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Ala-504 is a determinant of substrate binding affinity in the mouse Na^+/dicarboxylate cotransporter

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Abstract

The Na^+/dicarboxylate cotransporters from mouse (mNaDC1) and rabbit (rbNaDC1) differ in their ability to handle adipate, a six-carbon terminal dicarboxylic acid. The mNaDC1 and rbNaDC1 amino acid sequences are 75% identical. The rbNaDC1 does not transport adipate and only succinate produced inward currents under two-electrode voltage clamp. In contrast, oocytes expressing mNaDC1 had adipate-dependent inward currents that were about 60% of those induced by succinate. In order to identify domains involved in adipate transport, we examined the functional properties of a series of chimeric transporters made between mouse and rabbit NaDC1. We find that multiple transmembrane helices (TM), particularly TM 8, 9, and 10, are involved in adipate transport. In TM 10 there is only one amino acid difference between the two proteins, corresponding to Ala-504 in mouse and Ser-512 in rabbit NaDC1. The mNaDC1-A504S mutant had decreased adipate-dependent currents relative to succinate-dependent currents and an increase in the $K_{0.5}$ for both succinate and glutarate. We conclude that multiple amino acids from TM 8, 9 and 10 contribute to the transport of adipate in NaDC1. Furthermore, Ala-504 in TM 10 is an important determinant of $K_{0.5}$ for both adipate and succinate.

Keywords: Adipate; Succinate; NaDC1; Sodium; *Xenopus* oocytes; Substrate specificity

1. Introduction

Transport of citric acid cycle intermediates such as succinate and citrate across the plasma membrane is mediated by the Na^+/dicarboxylate cotransporters (NaDCs) belonging to the SLC13 gene family [1]. The low affinity transporter, NaDC1, is localized to the brush border membranes of renal proximal tubules and gastrointestinal tract. In contrast, the high affinity transporter, NaDC3, is expressed on the basolateral membrane of renal proximal tubule, as well as liver, placenta and brain. The physiological roles of NaDC1 involve absorption of dietary dicarboxylates in the intestine and reabsorption of filtered dicarboxylates in the kidney. NaDC3 and NaDC1 participate in organic anion secretion by contributing dicarboxylates to the organic anion exchangers [2]. NaDC1 in the kidney may affect the development of kidney stones through its regulation of urinary citrate concentrations [3]. Moreover, studies in flies and worms show a potential involvement of NaDCs in longevity since mutations in the genes encoding dicarboxylate transporter homologs in these organisms increased their lifespan [4,5].

Although the NaDC1 and NaDC3 transporters have overlapping substrate specificity, there are some notable differences between them. In general, the NaDC1 transporters prefer four-carbon dicarboxylates, such as succinate [6], whereas the NaDC3 transporters carry not only succinate but also longer and bulkier dicarboxylates such as glutarate and 2,3-dimethylsuccinate [7–9]. Interestingly, there are some species differences among the NaDC1 transporters, with the mouse NaDC1 having a substrate specificity similar to that of the NaDC3′s [10]. Recently, we constructed chimeras between the mouse and rabbit NaDC1 to show that putative transmembrane helices (TM) 3, 4, 7, and 8 are involved in glutarate transport in mNaDC1, with TM 3–4 making the greatest contribution to glutarate-dependent currents [11]. We also found that Gly-161 next to TM 4 affects the $K_{0.5}$ for glutarate but not for succinate [11].

Our preliminary experiments showed that the mouse and rabbit NaDC1 orthologs also differ in their ability to handle adipate, a six-carbon terminal dicarboxylate. The rabbit does not transport adipate, whereas the mouse transports adipate quite well. Therefore, the purpose of the present study was to identify domains and

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amino acids that determine adipate transport. Similar to our previous study, we found that multiple TMs are involved in producing adipate-induced inward currents. However, the main determinants of adipate transport are found in the C-terminal half of the protein, particularly TM 10 with contributions from TM 8 and 9, very different from the domains involved in glutarate transport [11]. Within TM 10, Ala-504 appears to be a determinant of adipate transport. Mutation from the mouse to the rabbit sequence at this position, mNaDC1-A504S, results in increases in the $K_{0.5}$ for both adipate and succinate, suggesting that Ala-504 is important for substrate binding in NaDC1.

2. Methods

2.1. Chimeric NaDC1 transporters

The NaDC1 chimeras consisting of mouse (m) NaDC1 (GenBank™ AF201903) and rabbit (rb) NaDC1 (GenBank™ U12186) are named using the number of transmembrane helices (TM) from mNaDC1 at the N-terminus and the letters M and R representing mouse and rabbit NaDC1, respectively. The current 11-TM secondary structure model of NaDC1 is based hydropathy analysis [12] and the location of N-glycosylation sites and epitopes [13,14]. The chimeras in this study were the same as those in our previous study, constructed using endogenous or introduced restriction sites followed by subcloning [11].

2.2. Site-directed mutagenesis

Mutagenesis in transmembrane helix (TM) 10 was carried out using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer's directions. Briefly, the heat-denatured parental NaDC1 cDNA was annealed with both sense and anti-sense primers containing a desired mutation. After extension by polymerase chain reaction, the plasmid incorporating the mutagenic NaDC1 was transformed into the XL-1 blue strain of *E. coli*. The final mutants were verified by sequencing at the Protein Chemistry Laboratory of the University of Texas Medical Branch.

2.3. Preparation of Xenopus oocytes and injection of cRNA

Female *Xenopus laevis* were purchased from *Xenopus* I (Dexter, MI). Stage V and VI oocytes were dissected and incubated with collagenase as described previously [11]. The cRNA was made by in vitro transcription with the T7 mMessage mMachine kit (Ambion) using a linearized plasmid template containing cDNA of wild-type, chimeric, or mutant NaDC1. The cRNA was stored at −80 °C until use. The oocytes were injected with 46–50 nl of cRNA the day after defolliculation and cultured at 18 °C in Barth's medium supplemented with 5% heat-inactivated horse serum, 2.5 mM pyruvate, 100 μg/ml gentamycin sulfate, and either 50 μg/ml tetracycline or a mixture of 5 μg/ml ceftazidime (GlaxoSmithKline) and 100 U/ml penicillin–100 μg/ml streptomycin (Gibco). Experiments were performed three to 6 days after injections, and culture vials and medium were changed daily.

2.4. Electrophysiology

Substrate-induced inward currents mediated by wild-type, chimeric, or mutant NaDC1 transporters were measured using the two-electrode voltage clamp (TEVC) method as described previously [11]. The electrode resistance was less than 0.5 MΩ. Test voltage pulses of 100 ms between + 50 and −150 mV in decrements of 20 mV were controlled using the pClamp6 program (Axon Instruments, Inc.). The holding membrane potential was set at −50 mV between the test pulses. The average of three measurements was recorded for the individual test pulses. For the experiments, the pulse protocol was applied first to oocytes equilibrated in choline buffer, then after addition of sodium buffer. The test solutions containing substrates in sodium buffer were then perfused over the oocytes. After measuring the substrate-induced currents, choline buffer was used to wash away the substrates, and subsequent experiments were performed following recovery of the initial currents in choline buffer. Experiments were repeated with oocytes from at least 3 different frogs.

The difference between steady-state currents with and without substrate in sodium buffer was defined as the substrate-dependent currents. The kinetic parameters were analyzed using SigmaPlot software (Jandel Scientific), and the steady-state substrate-evoked currents were fitted to the Michaelis–Menten equation: $I = I_{max} \times [S]/(K_{0.5} + [S])$, where I is the current, I_{max} is the maximum current at saturating substrate concentration, $[S]$ is the substrate concentration, and $K_{0.5}$ is the substrate concentration producing half of the maximum currents. Statistical analysis was done using the SigmaStat program (Jandel Scientific).

3. Results

3.1. Voltage-dependent currents in wild-type NaDC1 transporters

In our previous studies, we found that mNaDC1 and rbNaDC1 have functional differences in glutarate transport, although glutarate is only one carbon longer than succinate [6,10,11,15]. Therefore, in

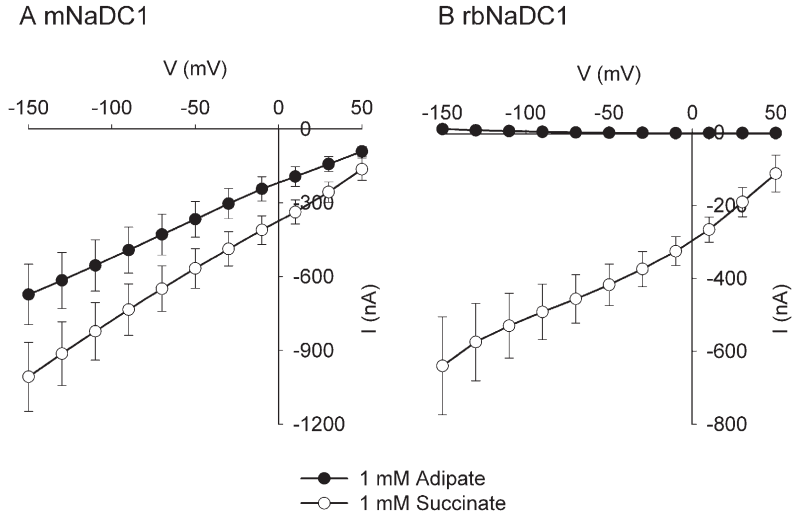

Fig. 1. The voltage dependence of steady-state substrate-dependent currents in mNaDC1 and rbNaDC1 expressed in *Xenopus* oocytes. Adipate- and succinate-induced currents in mNaDC1 (A) and rbNaDC1 (B) are shown as a function of different voltages. The concentration of the substrates was 1 mM. The mNaDC1-mediated inward currents were seen in the presence of both adipate and succinate at all tested voltages. In contrast, rbNaDC1-mediated inward currents were only detected in the presence of succinate. The data shown are mean ± S.E.M., $n = 14$ frogs.