Functional roles of cationic amino acid residues in the sodium-dicarboxylate cotransporter 3 (NaDC-3) from winter flounder

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Hagos, Yohannes, Jürgen Steffgen, Ahsan N. Rizwan, Denis Langheit, Ariane Knoll, Gerhard Bureckhardt, and Birgitta C. Bureckhardt. Functional roles of cationic amino acid residues in the sodium-dicarboxylate cotransporter 3 (NaDC-3) from winter flounder. Am J Physiol Renal Physiol 291: F1224–F1231, 2006. First published May 30, 2006; doi:10.1152/ajprenal.00307.2005.—In the present study, we determined the functional role of 15 positively charged amino acid residues at or within 1 of the predicted 11 transmembrane helices of the flounder renal sodium-dicarboxylate cotransporter fNaDC-3. Using site-directed mutagenesis, histidine (H), lysine (K), and arginine (R) residues of fNaDC-3 were replaced by alanine (A), isoleucine (I), or leucine (L). Most mutants showed sodium-dependent, lithium-inhibitable \(^{14}\)C-succinate uptake and, in two-electrode voltage-clamp (TEVC) experiments, \(K_m\) and \(\Delta V_{\text{max}}\) values comparable to wild-type (WT) fNaDC-3. The replacement of R109 and R110 by alanine and isoleucine (RR109/110AI) prevented the expression of fNaDC-3 at the plasma membrane. When the lysines at positions 232 and 235 were replaced by isoleucine (KK232/235II), the transporter was expressed but showed small transport rates and succinate-induced currents. K114I, located within transmembrane helix 4, showed \(^{14}\)C-succinate uptake similar to WT but relatively small inward currents. When K114 was replaced by arginine, glutamic acid (E), or glutamine (Q), all mutants were expressed at the cell surface. In \(^{14}\)C-succinate uptake and TEVC experiments performed simultaneously on the same oocytes, uptake was similar to or higher than WT, whereas succinate-induced currents were either comparable (K114R) to, or considerably smaller (K114E, K114I, K114Q) than, those evoked by WT. These results suggest that a positively charged residue at position 114 is required for electrogenic sodium-dicarboxylate cotransport.

In renal proximal tubules, NaDC-3 is located at the basolateral cell membrane (9, 33) and serves two functions. First, NaDC-3 maintains an outwardly directed gradient of \(\alpha\)-ketoglutarate driving the organic anion/dicarboxylate exchangers OAT1 (29, 35) and OAT3 (1, 31) and, thus, secretion of a large number of organic anions including widely used drugs and environmental toxins (2). Second, NaDC-3 as well as NaDC-1 provide proximal tubule cells with di- and tricarboxylates required for energy metabolism and gluconeogenesis (34). Studies in human and rat kidneys of different ages have demonstrated a higher abundance of NaDC-3 protein in older subjects (33). Although this may be beneficial for the supply of energy, the production of oxygen radicals is also increased, which may lead to damage of proximal tubule cells. In this respect, it is interesting that NaDC-3 exhibits a significant homology with INDY, the sodium-independent dicarboxylate transporter of Drosophila melanogaster (11, 14). A functional defect of INDY by mutation resulted in life span extension of the fly (27).

NaDC-3s have been cloned from a variety of species, including humans, the rat, mouse, flounder, and Xenopus laevis (4, 16, 20, 30, 32). They are members of the SLC13 gene family of the sodium-coupled anion transporters (15), and their gene is termed SLC13A3. This gene family includes also the NaDC-1s, sodium-dependent dicarboxylate transporters located at the luminal membrane of proximal tubule cells (15, 19). In most species, NaDC-1 shows a lower affinity for succinate than does NaDC-3 (17). In addition, the sodium-coupled citrate transporter NaCT (SLC13A5), which has a higher affinity for citrate than for succinate, also belongs to this gene family (12, 19). The predicted secondary structure of all SLC13 members revealed 11 transmembrane (TM) domains with an intracellular NH2 terminus and an extracellular COOH terminus (18, 37). On functional expression in X. laevis oocytes (21, 30) or cell lines (13, 24), NaDC-1s as well as NaDC-3s from different species showed sodium-dependent substrate uptake or substrate-associated inward currents (21, 30), indicating an electrogenic cotransport of three sodium ions with one divalent succinate. This stoichiometry was proven by simultaneous measurements of succinate uptake and succinate-induced current in the same oocyte (5, 13, 28).

The aim of the present study was to explore the potential functional role of positively charged amino acid residues at or within one of the putative TM domains of NaDC-3 from winter flounder kidney. Basic amino acids at the borders of TM helices may be involved in proper positioning of these seg-

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ments within the lipid bilayer, and those within TM helixes may form salt bridges and/or interact with charged substrates. By site-directed mutagenesis, we replaced 15 positively charged amino acids [arginine (R), histidine (H), and lysine (K); for location, see Fig. 1] and functionally characterized the mutants by measuring [$^{14}$C]succinate uptake and succinate-induced inward currents.

**MATERIALS AND METHODS**

**Expression of NaDC-3 and mutants.** Stage V and VI oocytes from *X. laevis* (Nasco, Ft. Atkinson, WI) were prepared by overnight treatment in control solution (in mM: 110 NaCl, 3 KCl, 2 CaCl$_2$, 5 HEPES/Tris, pH 7.5) supplemented with collagenase (type CLSII, Biochrom, Berlin, Germany). Subsequently, after being washed with Ca$^{2+}$/H$^{+}$-free control solution to remove adhering follicle cells, the oocytes were injected with 23 nl of 1 g/µl cRNA prepared either from the wild-type (WT) clone, from one of the mutants, or an equivalent volume of H$_2$O (“mock” oocytes). Afterward, injected oocytes were incubated for 3 days at 16–18°C in control solution supplemented with gentamicin (12 mg/ml) and 2.5 mM sodium pyruvate. The medium was changed daily, and damaged oocytes were discarded.

**In vitro cRNA synthesis.** The cDNA of WT winter flounder NaDC-3 (fNaDC-3) and of the mutants was used for in vitro cRNA synthesis. Initially, plasmids of WT and mutants were linearized with Not I restriction. In vitro transcription was carried out using a T7 mMessage mMachine kit (Ambion, Austin, TX). After purification, the cRNA by phenol-chloroform extraction, the cRNA was resuspended in water and adjusted to a final concentration of 1 g/µl.

**Site-directed mutagenesis.** The basic amino acids (arginine, histidine, and lysine) located within or close to the putative TM domains of fNaDC-3 were mutated to neutral amino acids [alanine (A), isoleucine (I), or leucine (L)]. We generated single mutants or, in the case of two adjacent positively charged amino acid residues, double mutants. The lysine at position 114 was replaced by isoleucine and, additionally, by glutamine (Q), glutamate (E), or arginine (R). All mutations were performed by using a QuickChange site-directed mutagenesis kit (Stratagene, Cambridge, UK). To verify the success of site-directed mutagenesis, both strands of the mutants were sequenced by dye-terminated cycle sequencing using specific fNaDC-3 primers. After purification, the PCR products were sequenced using an ABI automatic sequencer (ABI 377, Applied Biosystems, Weiterstadt, Germany). Table 1 includes the primers used for inserting the mutation in the WT NaDC-3 cDNA sequence.

**Electrophysiological studies.** These studies were carried out 3 days after cRNA injection at room temperature. Current recordings were made using the two-electrode voltage-clamp technique (TEVC) with a commercial amplifier (OC725, Warner, Hamden, CT). Microelectrodes were filled with 3 M KCl and had resistances of 1–10 MΩ. The resting membrane potential of the oocytes ranged between −28 and −46 mV, and holding currents to achieve a potential of −60 mV were in the range of −10 to −40 nA. Steady-state currents were obtained during 5-s voltage pulses from −60 mV to potentials between −90 and 0 mV in 10-mV steps. The current-voltage (I-V) relationships for

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**Figure 1. Model of putative secondary structure of flounder renal sodium-dicarboxylate cotransporter (fNaDC-3).** Prediction of the secondary structure of fNaDC-3 was performed with TopPred 2 (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html). Amino acids are indicated by single letter code. Each of the highlighted amino acids was mutated by site-directed mutagenesis.

**Table 1. Primers used for site-directed mutagenesis**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sense Primer Sequence</th>
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</thead>
<tbody>
<tr>
<td>HK14/15AA</td>
<td>GCTGTTGTCGCTGCGCCGCACCCTGATCCTCG</td>
</tr>
<tr>
<td>K36L</td>
<td>CGCTGCCGGAATGGGAAAGGTGCTTTAGG</td>
</tr>
<tr>
<td>R39I</td>
<td>GGCGGAAAAGGAGAATGTTTTACCTG</td>
</tr>
<tr>
<td>K78I</td>
<td>GGAATCTTGGGTGTTGGAATGACCTG</td>
</tr>
<tr>
<td>RK110/110A</td>
<td>GGATCTGGGCTCCATCAGATGTGGCTCAG</td>
</tr>
<tr>
<td>K114I</td>
<td>GCCGAGATGCTTGGATGTGGTCAGTTGG</td>
</tr>
<tr>
<td>K122A</td>
<td>GATCTGGGACTGGCCGCTGAGCTGG</td>
</tr>
<tr>
<td>KK232/235II</td>
<td>CCGAGATTACCTGATTTGTTGAGTATTCAT</td>
</tr>
<tr>
<td>KK374/375II</td>
<td>CCGAGATGCTGAGCTGGCCGCTGAGCT</td>
</tr>
<tr>
<td>R503I</td>
<td>GCTGTTGTCGCTGCGCCGCACCCTGATCCTCG</td>
</tr>
</tbody>
</table>

Bold and underlined nucleotides reflect the position of the mutation.
substrate-induced currents, \( \Delta I \), were determined by subtraction of the steady-state currents in the absence of the substrate from currents in the presence of the substrate, respectively. For kinetic experiments, oocytes were superfused with buffers of succinate concentrations up to 10 mM, and the induced currents were recorded at −60 mV. The kinetic parameters, \( K_m \) and \( \Delta I_{\text{max}} \), were obtained by Eadie-Hofstee analysis.

**Sucinate uptake experiments.** Expression of WT NaDC-3 and mutants in oocytes was confirmed by comparing the uptake of radio-labeled succinate between cRNA- and water-injected mock oocytes. Mutants in oocytes was confirmed by comparing the uptake of radio-labeled succinate between cRNA- and water-injected mock oocytes. Uptake of \([^{14}\text{C}]\)succinate (15–60 mCi/mmol; NEN Life Science) was measured under voltage-clamp conditions in oocytes expressing WT and mutant transporters. Oocytes were clamped at −60 mV, and the induced currents were recorded at 300 m/s pacing rate. The sodium-free buffer was obtained by replacing NaCl by tetramethylammonium chloride (TMACl). In this case, the pH was adjusted to 7.4 by 1 M TMAOH. \([^{14}\text{C}]\) Succinate uptake was stopped by aspiration of the incubation medium and several washes of the oocytes in ice-cold control solution. Oocytes were lysed in 1 M NaOH, and liquid scintillation counting was performed as described elsewhere (8). In a second set of experiments, uptake of \([^{14}\text{C}]\)succinate was determined after 30 min at room temperature in control solution containing in addition 18 \( \mu \)M labeled succinate. The sodium-free buffer was obtained by replacing NaCl by tetramethylammonium chloride (TMACl). This case, the pH was adjusted to 7.4 by 1 M TMAOH. \([^{14}\text{C}]\) Succinate uptake was stopped by aspiration of the incubation medium and several washes of the oocytes in ice-cold control solution. Oocytes were lysed in 1 M NaOH, and liquid scintillation counting was performed as described elsewhere (8).

** Immunohistochemistry of NaDC-3 surface expression.** Surface staining of WT and mutants was performed with a specific antibody generated in rabbits using a fNaDC-3-specific antigen (CKSP-KDSDS2; Eurogentec, Seraing, Belgium). Manually devitellinized oocytes were incubated for 5–10 min in 200 mM potassium aspartate, followed by an overnight fixation at −20°C in Dent’s solution (80% methanol/20% DMSO). After removal of the fixation solution, oocytes were incubated overnight at −4°C in 10% goat serum containing the anti-fNaDC-3 antibody in a dilution of 1:50. Afterward, the primary antibody was washed out with PBS (in mM: 140 NaCl, 4 KCl, 2 KH2PO4), and the oocytes were incubated with the secondary antibody (Alexa Fluor 488 goat anti-rabbit) at a dilution of 1:200 (Molecular Probes, Eugene, OR) for 1 h. To remove nonspecific labeling of the secondary antibody, the oocytes were washed several times with PBS and fixed for 30 min with 3.7% paraformaldehyde. The oocytes were embedded in acrylamide (Technovit 7100, Kulzer, South Bend, IN) according to the manufacturer’s instructions. Five-micrometer sections from the embedded oocytes were analyzed with a fluorescence microscope (Zeiss Axiolab S100TV, Jena, Germany) supported by digital imaging (Metamorph software, Universal Imaging, Jena, Germany).

**Chemicals.** Unless otherwise specified, all chemicals were of analytical grade and purchased from Sigma (Taufkirchen, Germany) or Merck (Darmstadt, Germany).

**Data analysis.** Data are expressed as means ± SE. All experiments were repeated with oocytes from at least two different frogs. Student’s t-test was used to reveal statistical significance at \( P < 0.01 \).

**RESULTS**

**Function of WT and mutant fNaDC3.** To investigate whether positively charged amino acids located in or near putative TM domains are important for function, we generated the mutants shown in Fig. 1. Initially, we tried to generate alanine mutants, but for unknown reasons some mutations containing the DNA codon for alanine did not form colonies after transformation of the mutant plasmid into *Escherichia coli* and incubation overnight. Therefore, we replaced the codons for other neutral amino acids such as isoleucine, resulting in successful growth of the colonies.

As shown in Table 2, most mutants were functional, i.e., they showed \([^{14}\text{C}]\) succinate uptake in the presence of 110 mM NaCl. Thereby, succinate uptake for the mutants HK14/15AA, K36L, R39I, K78I, K122A, R503I, and K548A was not significantly different from that observed for WT. The mutants RR109/110AI, KK232/235II, and KK374/375II revealed significantly higher \([^{14}\text{C}]\) succinate uptake rates compared with WT (\( P < 0.01 \)). When NaCl was completely replaced by TMACl, succinate uptake by WT and the investigated mutants dropped to 10% or less of the uptake in the presence of sodium (Table 2, 110 mM TMACl). Hence, all tested mutants retained their sodium dependence. Where tested, 10 mM LiCl in the presence of sodium inhibited succinate uptake by WT and mutant fNaDC3 (Table 2, 10 mM LiCl, 100 mM NaCl), and some residual uptake of succinate was observed in the presence of 110 mM LiCl (Table 2, 110 mM LiCl), suggesting unaltered interaction with lithium of the tested mutants.

** WT fNaDC-3 cotransports three Na+ with one divalent succinate and, hence, generates an inward current, \( \Delta I \), in voltage-clamped oocytes (3). Here, we used the TEVC method to determine the kinetic parameters, \( K_m \) and \( \Delta I_{\text{max}} \), of WT fNaDC-3 and its mutants. In each experiment, various succinate concentrations (\( \text{[S]} \)) were used, and the data were analyzed according to Eadie-Hofstee (linear plots of \( \Delta I \) against \( \text{[S]}/I \)). For each mutant, the experiments were performed at least three times with oocytes from different donors. As summarized in Table 3, WT fNaDC-3 showed a mean \( K_m \) of 22 \( \mu \)M and an \( \Delta I_{\text{max}} \) of −55 nA; the minus sign denotes an inward current. Most mutants showed an unchanged \( K_m \). Mutant RR109/110AI did not show any detectable current, and with

<table>
<thead>
<tr>
<th>Mutant</th>
<th>110 mM NaCl</th>
<th>110 mM TMACl</th>
<th>10 mM LiCl plus 100 mM NaCl</th>
<th>110 mM LiCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>fNaDC-3 (WT)</td>
<td>100.0 ± 5.5</td>
<td>5.6 ± 1.2</td>
<td>43.3 ± 6.3</td>
<td>23.1 ± 1.6</td>
</tr>
<tr>
<td>HK14/15AA</td>
<td>95.7 ± 19.3</td>
<td>10.0 ± 3.0</td>
<td>36.6 ± 3.0</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>K36L</td>
<td>73.1 ± 4.7</td>
<td>0.7 ± 0.1</td>
<td>38.3 ± 2.9</td>
<td>15.0 ± 1.8</td>
</tr>
<tr>
<td>R39I</td>
<td>114.0 ± 7.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>K78I</td>
<td>103.6 ± 10.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RR109/110AI</td>
<td>5.6 ± 1.4</td>
<td>1.53 ± 0.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>K114I</td>
<td>138 ± 16.7</td>
<td>7.5 ± 2.1</td>
<td>52.2 ± 12.0</td>
<td>10.9 ± 3.6</td>
</tr>
<tr>
<td>K122A</td>
<td>130 ± 10.7</td>
<td>0.4 ± 0.1</td>
<td>41.0 ± 3.9</td>
<td>21.6 ± 1.5</td>
</tr>
<tr>
<td>KK232/235II</td>
<td>28.2 ± 4.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>KK374/375II</td>
<td>66.8 ± 5.7</td>
<td>0.6 ± 0.1</td>
<td>29.1 ± 1.6</td>
<td>7.7 ± 0.4</td>
</tr>
<tr>
<td>R503I</td>
<td>124.7 ± 10.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>K548A</td>
<td>97.6 ± 6.3</td>
<td>0.9 ± 0.1</td>
<td>69.9 ± 2.4</td>
<td>10.4 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6–8 oocytes from 3–4 donors. All experiments were standardized by setting the wild-type (WT) \([^{14}\text{C}]\) succinate uptake at each day to 100%. Uptake of 18 \( \mu \)M \([^{14}\text{C}]\) succinate was determined for 30 min in oocytes expressing WT or mutants indicated in the first column. In 40 independent experiments using oocytes injected with an equal amount of cRNA, succinate uptake in WT was 2750 ± 36.6 pmol/30 min−1 oocyte−1, and mock oocytes showed a nonspecific succinate uptake of 6.2 ± 0.9 pmol/30 min−1 oocyte−1. Uptake buffer contained either 110 mM NaCl, 110 mM tetramethylammonium chloride (TMACl), 10 mM LiCl plus 100 mM NaCl, or 110 mM LiCl, respectively. ND, not determined. *\( P < 0.01 \) vs. WT fNaDC-3.
Table 3. Kinetic parameters determined by the 2-electrode voltage-clamp technique

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$, μM</th>
<th>$\Delta I_{\text{max}}$, nA</th>
<th>$n_{\text{m}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>fNaDC-3 (WT)</td>
<td>21.6 ± 10.6</td>
<td>−55 ± 37</td>
<td>20/17</td>
</tr>
<tr>
<td>HK14/15AA</td>
<td>21.3 ± 8.1</td>
<td>−66 ± 18</td>
<td>7/4</td>
</tr>
<tr>
<td>K36L</td>
<td>8.9 ± 5.3</td>
<td>−40 ± 20</td>
<td>3/3</td>
</tr>
<tr>
<td>R39I</td>
<td>27.8 ± 13.2</td>
<td>−121 ± 25</td>
<td>6/4</td>
</tr>
<tr>
<td>K78I</td>
<td>36.0 ± 5.9</td>
<td>−95 ± 51</td>
<td>4/3</td>
</tr>
<tr>
<td>K114I</td>
<td>−12.9 ± 0.9</td>
<td>9/4</td>
<td></td>
</tr>
<tr>
<td>K122A</td>
<td>23.3 ± 4.9</td>
<td>−41 ± 10</td>
<td>6/4</td>
</tr>
<tr>
<td>KK232/235II</td>
<td>−7.4 ± 4.9</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>KK374/375II</td>
<td>6.0 ± 4.7</td>
<td>−68 ± 40</td>
<td>3/3</td>
</tr>
<tr>
<td>R503I</td>
<td>19.6 ± 6.9</td>
<td>−67 ± 1</td>
<td>4/3</td>
</tr>
<tr>
<td>K548A</td>
<td>27.0 ± 15.2</td>
<td>−104 ± 47</td>
<td>5/3</td>
</tr>
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</table>

Values are means ± SE. $n$, No. of oocytes; $m$, number of donors. Succinate-induced currents were measured at various succinate concentrations at −60 mV. $K_m$ and $\Delta I_{\text{max}}$ values were determined from Eadie-Hofstee plots. For the mutants K114I and KK232/235II, a saturation of the induced currents was not observed up to 10 mM succinate. In these 2 cases, $\Delta I_{\text{max}}$ is the current at 10 mM succinate. RR109/110AI is not included, because it did not show succinate-induced currents. $^*P < 0.01$ vs. WT fNaDC-3.

Mutants K114I and KK232/235II it was not possible to determine $K_m$ and $\Delta I_{\text{max}}$ due to very low succinate-induced currents (Table 3). Among the mutants, we chose the substitutions at position 114 to further characterize.

Mutants RR109/110AI and KK232/235II. To determine whether the loss in transport activity (cf. Table 2) was due to defects in expression, immunohistochemical investigations were performed using polyclonal rabbit antibodies raised against a fNaDC-3-specific peptide. Whereas water-injected oocytes were negative due to the absence of endogenous NaDC-3 protein, WT-expressing oocytes showed a staining of the transporter protein at the surface of the oocyte (Fig. 2). In contrast, mutant RR109/110AI protein did not appear at the plasma membrane (Fig. 2, bottom left), which explains the very slow $[^{14}\text{C}]$succinate uptake. As shown in Fig. 2 (bottom right), mutant KK232/235II protein did appear at the surface of the oocytes as visualized by staining, suggesting that the mutant is properly expressed but functionally defective.

Substitution of K114. The replacement of the positively charged lysine by uncharged isoleucine (K114I) resulted in a functional mutant (Table 2). However, in current-clamped oocytes, addition of succinate induced a relatively small depolarization (Fig. 3A). In WT-expressing oocytes, 1 mM succinate depolarized the membrane potential from −41.1 ± 3.1 to +6.4 ± 8.9 mV, i.e., by 47.5 mV. In K114I-expressing oocytes, the resting potential was comparable to that of WT (−41.6 ± 6.9 mV), but 1 mM succinate depolarized the oocytes by only 21.9 mV to −19.6 ± 4.6 mV. Next, we determined the I-V relationships of the succinate-induced currents. As shown in Fig. 3B, there is an almost linear relationship between the succinate-induced inward current and the clamp voltage in oocytes expressing WT fNaDC-3 (Fig. 3B, ●). Oocytes expressing mutant K114I again showed near-linear I-V relationships between succinate-induced current and clamp potential (Fig. 3B, ○). However, at each membrane potential, the observed current was considerably smaller for K114I than for WT.

We generated three additional mutants in which the lysine at position 114 was replaced by arginine, glutamic acid, and glutamine. The I-V relationships of K114E (Fig. 4A, ○), K114R (●), and K114Q (Fig. 4B, ○) are shown with those obtained in WT (Fig. 4, A and B, ●). K114R and K114Q showed potential-dependent, succinate-induced inward currents. The currents evoked by K114R were similar in magnitude to those induced by WT, whereas K114Q currents showed amplitudes much smaller than those of WT. The extrapolated reversal potential for these currents was approximately +70 mV, indicating that the currents were carried by sodium. The currents induced by K114E were small and reversed at −43.7 ± 4.3 mV.
To exclude the possibility that the decreased depolarization and currents were due to a lower degree of transporter expression, we first performed immunostaining. Figure 5 shows that all mutants, K114I, K114R, K114Q, and K114E, were present at the oocyte’s membrane. Then, we performed [14C]succinate uptake and current measurement simultaneously on the same oocytes. Each oocyte was clamped to \(-60 \text{ mV}\), and \(68 \mu M\) succinate (18 \(\mu M\) [14C]succinate plus 50 \(\mu M\) unlabeled succinate) was added to the perfusion chamber. Uptake and current were measured for 30 min under continuous voltage clamp. The experiment was repeated with different oocytes expressing either WT fNaDC3 or any of the mutants, or with water-injected “mock” oocytes. As shown in Fig. 6A, [14C]succinate uptake was markedly higher in WT and all mutants compared with mock oocytes, indicating functional expression. Uptake by K114I and K114Q exceeded that of WT, whereas K114R and K114E showed [14C]succinate uptake rates similar to WT. The succinate-induced inward current was much higher in WT-expressing oocytes than in mock cells (Fig. 6B). Mutant K114R showed an inward current of similar magnitude as WT. However, the mutants K114I, K114Q, and K114E exhibited significantly reduced inward currents, indicating a change in the electrogenicity of Na\(^{+}\)-succinate cotransport.

**DISCUSSION**

In renal proximal tubule cells, NaDC-3, the sodium-dependent dicarboxylate cotransporter, is located at the basolateral membrane. NaDC-3 carries a range of dicarboxylates, e.g., succinate, \(\alpha\)-ketoglutarate, and protonated tricarboxylates such as divalent citrate (2). The identification of dicarboxylate and cation binding sites in NaDC-3 and the TM helixes involved in forming the translocation path remain areas of investigation. Because the cosubstrate (Na\(^{+}\)) and the substrates (di- and tricarboxylates) are charged, binding and translocation may involve charged amino acid residues within the NaDC-3 molecule, probably located near to or within the transmembrane helices forming the as yet elusive transport pore.

As opposed to NaDC-3, considerable efforts have been undertaken to unravel amino acid residues involved in Na\(^{+}\) and succinate binding of the rabbit NaDC-1. Mainly, amino acids in TMs 7, 8, 9, and 10 as well as the extracellular loop between

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**Fig. 3.** Succinate-induced depolarization (A) and inward currents (B) in WT- and K114I-expressing oocytes. A: membrane potential \(V_m\) was measured under current-clamp conditions in the absence and presence of 1 mM succinate in control solution. Compared with WT, in K114I-expressing oocytes the succinate-induced depolarization was smaller. B: afterward, current-voltage relationships were obtained using the same oocytes. At all clamp potentials \(V_c\), the substrate-associated inward currents \(\Delta I\) were smaller in K114I-expressing \(\bigcirc\) than in WT-expressing oocytes \(\bullet\). Data were obtained from 10 (WT) and 9 (K114I) oocytes from 4 donors.

**Fig. 4.** Current-voltage relationships of WT, K114E, K114R, and K114Q, \(\Delta I\) induced by 1 mM succinate was plotted as a function of \(V_c\). A: currents evoked by K114E \(\bigcirc\) and K114R \(\bullet\) compared with WT currents \(\bullet\). B: similar experiments performed with K114Q \(\bigcirc\) and WT \(\bullet\). Values are means ± SE of 7 oocytes from 3 donors.
TM 9 and 10 of rbNaDC-1 are accessible from the outside, interact with succinate, and/or are involved in Na$^+$-induced conformational changes (19). The affinity for succinate was altered when the residues K84 (intracellular loop between TM2 and 3 of rbNaDC-1), R349 (TM7), S372, D373 (TM8), and E475 (TM9) were mutated (7, 22, 36). Activation by Na$^+$ was changed, when residues S372 and D373 in TM8, and E475, A481, and T482 in TM9 were replaced by other amino acids (7, 18, 36). After being replaced with cysteine, most of the residues at positions 480 – 493 in the fifth extracellular loop (7, 18, 36). Activation by Na$^+$ increased, and succinate increased accessibility, amino acids at the outer faces of TM9 and 10 and the connecting loop are involved in Na$^+$-induced binding of succinate.

In fNaDC-3, we mutated 4 arginines (R39, R109, R110, R503), 1 histidine (H14), and 10 lysines (K15, K36, K78, K114, K122, K232, K235, K374, K375, K548) within or near putative TM domains. Most mutants proved to be functional and exhibited Na$^+$-dependent $[^{14}C]$succinate uptake. Only the double mutants RR109/110AI and KK232/235II showed strongly reduced uptake rates. In TEVC experiments, all mutants except K114I and KK232/235II exhibited $K_m$ values between 6 and 36 μM (WT: 22 μM) and maximal inward currents between −40 and −121 nA (WT: −55 nA).

The first conclusion to be drawn from these data is that the replacement of H14, K15, K36, R39, K78, K122, K374, K375, R503, and K548 by neutral amino acid residues appears to have no gross influence on the function of flounder renal NaDC-3. With regard to K36, R503, and K548, our results are in agreement with the mutational analysis of K34, A496, and R542 present at the respective positions in rbNaDC-1, which also did not show any changes in succinate transport (22, 23). The amino acids corresponding to R39, K78, K122, K374, and K375 have not been mutated in earlier studies on rbNaDC-1.

The replacements of the amino acid residues R109 and R110 in RR109/110AI, of K232 and K235 in KK232/235II, and of K114 by neutral and acidic amino acids did interfere with transport capacity or electrogenicity of the cotransporter. The replacement of RR109/110 in the second extracellular loop by alanine and isoleucine (RR109/110AI) led to a complete loss of function. Immunofluorescence studies revealed that the RR109/110AI protein was not expressed and did not appear at the plasma membrane of the oocytes. The arginines at positions 109 and 110 of NaDC-3 seem to be important, because they are conserved either as RR or as KR through all NaDC-1s, NaDC-3s, and NaCTs (alignment not shown). Mutational analysis of rbNaDC-1 demonstrated that the replacement of R108 (which corresponds to R110 in NaDC-3) by alanine reduced $V_{\text{max}}$ without changing the affinity (22). The strong reduction of $V_{\text{max}}$ suggested a low expression level of this mutant at the oocyte membrane. The studies on rbNaDC-1 and fNaDC-3 demonstrate the relevance of basic amino acids close to TM4 for proper expression and targeting of the transporters to the plasma membrane.

The basic amino acids K232 and K235 are located within TM5. K235 is highly conserved through all NaDC-3s and NaCTs. NaDC-1s exhibit a glutamine at this position. The replacements of the amino acid residues R109 and 110 of NaDC-3 seem to be important, because they are conserved either as RR or as KR through all NaDC-1s, NaDC-3s, and NaCTs (alignment not shown). In hNaDC-1, rbNaDC-1, and all NaCTs, an arginine is located at that position. $[^{14}C]$succinate uptake mediated by K114I was slightly higher than that of WT. In contrast, succinate-induced depolarization and currents were considerably smaller than those observed for WT. Even when tested simultaneously in the same oocytes, $[^{14}C]$succinate uptake mediated by K114I was slightly higher than that of WT. Even when tested simultaneously in the same oocytes, $[^{14}C]$succinate uptake was still higher for K114I, but the induced inward current was significantly smaller for K114I than for WT fNaDC-3, suggesting an altered electrogenicity.

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Fig. 5. Immunohistochemistry of mutant K114-expressing oocytes. Devitellinized X. laevis oocytes expressing K114I, K114R, K114Q, and K114E were incubated with rabbit anti-NaDC-3 antibodies in a dilution of 1:50 followed by the secondary antibody Alexa Fluor 488 goat anti-rabbit in a dilution of 1:200. Sections (5 μm) were analyzed with a fluorescence microscope.
clamped at \( \text{Na}^{+} \text{K}^{+} \text{H}^{+} \) to succinate uptake not different from WT, and a substitution of lysine by glutamine (K114Q) or glutamic acid (K114E) led to a decrease in succinate-induced currents as was found for K114I and to find out the \( \text{Na}^{+} \) binding sites in NaDC-3.

For further characterization, we substituted lysine 114 with the basic amino acid arginine, the acidic amino acid glutamic acid, and the neutral amino acid glutamine. Mutant K114R revealed the same functional characteristics as WT with respect to succinate uptake and succinate-induced current. Substitution of lysine by glutamine (K114Q) or glutamic acid (K114E) led to a decreased succinate-induced current not different from WT, and a decrease in succinate-induced inward currents as was found for K114I. From these experiments it can be inferred that a positive charge must be present at position 114 for fully electrogenic Na\textsuperscript{+}-succinate cotransport.

A possible reason for decreased succinate-induced currents at unaltered \( [^{14}C] \) succinate uptake may be a change in the sodium:succinate stoichiometry. Whereas WT translocates three sodium ions with each succinate molecule, K114l may vary between a 2:1 and a 3:1 stoichiometry, leading to a decreased inward current and a decreased membrane depolarization. Alternatively, electroneutrality in the K114I mutant may result from a backflux of a cation (\( \text{Na}^{+}, \text{K}^{+}, \text{H}^{+} \)) during each transport cycle, or a symport of sodium, succinate, and a monovalent anion (\( \text{Cl}^{-}, \text{OH}^{-} \)). Clearly, more experiments are needed to define the exact transport mode of mutant K114I and to find out the \( \text{Na}^{+} \) binding sites in NaDC-3.

In conclusion, the mutational analysis of basic amino acid residues in fNaDC-3 documented the importance of the conserved R109 and R110 for the expression at the plasma membrane. K232 and K235 near TM5 are probably involved in substrate recognition and/or transport. K114 located in TM4 is essential for fully electrogenic Na\textsuperscript{+}-succinate cotransport.

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