential, V_h, of −60 mV. Oocytes were superfused at a rate of 4 ml/min with the test solutions at room temperature. To determine the charge to substrate coupling ratio, current evoked by 1 mM succinate was measured under voltage clamp at the initial membrane potential, and afterward tracer uptake of 1 mM succinate was determined for the same individual oocytes. Current was converted to the rate of net charge influx according to the equation: rate of net charge influx (mol/min) = $60 \times A/F$, where A represents current and F the Faraday's constant (9.65×10^4 Coulombs/mol).

Data Representation—All data are given as means ± S.E.

RESULTS AND DISCUSSION

Expression Cloning of fNaDC-3—A winter flounder cDNA library was screened for Na^+-dependent succinate uptake by injection of cRNA into oocytes. A single cDNA clone was isolated, which increased succinate transport 250-fold over controls (Fig. 1*A*). This stimulated uptake was completely sodium-dependent. Based on these results, the cDNA of this positive clone was named fNaDC-3, or flounder Na^+-dicarboxylate cotransporter 3.

Testing the membrane potential response in the presence of 1 mM succinate, we detected a depolarization of 52.9 ± 7.8 mV (n = 12 oocytes) in oocytes injected with cRNA from fNaDC-3 (Fig. 1*B*). When the same oocytes were investigated afterward in voltage clamp experiments (holding potential −60 mV), addition of 1 mM succinate induced a net inward current, I_{succ}, of −119 ± 24 nA (n = 12). Cell membrane conductance at −60 mV significantly ($p < 0.001$) increased from 0.4 ± 0.07 microsiemens under control conditions to 1.8 ± 0.26 microsiemens in the presence of 1 mM succinate. Control oocytes showed barely detectable changes in membrane potential, in inward current (Fig. 1*B*, H_2O), and in cell membrane conductance upon addition of 1 mM succinate. To determine the coupling ratio of substrate to charge influx, the same oocytes were first analyzed for succinate induced currents under voltage clamp conditions and then assayed individually for tracer uptake, both at 1 mM succinate. Current converted to the rate of net charge influx was 21.9 ± 2.8 pmol/min/oocyte, and uptake rate was 19.7 ± 2.4 pmol/min/oocyte (n = 9 oocytes). Thus, the transport of the divalent negatively charged succinate was most likely accompanied by movement of three Na^+ ions.

fNaDC-3 Sequence and Comparison with Related Data Base Sequences—The nucleotide and predicted amino acid sequences of fNaDC-3 are shown in Fig. 2*A*. The fNaDC-3 cDNA is 2384 nucleotides in length with a major open reading frame coding for a protein of 601 amino acids with a calculated molecular mass of 66.4 kDa. This reading frame contains several potential AUG codons, which lie in a favorable context for translation initiation according to Kozak's rules (25). Due to its 5′-proximal localization, the first of these was tentatively assigned as the first codon.

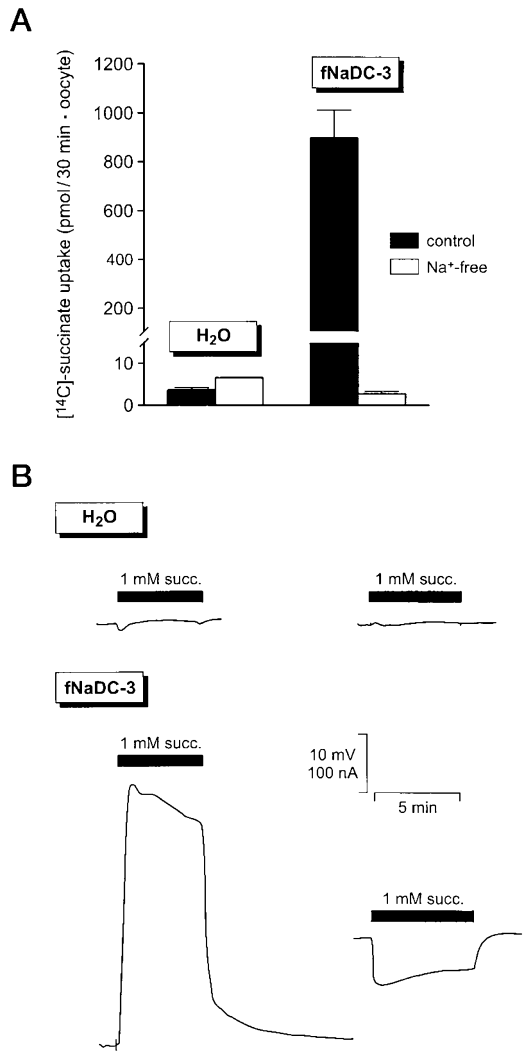

FIG. 1. **Succinate uptake (*A*) and succinate dependent currents (*B*) in oocytes expressing fNaDC-3.** Oocytes were injected with 40 ng each of fNaDC-3 cRNA. Control oocytes were injected with the same volume of water. *A*, after 3 days, uptake of [^{14}C]succinate (58 μM in the incubation medium) was measured within 30 min in the presence (*filled bars*) or absence of sodium (*open bars*). Uptake data are means + S.E. of 10 oocytes in a representative of four independent experiments. *B*, succinate (1 mM) evoked a depolarization (*left trace*) and an inward current (*right trace*) in fNaDC-3-injected oocytes. Water-injected oocytes showed no depolarization and no inward current upon 1 mM succinate. The data shown are from one representative oocyte out of 12 from 6 animals.

Secondary structure analysis using several different programs, *i.e.* Sosui (26), TMpred (27), and TopPred2 (28), predicts 12 transmembrane domains in overlapping regions. Only eight of these are identified by Kyte-Doolittle hydropathy analysis (29) as shown in Fig. 2*B*. However, assuming that at least one of the two potential *N*-glycosylation sites (N*X*S/T) at the C-terminal end of the protein (Asn^{586} and Asn^{598}) is actually used, both the N terminus and the C terminus would have to be localized extracellularly according to all these models. Such an orientation has been suggested previously for the Na^+-D-glucose cotransporter SGLT1 (30). There are several potential phosphorylation sites for protein kinase C and casein kinase II (*cf.* Fig. 2*A*). Only in the secondary structure predicted by Kyte-Doolittle analysis would any of these be localized on the cytosolic side of the membrane. However, to our knowledge, regulation of renal sodium-dicarboxylate cotransport by phosphorylation has not yet been demonstrated. On the other hand,

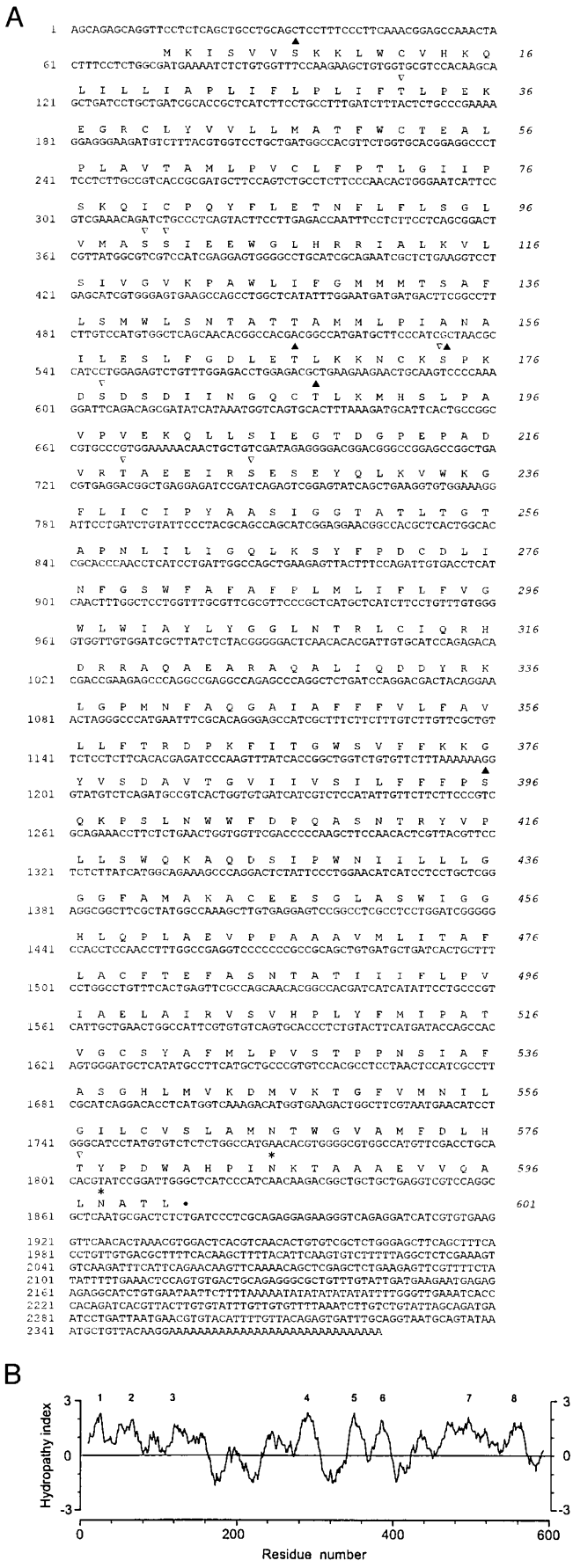

FIG. 2. **Nucleotide (*below*) and deduced amino acid sequence (*above*) (*A*) and Kyte-Doolittle hydropathy analysis (*B*) of fNaDC-3.** *A*, the nucleotides are numbered on the *left* and the amino acids in italics on the *right*. Potential *N*-linked glycosylation sites are indicated by *asterisks*. Putative protein kinase C (▲) and casein kinase II (▽) phosphorylation sites are indicated. ● denotes the first in-frame termination codon. The sequence has been submitted to the GenBank™ data base and was assigned the accession number AF102261. *B*, Kyte-Doolittle (29) hydropathy analysis using a window setting of 19 amino acids. The putative membrane-spanning domains are numbered.

C-terminal glycosylation sites are present in all Na^+-dicarboxylate cotransporters cloned to date, and glycosylation has indeed been shown for both the rabbit and human NaDC-1 (13, 14).

Aside from hypothetical proteins, a data base search revealed the highest homology of fNaDC-3 with six proteins, five of which represent Na^+-dicarboxylate cotransporters from kidney (human, rat, and rabbit NaDC-1) and intestine (rat Ri-19 and *X. laevis* NaDC-2) (Fig. 3), the other being the rat renal Na^+-sulfate cotransporter NaSi-1. Of these, the rat and rabbit NaDC-1 (15, 31), as well as NaSi-1 (32), have so far been associated with the luminal membrane of the epithelium. The degree of identity of the fNaDC-3 protein with the functional Na^+-dicarboxylate cotransporters is around 45% (in detail: 46% with *X. laevis* NaDC-2; 43, 44, and 43% with rat, rabbit, and human NaDC-1, respectively). The lower amino acid identity to the not yet functionally characterized Ri-19 (36%) might be attributable to possible frameshifts as discussed by Sekine *et al.* (15). On the other hand, the human renal NaDC-1 has a strikingly higher homology not only to rabbit and rat NaDC-1 (78 and 77% identity, respectively), but also to *X. laevis* intestinal NaDC-2 (66% identity). This suggests that the low homology of fNaDC-3 to other members of this transporter family might not be attributable to evolutionary distance alone.

Additionally, the sequence of fNaDC-3 shows almost no similarity to the other cloned dicarboxylate transporters between positions 166 and 237 (corresponding to amino acids 160–222 of rabbit NaDC-1 in the alignment). Interestingly, antisera raised against amino acids 164–223 of rabbit NaDC-1 only stained the luminal membrane of the proximal tubule (31). We conclude that the different immunoreactivity could be related to the lack of homology between the luminal and the basolateral transporter in this particular region of the protein. Taken together, the divergence of the fNaDC-3 sequence suggests that it could represent the basolateral Na^+-dicarboxylate cotransporter.

Functional Characterization of fNaDC-3 by Uptake Studies and Electrophysiological Measurements—In oocytes expressing fNaDC-3, uptake of succinate linearly increased with time up to 30 min ($r = 0.99$) with an uptake rate of 28.8 pmol/min × oocyte (three independent experiments with 10 oocytes per treatment each). To characterize the sodium dependence of fNaDC-3, we varied the sodium concentration from 0 to 110 mM (Fig. 4) by isoosmotic replacement with tetramethylammonium. [^{14}C]Succinate uptake exhibited sigmoidal relationship to the Na^+ concentration. Half-maximal activation of succinate uptake occurred at approximately 40 mM Na^+. Using the Michaelis-Menten equation, the best fit to the data could be obtained assuming a cotransport of 3 sodium ions, resulting in an apparent affinity constant of 39.6 ± 3.3 mM Na^+. When we replotted the data according to Hill (not shown), a straight line could be obtained with a slope of 2.5 ± 0.4, indicating positive cooperativity between multiple binding sites for Na^+. Together with the inward current induced by succinate (see Fig. 1*B*), these data indicate net movement of at least three sodium ions per succinate molecule. In the presence of 10 mM lithium, succinate uptake was inhibited by 66.4 ± 4.8% (three independent experiments, each carried out on 8–10 oocytes per treatment). Lithium (10 mM) also completely prevented mem-

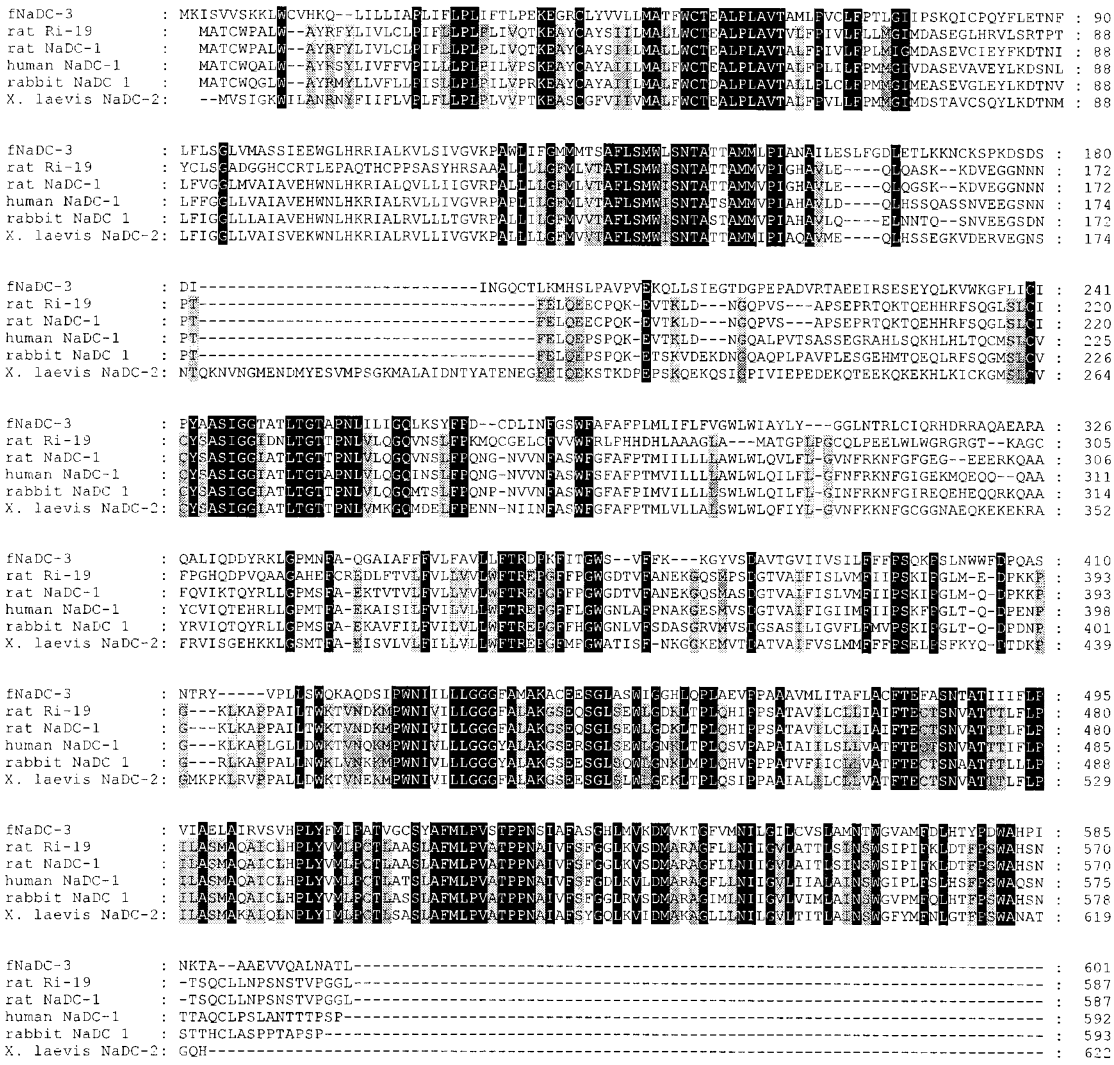

FIG. 3. **Comparison of the fNaDC-3 amino acid sequence with other known or presumed Na^+-dicarboxylate cotransporters.** The alignment was performed using the MAP program (36) with the PAM250 matrix. Amino acids conserved among all six sequences are *shaded black*, those identical between all previously published luminal Na^+-dicarboxylate cotransporters but different in fNaDC-3 are *shaded gray*.

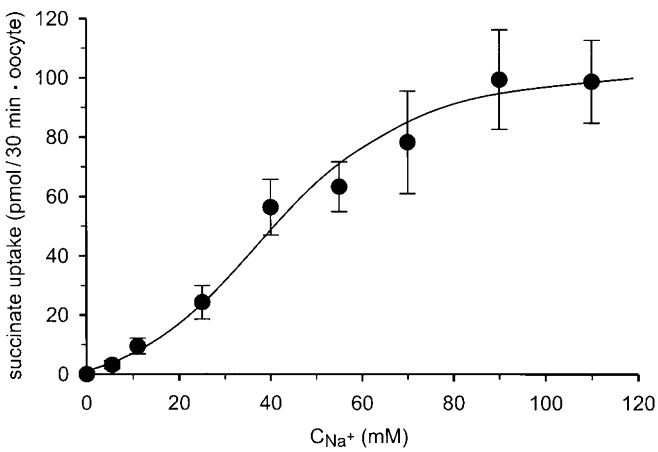

FIG. 4. **Dependence of fNaDC-3-mediated succinate uptake on Na^+ concentration.** Thirty-min uptakes of 16 μM succinate were measured in the presence of increasing Na^+ concentrations. Na^+ was isoosmotically replaced by tetramethylammonium. Results are means ± S.E. of four independent experiments with 6–10 oocytes per condition in each experiment. Uptake in the presence of 110 mM Na^+ was set to be 100%, and data were corrected for Na^+-independent uptake. Curve drawn by eye.

brane depolarization and the inward current induced by succinate (not shown). The inhibition by lithium, which is probably due to the replacement of one of the sodium ions by lithium, has been shown previously for dicarboxylate transport in the renal luminal (33, 34) and basolateral membranes (8, 10), as well as for the cloned NaDC-1 of rabbit (13, 35) and rat kidney (16). In contrast, lithium binds probably to all sodium binding sites of the intestinal transporter NaDC-2, albeit the V_{max} of succinate uptake in the presence of Li^+ was approximately one-third of that in the presence of Na^+ (18).

Succinate uptake in fNaDC-3-cRNA-injected oocytes showed saturation (Fig. 5) with a K_m value of 30.4 ± 13.5 μM succinate. Therefore, fNaDC-3 seems to encode for a high affinity succinate transporter similar to that detected in rabbit renal basolateral membrane vesicles, which has a K_m of 12 μM (6). In contrast, succinate uptake by brush-border membrane vesicles has a reported K_m of 0.61 mM (6). For the cloned luminal dicarboxylate transporters of rabbit and human kidney, NaDC-1 and hNaDC-1, expressed in oocytes, K_m values for succinate of 0.45 mM (13) and 0.36 mM (14), respectively, have been determined. Similarly, the calculated K_m value of the cloned dicarboxylate transporter from *X. laevis* intestine, NaDC-2, was 0.28 mM (18). Thus, one functional difference of fNaDC-3 from these low affinity transporters is its higher affinity for succinate.

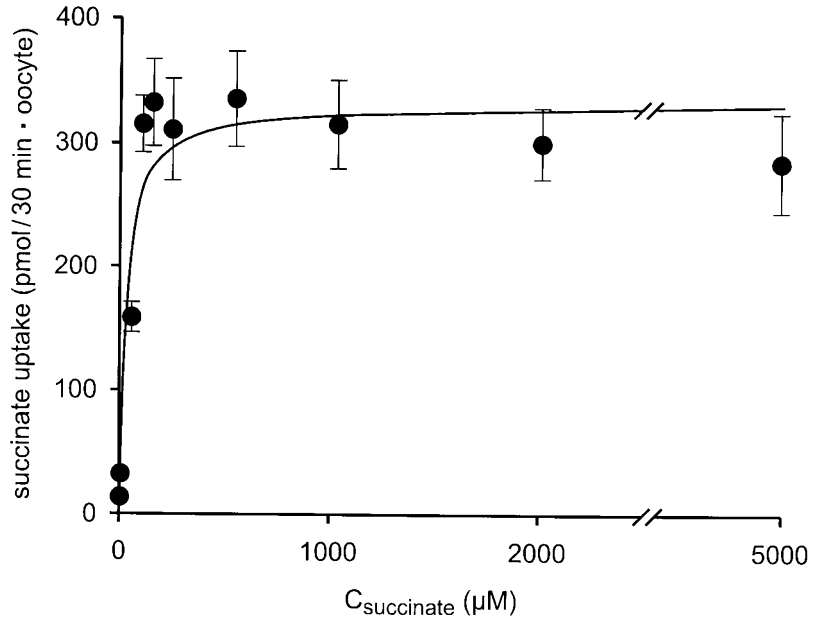

FIG. 5. **Kinetics of fNaDC-3-mediated succinate uptake.** The oocytes were injected with 20 ng of cRNA from fNaDC-3. 30-min uptake was measured in oocyte Ringer's solution containing various concentrations of succinate. Data are means ± S.E. of three independent experiments with 6–10 oocytes per condition. Fitting a Michaelis-Menten kinetics to the data, a K_m value of 30 ± 13 μM succinate was obtained.

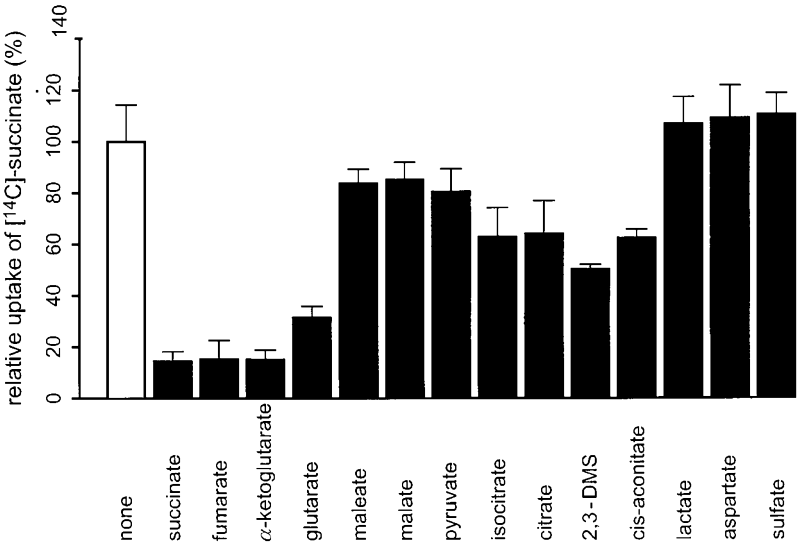

FIG. 6. **Inhibition of fNaDC-3-mediated succinate transport by other potential substrates.** Uptake of succinate (58 μM) was measured in the presence of 1 mM of the indicated test inhibitors for 30 min. 2,3-DMS = 2,3-dimethylsuccinate. Data are means + S.E. from three to six independent experiments with 5–10 oocytes per condition in each experiment.

Since the luminal and the basolateral dicarboxylate transporter also differ with respect to substrate specificity (see below), we characterized uptake of succinate in the presence of 1 mM of other potential substrates (Fig. 6). Inhibitory potency ranked in the following order: succinate = α-ketoglutarate = fumarate > glutarate > 2,3-dimethylsuccinate > *cis*-aconitate = citrate = isocitrate. On the other hand, there was only a small inhibition by malate, maleate, and pyruvate and no inhibition by lactate, sulfate, or aspartate. The expressed transporter preferred substrates with a *trans* configuration (fumarate) over those with a *cis* configuration (maleate), as has been reported previously for both the luminal and basolateral dicarboxylate transporters (6, 8, 10, 33).

Ullrich and co-workers (8, 9) reported that 2,3-dimethylsuccinate and cis-aconitate inhibit the basolateral, but not the luminal dicarboxylate transporter. In our experiments, the addition of 2,3-dimethylsuccinate or *cis*-aconitate caused a significant inhibition of succinate uptake. Testing the effects of 1 mM 2,3-dimethylsuccinate and *cis*-aconitate in electrophysiological measurements, we could also demonstrate inward currents of −128 ± 21 nA (n = 7) and −71 ± 20 nA (n = 4), respectively, evoked by these substances at −60 mV. Therefore, both substrates are actually translocated by fNaDC-3. In contrast, neither the cloned rabbit or human NaDC-1, nor *X. laevis* NaDC-2 were inhibited by dimethylsuccinate (13, 14, 18). Additionally, dimethylsuccinate did not evoke a significant current in oocytes injected with cRNA from rabbit NaDC-1 (35) or rat SDCT1 (16). The effect of *cis*-aconitate on the cloned luminal transporters has not been tested so far. Thus, fNaDC-3 resembles basolateral dicarboxylate transport in mammalian renal tubules (8) in its response to 2,3-dimethylsuccinate and *cis*-aconitate.

In experiments with membrane vesicles, the basolateral and the luminal dicarboxylate transporters also differed with respect to the pH dependence of succinate and citrate uptake. In the basolateral membrane, uptake of succinate was significantly stimulated by raising pH from 5.5 to 7.5, whereas succinate uptake by brush-border membrane vesicles was not influenced by pH (6, 10). Therefore, we compared succinate-mediated current at pH 7.5 with current at pH 6.5 and pH 5.5. At a holding potential of −60 mV, maximum current was found at pH 7.5 (−70.3 ± 7.9 nA), and was significantly ($p < 0.05$, n = 7 each) decreased at pH 6.5 (−59.6 ± 8.9 nA) and pH 5.5 (−57.7 ± 6.2 nA). In contrast, succinate transport in oocytes

injected with cRNA coding for the luminal transporters rabbit NaDC-1, hNaDC-1, SDCT1, or *X. laevis* NaDC-2 was independent of pH changes (13, 14, 16, 18). Thus, the pH dependence of succinate transport by fNaDC-3 differs from the previously cloned luminal dicarboxylate carriers, but resembles that of the renal basolateral transporter.

It has been well established that both the luminal and the basolateral dicarboxylate transporters accept citrate as a substrate (6, 10). However, a specific difference exists with respect to the dependence of citrate uptake on pH. Lowering pH increased citrate uptake by the luminal dicarboxylate transporter (6, 12), but did not significantly affect (6) or slightly decreased (12) basolateral citrate uptake. Therefore, we compared the citrate-evoked current at pH 5.5, 6.5, and 7.5 in oocytes injected with fNaDC-3 cRNA. There was a slight, but significant ($p < 0.05$, $n = 7$ each), decrease of citrate current from -58 ± 6.9 nA to -48 ± 6.8 nA when pH was lowered from pH 7.5 to pH 5.5, with no significant difference between the current evoked by citrate at pH 7.5 and pH 6.5 $-$ 54 $\pm$ 6.2 nA). Additionally, in six independent uptake experiments there was no significant difference in the inhibition of succinate uptake by 1 mM citrate at pH 6.0 and 7.5. The predominant ionic species of citrate at pH 7.5 is trivalent, whereas at pH 6.0 the divalent citrate dominates ($pK_3 = 6.4$). If citrate were transported in the trivalent form together with three sodium ions, transport should be electroneutral. However, we registered a depolarization and an inward current in the presence of 1 mM citrate. Depolarization and an inward current were also observed with another tricarboxylate, *cis*-aconitate. This can be explained either by a symport of even four sodium ions with one trivalent citrate or *cis*-aconitate molecule or only transport of the divalent tricarboxylate. In the latter case, the binding site of the transporter could be influenced by lowering pH such that affinity for both succinate and citrate were reduced. Whether this assumption is valid was not tested further.

In uptake experiments with the cloned luminal dicarboxylate transporters, citrate uptake was strikingly higher at pH 6.0 than at pH 7.5 (13, 14, 18). Consistent with these findings, electrophysiological measurements with the cloned luminal transporter of rat kidney (16) revealed a much higher citrate-induced current at pH 6.5 than at pH 7.5. Again, fNaDC-3 differs from the other cloned dicarboxylate transporters, while resembling basolateral transport.

In summary, a sodium-dicarboxylate cotransporter, fNaDC-3, has been cloned from flounder kidney. fNaDC-3 differs from the luminal dicarboxylate transporters cloned from rabbit and human kidney (NaDC-1) and *X. laevis* intestine (NaDC-2) with respect to substrate affinity, inhibition by dimethylsuccinate, and pH dependence of succinate and of citrate uptake. Inhibition by dimethylsuccinate and the effect of pH on succinate or citrate uptake also contrast with the data obtained for the luminal dicarboxylate transporter (rNaDC-1 or SDCT1) of rat kidney. Therefore, fNaDC-3 should to be regarded as a new type of Na^+-dicarboxylate cotransporter similar to the basolateral transporters characterized in rat and rabbit kidney.

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