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Electrophysiological Characterization of Human and Mouse Sodium-Dependent Citrate Transporters (NaCT/SLC13A5) Reveal Species Differences with Respect to Substrate Sensitivity and Cation Dependence

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ABSTRACT

The citric acid cycle intermediate citrate plays a crucial role in metabolic processes such as fatty acid synthesis, glucose metabolism, and β-oxidation. Citrate is imported from the circulation across the plasma membrane into liver cells mainly by the sodium-dependent citrate transporter (NaCT; SLC13A5). Deletion of NaCT from mice led to metabolic changes similar to caloric restriction; therefore, NaCT has been proposed as an attractive therapeutic target for the treatment of obesity and type 2 diabetes. In this study, we expressed mouse and human NaCT into *Xenopus* oocytes and examined some basic functional properties of those transporters. Interestingly, striking differences were found between mouse and human NaCT with respect to their sensitivities to citric acid cycle intermediates as substrates for these transporters. Mouse NaCT had at least 20- to 800-fold higher affinity for these intermediates than human NaCT. Mouse NaCT is fully active at physiologic plasma levels of citrate, but its human counterpart is not. Replacement of extracellular sodium by other monovalent cations revealed that human NaCT was markedly less dependent on extracellular sodium than mouse NaCT. The low sensitivity of human NaCT for citrate raises questions about the translatability of this target from the mouse to the human situation and raises doubts about the validity of this transporter as a therapeutic target for the treatment of metabolic diseases in humans.

Introduction

The sodium-dependent citrate transporter (NaCT; also known as SLC13A5) is a member of the mammalian solute carrier gene family 13 (SLC13). This gene family further consists of two sodium-coupled dicarboxylate transporters, NaDC1 (SLC13A2) and NaDC3 (SLC13A3), and two sodium-coupled sulfate transporters, NaS1 (SLC13A1) and NaS2 (SLC13A4) (Markovich and Murer, 2004; Pajor, 2006, 2014; Markovich, 2012; Bergeron et al., 2013; Willmes and Birkenfeld, 2013). NaCT has been cloned from various species, including humans (Inoue et al., 2002a), rats (Inoue et al., 2002b), and mice (Inoue et al., 2003). In mammals, NaCT is expressed in the liver (Inoue et al., 2002a,b; Gopal et al., 2007) and in the brain (Inoue et al., 2002a,b; Yodoya et al., 2006) and it transports primarily the tricarboxylate citrate from the circulation into hepatocytes and neurons, where it regulates metabolic processes. The recent crystal structure reported for a bacterial homolog of human NaCT, named VcINDY (Mancusso et al., 2012), provided insight on how proteins of this class of transporters bind their substrates and transport them across the cell membrane (Mulligan et al., 2014).

Citrate is a crucial intermediate of the citric acid cycle and plays a major role in determining the metabolic status of the cell. Cytoplasmic citrate is the prime carbon source for the synthesis of fatty acids, triacylglycerols, cholesterols, and low-density lipoproteins. In addition, citrate leads to the activation of fatty acid synthesis and affects glycolysis and β-oxidation (Spencer and Lowenstein, 1962; Bloch and Vance, 1977; Ruderman et al., 1999). Loss of function of NaCT in mammals and NaCT homologs in other species increased the life span in different species by mechanisms similar to caloric restriction. In *Drosophila*, loss-of-function mutations in the fly homolog of NaCT (named INDY for "I'm Not Dead Yet") result in an 80–100% increase in the average lifespan of both adult male and female *Drosophila* flies (Rogina et al., 2000). Studies in *Caenorhabditis elegans* also revealed that the disruption of transporters with similar transport properties as NaCT extends the animal's lifespan (Fei et al., 2003). Moreover, studies with NaCT knockout mice showed that deletion of SLC13A5 in mice results in protection from adiposity and insulin resistance. These findings underscore the importance of NaCT for normal metabolic function and suggest that NaCT might be a promising therapeutic target for the treatment of metabolic diseases such as obesity and diabetes (Birkenfeld et al., 2011). In addition to playing an important role in the liver, it was recently shown that mutations in human

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ABBREVIATIONS: NaCT, sodium-dependent citrate transporter; NFR, normal frog ringer.

SLC13A5 cause autosomal recessive epileptic encephalopathy with seizure onset in the first days of life, indicating that NaCT is important for the development and function of the human brain (Thevenon et al., 2014).

Although tricarboxylate citrate is regarded to be the main substrate of NaCT, dicarboxylate intermediates of the citric acid cycle, such as succinate, fumarate, and malate, can also serve as substrates of NaCT (Inoue et al., 2002a). Thus far, concentration-response relationships for each of the citric acid cycle intermediates have not been systematically investigated and it is currently unknown whether all of the citric acid cycle intermediates, or only a subset, are indeed substrates of NaCT. Current knowledge of substrate transport by NaCT is mainly gathered from biochemical experiments using radiolabeled substrate uptake assays. NaCT is a so-called symporter that couples the transport of substrate to the transport of Na^+ ions. It has been deducted that for each trivalent citrate or divalent succinate molecule transported by NaCT, four sodium ions are cotransported, resulting in a net transport of positive charges through the membrane with each substrate molecule passing the membrane (Inoue et al., 2004). Both rodent and human NaCT can be functionally expressed in *Xenopus* oocytes and, because of the electrogenic nature of NaCT, transport activity can be detected by applying the two-microelectrode voltage clamp technique (Inoue et al., 2004; Brauburger et al., 2011; Gopal et al., 2015). Studying the functional and pharmacological properties of transporters by this technique has advantages over using radiolabeled substrate uptake assays, because transporter activity can be measured more directly and in real time. Despite these advantages, this approach has not yet been used extensively to study the functional and pharmacological properties of NaCT.

In this study, we expressed human and mouse NaCT in *Xenopus* oocytes and applied the voltage clamp technique to measure concentration-response curves for the various intermediates of the citric acid cycle. In addition, we substituted sodium ions in the recording solution with other monovalent cations (potassium, choline, and lithium) and we characterized in more detail the modulation of human and rat NaCT by lithium ions.

Materials and Methods

Stage V and VI *Xenopus* oocytes were prepared using standard procedures, and oocyte expression and electrophysiological recordings were performed as described previously (Zwart et al., 2002). The oocytes used in this study were from adult female *Xenopus laevis* frogs, which were purchased from *Xenopus* Express (Vernassal, France). The frogs were kept in the laboratory and the care and use of the frogs complied with the guidelines of the 1986 Scientific Procedures Act of the United Kingdom. The sequences of human and mouse NaCT cDNAs were similar to those deposited in the GenBank sequence database with accession numbers NM_177550.4 and NM_001004148.4, respectively. These sequences were ligated into pcDNA3.1 (Invitrogen, Carlsbad, CA). After enzymatic linearization of the plasmids, capped human and mouse NaCT mRNAs were prepared using the mMessage mMachine Kit (Ambion, Paisley, UK), and dissolved in distilled water at a concentration of 1 mg/ml (spectrophotometric determinations). mRNA was injected into the cytoplasm of oocytes in a volume of 50 nl per oocyte, using a Nanoject Automatic Oocyte Injector (Drummond, Broomall, PA). An alternative clone of human NaCT in pCMV6-XL5 was purchased from Origene (Rockville, MD). This DNA was diluted to 1 mg/ml and approximately 20 nl per oocyte of this dilution was directly injected into nuclei of oocytes. After injection, oocytes were incubated at 18°C for 3–5 days in a modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES, and 5 mg/l neomycin (pH 7.6). Recordings were performed 3–5 days postinjection. Oocytes were placed in a 0.1-ml recording chamber and perfused with normal frog ringer (NFR) (Ecocyte Biosciences, Castrup-Rauxel, Germany) at a rate of 10 ml/min. NFR contained 90 mM NaCl, 2 mM KCl, 5 mM HEPES, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ (pH 7.2).

Two-Electrode Voltage Clamp Recording and Data Analysis. Oocytes were impaled by two microelectrodes filled with 3 M KCl (0.5–2.0 MΩ) and voltage clamped at −60 mV using a Geneclamp 500B amplifier and PCLAMP 10 software (Axon Instruments, Sunnyvale, CA). Typically, traces were filtered at 10 Hz during recording and were digitized at 50 Hz using the DigiData 1200 interface (Axon Instruments). All experiments were carried out at room temperature. Concentration-response curves for the different substrates were obtained by normalizing substrate-induced responses to the control responses induced by 3 mM citrate or 30 μM citrate for human and mouse NaCT, respectively. An interval of 2 minutes was allowed between substrate applications, because this was found to be sufficient to ensure reproducible recordings.

Concentration-response curves were fitted by a nonlinear least-squares algorithm according to eq. 1:

$$I = I_{\max}/\left(1 + \left\{\mathrm{EC}_{50}/[\mathrm{conc}]\right\}^n\right) \qquad (1)$$

in which $I_{\max}$ is the maximum obtainable peak current, EC_{50} is the concentration of the substrate that elicits 50% of the maximum obtainable peak current, and n is the slope factor. Curve fitting was performed using GraphPad Prism 6.02 software (GraphPad Software, San Diego, CA). Results are expressed as means ± S.E. Statistical significance of log EC_{50} and $E_{\max}$ values were taken as $P < 0.05$ by a *t* test or one-way analysis of variance (followed by a post hoc Tukey test).

Ion Substitution. In some experiments, extracellular Na^+ ions were replaced by K^+, $choline^+$, or Li^+ ions. This was done by substituting 90 mM NaCl with 90 mM KCl, choline chloride, or LiCl, respectively. In addition, the pH of these solutions was adjusted by using KOH instead of NaOH.

Chemicals. Most of the substrates used in this study were purchased as salt forms [α-ketoglutaric acid sodium salt, citric acid trisodium salt, sodium fumarate dibasic, L-(−)malic acid sodium salt, and DL-isocitric acid trisodium salt hydrate], but oxaloacetic acid and succinic acid were purchased as free acids. The substrates and all of the chemicals to prepare the Barth's solutions, lithium chloride, and choline chloride were purchased from Sigma-Aldrich (Poole, UK). The recording solution was NFR and was purchased from Ecocyte Biosciences. Concentrated stock solutions of 1 M of each substrate were prepared in distilled water. Stock solutions were aliquoted, frozen at −20°C, and thawed on the day of the experiment. The pH of the final dilutions of oxaloacetate and succinate was adjusted to 7.2 with NaOH.

Results

Substrate Sensitivity. Various concentrations of citrate were applied to oocytes expressing human and mouse NaCTs (Fig. 1, A and C, respectively). In both cases, citrate induced inward currents, the amplitudes of which increased with increasing citrate concentrations. A large difference was observed between human and mouse NaCT. The threshold of activation of human NaCT was about 100 μM citrate and the current amplitudes still increased at citrate concentrations as high as 30 mM (Fig. 1A). Control experiments in which citrate

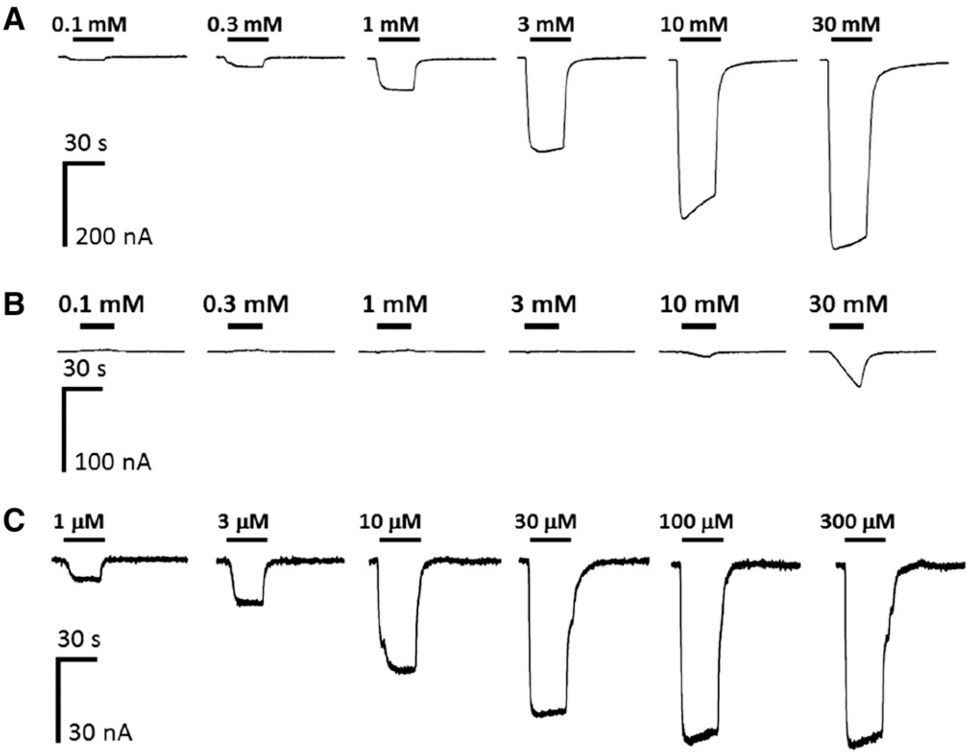

Fig. 1. Citrate concentration-dependent responses of human and mouse NaCT. (A) Application of increasing concentrations of citrate to oocytes expressing human NaCT induces ion currents with increasing amplitude. Upon citrate application, the maximum amplitude of ion currents is reached within a couple of seconds and when citrate is removed from the oocyte the currents return to baseline level. (B) The same citrate concentrations applied to oocytes expressing human NaCT were also applied to uninjected oocytes. Citrate induced small nonspecific inward currents only at the highest concentrations (10 and 30 mM). (C) Application of increasing concentrations of citrate to oocytes expressing mouse NaCT induce ion currents with increasing amplitude. Bars above the responses indicate the time of application of citrate and the concentration of citrate applied is indicated above the bars.

was applied to uninjected oocytes revealed that citrate did not activate possible transporters that are endogenous to the oocytes (Sobczak et al., 2010). Small nonspecific inward currents were observed upon citrate application only at the very highest concentrations of citrate tested (10 and 30 mM) (Fig. 1B). In contrast with human NaCT, the threshold of activation of mouse NaCT by citrate was at concentrations as low as 1 μM citrate and maximum activation of mouse NaCT was seen at 300 μM (Fig. 1C). At a maximal effective concentration of citrate, much larger currents were obtained in oocytes expressing human NaCT than in oocytes expressing mouse NaCT. The reason for this discrepancy in expression levels is not known, since both the human and mouse NaCT sequences were inserted in the same way in the same expression vector. Pharmacological properties of human and mouse NaCT are not likely to be affected by a difference in cell surface expression. Concentration-response curves for citrate transport through human and mouse NaCT were fitted and the averaged curves are shown in Fig. 3. The values for the estimated parameters for EC_{50}, E_{max}, and slope factor are summarized in Table 1. The large difference in EC_{50} values for citrate between human and mouse NaCT shows that the citrate sensitivity of human NaCT is much lower than for its murine counterpart. This raised the possibility that something might have been wrong with the human NaCT sequence. To verify this, we sequenced both the coding regions of human and rat NaCT in our plasmids and found that based on alignment results, they are exactly the same as the National Center for Biotechnology Information sequences (NM_177550.4 and NM_001004148.4). To further exclude this possibility, we also obtained an independent human NaCT cDNA clone from a commercial source (see *Materials and*

TABLE 1

Substrate sensitivities of human and mouse NaCT

Responses of the various substrates were normalized to control responses to 3 mM and 30 μM citrate human and mouse NaCT, respectively. Data are presented as means ± S.E. and means (95% confidence intervals). In the fields in which no values are given (indicated with dashes), the responses to the substrate were either too small to fit a curve to the data, or the concentration-response curves did not saturate at the highest concentrations of the substrates tested. Conservative estimates for "fold difference" values were calculated by using the upper value for the 95% confidence interval of the mouse EC_{50} and the lower value for the 95% confidence interval for the human EC_{50}; therefore, the true "fold difference" is likely even greater than the reported values. All substrates tested were significantly more potent on mouse NaCT compared with human NaCT (t test, $P < 0.001$).

Compound	Human NaCT				Mouse NaCT				Fold Difference
	EC_{50}	E_{max}	n_H	n	EC_{50}	E_{max}	n_H	n	
	mM	%			*μM*	%			
Citrate	3.5 (3.3–3.7)	217 ± 3	1.27 ± 0.04	4	7.2 (6.5–8.0)	116 ± 2	1.24 ± 0.06	3	>400
Oxaloacetate	1.6 (0.3–9.0)	19 ± 4	0.70 ± 0.26	3	12 (9–18)	93 ± 4	1.02 ± 0.13	3	>20
Malate	6.6 (6.2–7.1)	153 ± 3	2.18 ± 0.06	3	22 (17–29)	102 ± 4	1.03 ± 0.10	3	>200
α-Ketoglutarate	18.0 (3.9–84)	334 ± 119	1.11 ± 0.28	3	—	—	—	3	—
Succinate	9.3 (7.4–12)	92 ± 4	1.01 ± 0.05	3	5.5 (3.3–9.2)	80 ± 5	1.10 ± 0.25	3	>800
Fumarate	20 (19-21)	108 ± 1	1.01 ± 0.02	3	26 (14–46)	119 ± 12	1.24 ± 0.31	3	>400
Isocitrate	10.4	6 ± 2	—	3	34 (6–180)	21 ± 7	1.55 ± 1.48	3	>60

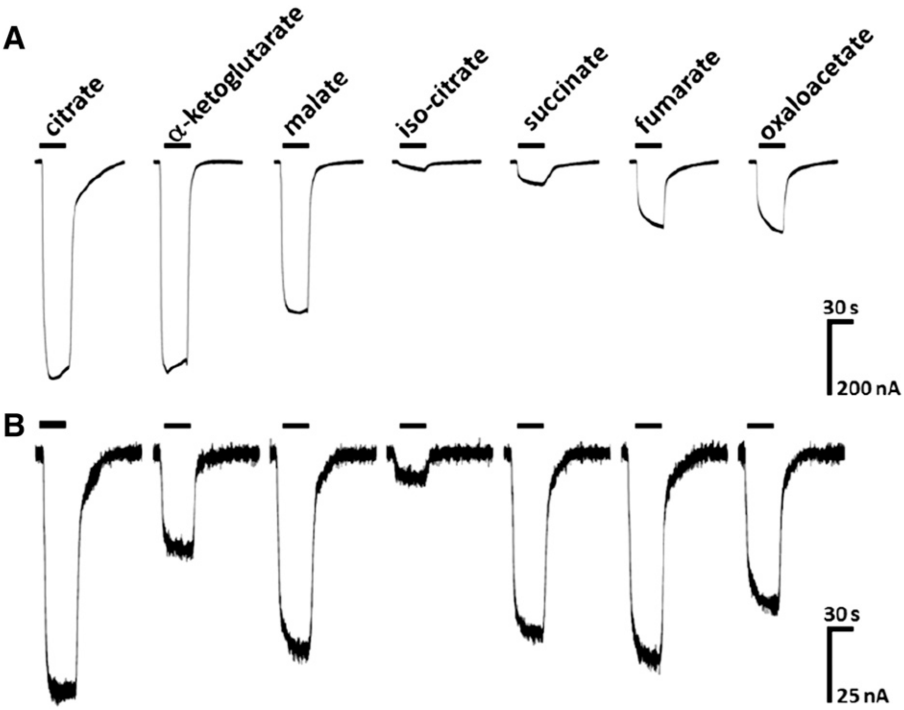

Fig. 2. Various citric acid cycle intermediates serve as substrates for human and mouse NaCT. (A) *Xenopus* oocytes expressing human NaCT were perfused with high concentrations (30 mM) of citric acid cycle intermediates. Each intermediate activated the transporter and the current amplitude varied with the intermediate tested. (B) Oocytes expressing mouse NaCT were perfused with near maximum effective concentrations (300 μM) of citric acid cycle intermediates. Each intermediate activated the transporter and the current amplitude varied with the intermediate tested.

Methods). This alternative clone was injected and expressed in oocytes, and we found that it also had the same low sensitivity to citrate as our own clone (unpublished data).

The various citric acid cycle intermediates were then tested for their ability to be transported by human and mouse NaCT. Figure 2 shows responses evoked by application of a high concentration of each of the citric acid cycle intermediates on human and mouse NaCT, respectively. The results show that all of the citric acid cycle intermediates are substrates of human and mouse NaCT. Full concentration-response curves were measured from both human and mouse NaCT with each of the citric acid cycle intermediates (Fig. 3). The estimated values for EC_{50}, E_{max}, and slope factors were obtained by fitting concentration-response curves to the data and are summarized in Table 1. All of the substrates are significantly less potent on human NaCT compared with mouse NaCT. The results of the comparison of E_{max} values for the different substrates within each species are summarized in Tables 2 and 3 for human and mouse NaCT, respectively.

Ion Substitution. An intrinsic property of NaCT is its dependence on extracellular sodium ions. To investigate whether the functioning of human and mouse NaCT depends fully on extracellular sodium ions, we replaced all extracellular sodium by alternative cations. As shown in Fig. 4A, ion substitution did not fully abolish 10 mM citrate–induced ion currents in oocytes expressing human NaCT. Substantial ion currents were observed and the current amplitudes of 10 mM citrate–induced responses were 28 ± 4% (n = 3), 32 ± 2% (n = 3), and 21 ± 4% (n = 3), when sodium was replaced by potassium, choline, and lithium, respectively. Mouse NaCT behaved much differently. Substitution of extracellular sodium by potassium or choline fully abolished the functioning of mouse NaCT, because there were no responses seen to 0.1 mM citrate when the recording solutions contained these cations. When sodium ions were replaced by lithium ions, 0.1 mM citrate induced a small ion current with an amplitude of 14 ± 7% (n = 3) compared with responses by the same concentration of citrate in the presence of sodium ions. Therefore, although the effect of sodium ion substitution by lithium ions

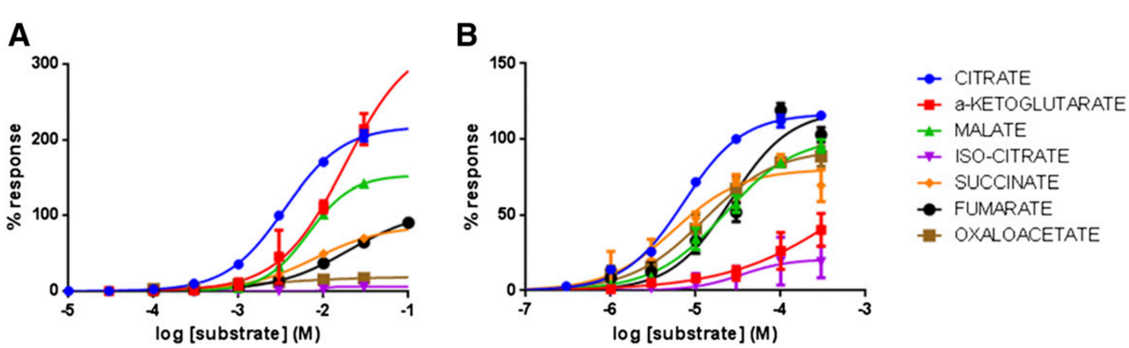

Fig. 3. Concentration-response curves of the citric acid cycle intermediates to activate human NaCT (A) and mouse NaCT (B).Concentration-response curves were obtained for each citric acid cycle intermediate and current response amplitudes were normalized to the amplitude of a 3 mM (human) and 30 μM (mouse) citrate-induced response in the same oocyte.

TABLE 2

Results of statistical comparison of E_{max} values of the different substrates for human NaCT

Data are presented as results (P values) of one-way analysis of variance followed by the post hoc Tukey test.

Human	Citrate	Oxaloacetate	Malate	α-Ketoglutarate	Succinate	Fumarate
Oxaloacetate	<0.05					
Malate	<0.05	<0.05				
α-Ketoglutarate	n.s.	n.s.	n.s.			
Succinate	<0.05	<0.05	<0.05	n.s.		
Fumarate	<0.05	<0.05	<0.05	n.s.	n.s.	
Isocitrate	<0.05	n.s.	<0.05	n.s.	<0.05	<0.05

n.s., not significant.

was similar for human and mouse NaCT, marked differences between human and mouse NaCT were observed when sodium ions were substituted by potassium or choline ions.

Lithium Modulation. Biochemical experiments with radiolabeled substrate uptake assays revealed that the function of human NaCT was potentiated by lithium ions in the presence of low concentrations of citrate and was inhibited by lithium ions in the presence of high concentrations of citrate. On the other hand, mouse NaCT was only inhibited by lithium ions, regardless of the citrate concentration used (Inoue et al., 2003). We asked the question of whether this phenomenon could also be detected when transporter activity was monitored electrophysiologically. In the experiment described above, lithium ions completely replaced the sodium ions in the recording solution. To study lithium modulation in more detail, various concentrations of lithium chloride were added to the normal sodium-containing recording solution. Figure 5A shows that when human NaCT was activated by the relatively low concentration of 0.1 mM citrate, the citrate responses were potentiated by lithium in a concentration-dependent manner. When human NaCT was activated by the higher concentration of 3 mM citrate, the effect of lithium was reversed and lithium inhibited the citrate-induced responses (Fig. 5B). Figure 5C shows the concentration-response relationship of lithium action on human NaCT in the presence of four different citrate concentrations. We found that mouse NaCT was also potentiated by lithium at relatively low citrate concentrations (Fig. 5D), and this potentiating effect disappeared when the transporter was activated by relatively high concentrations of citrate (Fig. 5D). In contrast with the effects of lithium on human NaCT, no inhibition of mouse NaCT was observed when the transporter was activated by the relatively high concentration of 0.1 mM citrate. Concentration-response curves for the lithium effects on mouse NaCT are depicted in Fig. 5F. Estimated values for E_{max}, EC_{50}/IC_{50}, and slope factors obtained by fitting lithium concentration-response curves to the data are summarized in Table 4.

Discussion

In this study, human NaCT and mouse NaCT were heterologously expressed in *Xenopus* oocytes, and the ability of the citric acid cycle intermediates to act as substrates of these transporters was systematically investigated using the two-electrode voltage clamp technique. The results show that not only citrate but all of the citric acid cycle intermediates tested served as substrates of human and mouse NaCT. Striking differences were found between human and mouse NaCT with respect to substrate sensitivity. Mouse NaCT was at least 400 times more sensitive to citrate than its human counterpart. Mouse NaCT was also much more sensitive to the other intermediates investigated. The difference between human and mouse NaCT ranged from at least 20-fold for oxaloacetate to 800-fold for succinate. A smaller (approximately 16-fold) difference in citrate sensitivity between human and mouse NaCT was previously detected in radiolabeled substrate uptake experiments. In these assays, K_i values for citrate were 604 μM for human NaCT (Inoue et al., 2002a) and 38 μM for mouse NaCT (Inoue et al., 2004), respectively.

In addition to the striking general difference in sensitivity for substrates between human and mouse NaCT, there are also more subtle differences that are worth mentioning. The rank order of substrates to activate human NaCT and mouse NaCT are different. For humans, the rank order was citrate > malate ~ α-ketoglutarate > succinate ~ fumarate > oxaloacetate ~ isocitrate. The rank order of substrates to activate mouse NaCT was citrate > succinate ~ fumarate ~ oxaloacetate ~ malate > α-ketoglutarate ~ isocitrate. Compared with citrate, oxaloacetate, fumarate, and succinate are relatively better substrates for mouse NaCT than for human NaCT, whereas α-ketoglutarate is a relatively better substrate for human NaCT than for mouse NaCT. Although isocitrate is a tricarboxylate, it is a very poor substrate for both human and mouse NaCT.

In addition to the striking differences in substrate sensitivity, human and mouse NaCT also differed markedly in their dependencies on extracellular cations to function. Mouse NaCT did not transport citrate when sodium ions were replaced by potassium or choline ions, whereas human NaCT was more promiscuous and substantial transporter activity was observed when sodium was replaced by either potassium or choline. In the presence of lithium ions, human NaCT and mouse NaCT were activated by citrate; however, in both cases, citrate-induced responses were much smaller than in normal solution containing sodium ions.

The observation that human NaCT is not very sensitive to citrate and other substrates raises questions about the

TABLE 3

Results of statistical comparison of E_{max} values of the different substrates for mouse NaCT

Data are presented as results (P values) of one-way analysis of variance followed by the post hoc Tukey test.

MOUSE	Citrate	Oxaloacetate	Malate	Succinate	Fumarate
Oxaloacetate	<0.05				
Malate	<0.05	n.s.			
Succinate	<0.05	n.s.	n.s.		
Fumarate	n.s.	n.s.	n.s.	n.s.	
Isocitrate	<0.05	<0.05	<0.05	<0.05	<0.05

n.s., not significant.

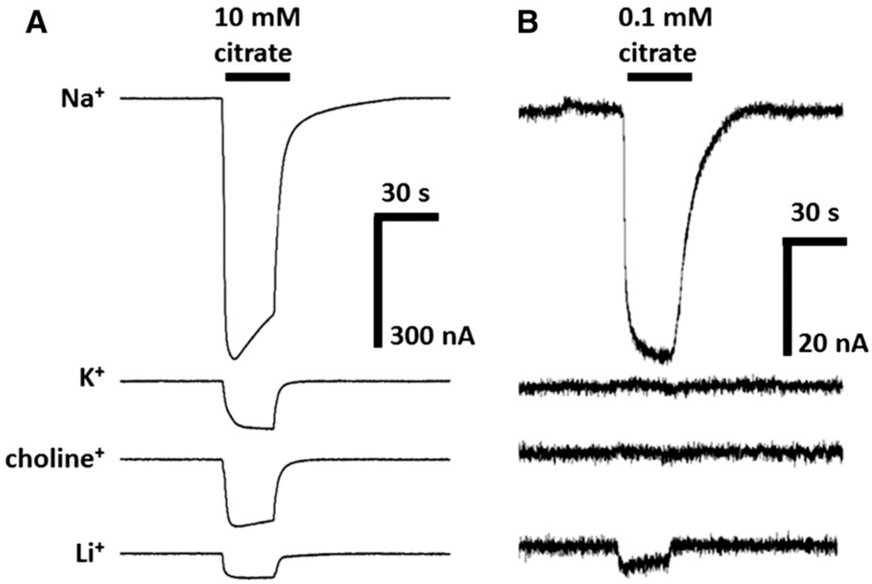

Fig. 4. Effect of sodium substitution by potassium, choline, and lithium on citrate-induced inward currents. (A) Effects of ion substitution on human NaCT. A near maximum effective concentration of 10 mM citrate induces a large inward current in oocytes when recorded in standard, sodium-containing recording solution. When all sodium was replaced by potassium, choline, or lithium, 10 mM citrate still induced ion currents, but their amplitudes were smaller than in sodium-containing recording solution. (B) Effects of ion substitution on mouse NaCT. A near maximum effective concentration of 0.1 mM citrate induces an inward current in oocytes when recorded in standard, sodium-containing recording solution. When all sodium was replaced by potassium or choline, 0.1 mM citrate did not activate mouse NaCT. When all sodium was replaced by lithium, 0.1 mM citrate evoked a small response.

physiologic role of NaCT in humans. Plasma concentrations of citrate in humans and rodents are in the range of 100–180 μM (Krebs, 1950; Shearer et al., 1971; Pugliese et al., 2002; Fraenkl et al., 2011). These concentrations of citrate fully activate mouse NaCT but only marginally activate human NaCT. In addition, the plasma concentrations of other carboxylates, such as fumarate (approximately 25 μM), malate (34 μM), succinate (42 μM), and α-ketoglutarate (55 μM), are sufficient to activate mouse NaCT but human NaCT will be unaffected by these plasma concentrations (Krebs, 1950). For these reasons, it is unclear whether human NaCT plays a significant role in hepatic uptake of citrate and other carboxylic acids. It might be possible that in vivo the NaCT protein interacts with other protein partners that regulate its function and influence the sensitivity of human NaCT for its substrates. Studies with NaCT knockout mice suggested that NaCT might be a promising target for the treatment of metabolic diseases such as obesity and type 2 diabetes. The finding that human NaCT is much less sensitive to citrate than its murine counterpart raises questions of whether NaCT is a valid target in humans and about the role NaCT plays in human physiology.

Despite previous publications that have suggested that lithium ions potentiate NaCT for humans (and other primates) while NaCT from mice (and other rodents) are not (Inoue et al., 2003; Gopal et al., 2015), we have found that both human and mouse NaCT could be potentiated by lithium ions. we found that human and mouse NaCT could both be potentiated by lithium ions. Importantly, our results demonstrate that potentiation by lithium only occurred when the transporter was exposed to relatively low concentrations of citrate. These citrate concentrations are relative to the EC_{50} values of citrate, which are very different for humans and mice. Potentiation by lithium was abolished at higher citrate concentrations, whereas the effect of lithium was reversed at high citrate concentrations and human NaCT was even inhibited by lithium. The discrepancy between our results and the reported effects of lithium on human and rodent NaCT are likely caused by the strong dependence of the potentiating effect of lithium on the citrate concentration. The potentiating effect of lithium on human NaCT is of clinical and therapeutic relevance because lithium is widely used to treat affective disorders such as bipolar disorder. Plasma concentrations of lithium in patients treated with the substance range from 0.8 to 2.0 mM (Sproule, 2002). Our results and the findings reported in the literature (Inoue et al., 2003; Gopal et al., 2015) show that potentiation of human NaCT is optimal at lithium concentrations reached in human plasma of patients treated with lithium. In addition, although human NaCT is not very sensitive to citrate, our results show that lithium potentiates only at relatively low citrate concentrations (Fig. 5C; 100–300 μM), resulting in a substantial increase in citrate uptake at concentrations of citrate that are normally near threshold. Uptake of citrate will be even further increased when lithium is administered as lithium-citrate. Weight gain is one of the side effects of lithium therapy. As pointed out by Gopal et al. (2015), this weight gain is likely caused by an enhanced NaCT-mediated uptake of citrate in the liver, which results in an increased synthesis of fatty acids and cholesterol.

A bacterial homolog of NaCT, VcINDY, was recently crystallized and its high-resolution structure was published (Mancusso et al., 2012). Since it is expected that this bacterial homolog will provide a basis for the understanding of the functioning of the whole SLC13 family of solute transporters, basic functional properties of VcINDY have been investigated (Mulligan et al., 2014). The latter study revealed that VcINDY is a high-affinity, sodium-dependent electrogenic transporter. Besides transporting succinate, it also transports malate, fumarate, oxaloacetate, and α-ketoglutarate (to a lesser extent). Lithium ions can substitute for sodium, but succinate in lithium is much less efficacious than in sodium. Interestingly, VcINDY hardly transports any citrate. Similarities and differences are evident between the functional properties reported for VcINDY and human and mouse NaCT observed in this study. Concerning the similarities, like VcINDY, human NaCT and mouse NaCT are both electrogenic, they transport all of the citric acid cycle intermediates that are transported by VcINDY, lithium ions can replace sodium, and transport of substrate is still taking place (although with a much smaller efficacy). Like VcINDY, mouse NaCT has a high-affinity for citric acid cycle intermediates, but human NaCT has a low

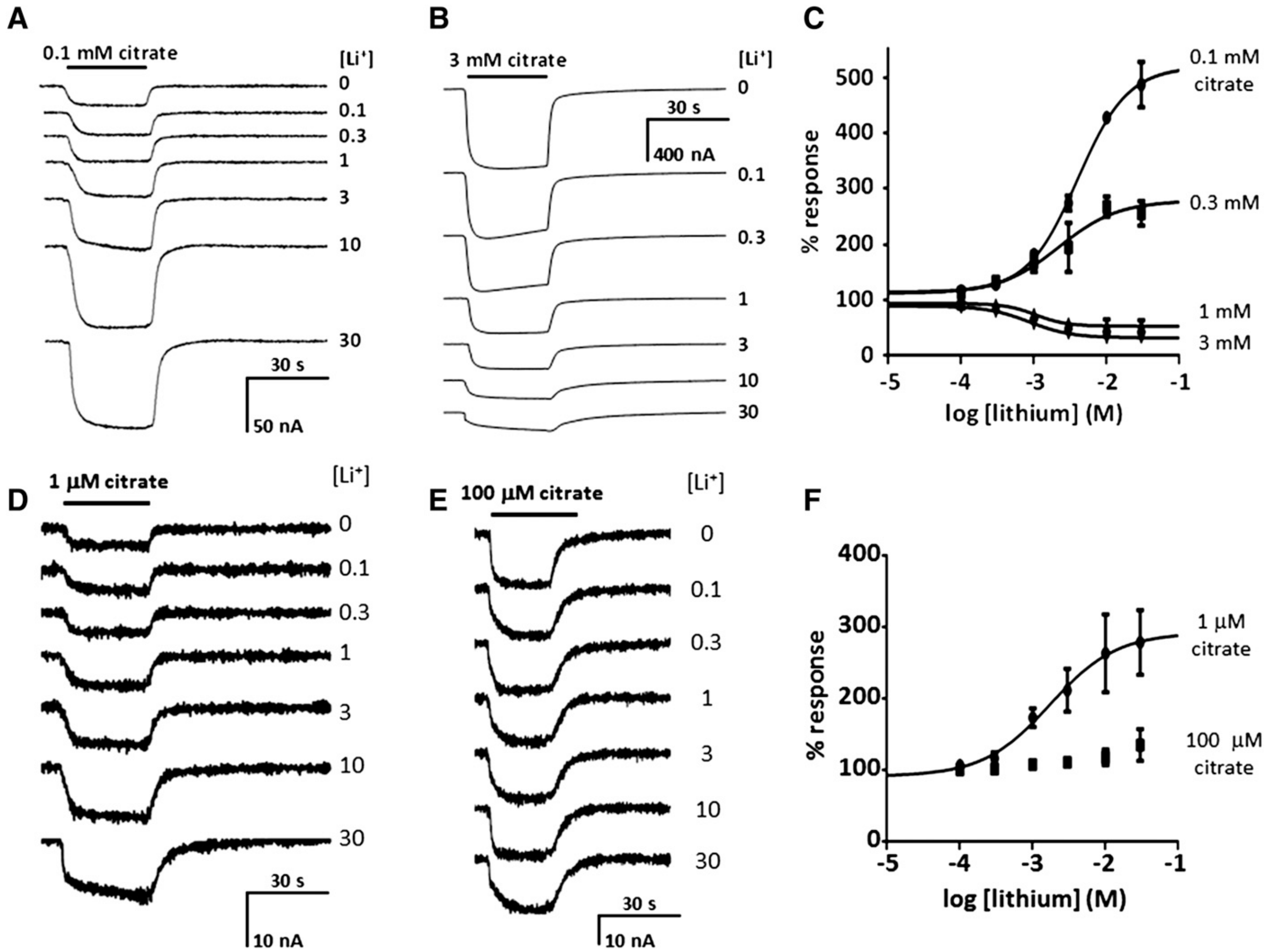

Fig. 5. Modulation of human and mouse NaCT by lithium ions. (A) Potentiation of human NaCT at a relatively low citrate concentration. Citrate was applied alone or coapplied with various concentrations of lithium as indicated. Lithium increased the citrate-induced ion currents in a concentration-dependent manner. (B) Inhibition of human NaCT at a higher concentration of citrate. Lithium decreased citrate-induced ion currents in a concentration-dependent manner when it was coapplied with 3 mM citrate and no full inhibition was obtained at the highest concentrations of lithium tested. (C) Potentiation and inhibition of human NaCT by lithium depends on citrate concentration. Concentration-response curves for lithium were measured using four different concentrations of citrate to activate the transporter. Lithium potentiated citrate-induced ion currents when the citrate concentration was relatively low. Increasing the citrate concentration from 0.1 to 0.3 mM reduced the maximum amount of potentiation of the citrate responses by lithium. When the lithium concentration was further increased to 1 or 3 mM, the effect of lithium was reversed from potentiation into inhibition. The estimated values for E_{max}/E_{min}, EC_{50}/IC_{50}, and Hill slopes obtained the curve-fitting procedure are summarized in Table 4. (D) Potentiation of mouse NaCT at a relatively low citrate concentration. Citrate was applied alone or coapplied with various concentrations of lithium as indicated. Lithium increased the citrate-induced ion currents in a concentration-dependent manner. (E) Potentiation of mouse NaCT was abolished at the higher concentration of 100 μM citrate. (F) The degree of potentiation of mouse NaCT by lithium depends on citrate concentration. Concentration-response curves for lithium were measured using two different concentrations of citrate to activate the transporter. Lithium potentiated citrate-induced ion currents when the citrate concentration was relatively low. Increasing the citrate concentration from 1 to 100 μM reduced the maximum amount of potentiation of the citrate responses by lithium to almost zero.

affinity for citric acid cycle intermediates. Furthermore, like VcINDY mouse NaCT is strongly sodium dependent, but the human ortholog is much less dependent on sodium ions to function. A main difference between VcINDY and human and mouse NaCT is that NaCT of both species is activated by citrate, whereas citrate is not a substrate that activates VcINDY. These observations show that the crystal structure and function of VcINDY can account for some, but not all, of the functional properties of mammalian transporters belonging to the SLC13 family.

In conclusion, human NaCT differs strongly from mouse NaCT in terms of substrate sensitivity and cation dependence. Human NaCT is activated by nonphysiologic citrate concentrations, whereas the sensitivity of mouse NaCT to citrate is in the same range as plasma citrate concentrations. Furthermore, activity of mouse NaCT fully depends on extracellular sodium ions, whereas human NaCT is much less

TABLE 4

Modulation of human and mouse NaCT by lithium

Data are expressed as means ± S.E. except for EC_{50}/IC_{50} values, which are expressed as means (95% confidence intervals). In the fields in which no values are given (indicated by dashes) the effects of lithium were too small to fit a curve to the data.

	[Citrate]	E_{max}	EC_{50}/IC_{50}	n_H	n
	µM	*%*	*mM*		
Human NaCT	100	536 ± 24	4.0 (2.9–5.6)	1.08 ± 0.11	3
Human NaCT	300	287 ± 31	2.0 (0.6–6.7)	0.86 ± 0.26	3
Human NaCT	1000	52 ± 4	0.9 (0.4–1.6)	1.34 ± 0.42	3
Human NaCT	3000	33 ± 3	0.9 (0.7–1.1)	1.49 ± 0.22	3
Mouse NaCT	1	292 ± 63	1.7 (0.3–11.7)	0.95 ± 1.01	3
Mouse NaCT	100	—	—	—	4

dependent on sodium because significant citrate activation is seen when sodium is replaced by potassium or choline. The weak sensitivity of human NaCT to citrate raises questions about the physiologic role of NaCT in the human liver and the validity of this transporter as a target for metabolic diseases.

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Authorship Contributions

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Conducted experiments: Zwart, Peeva.

Performed data analysis: Zwart, Peeva.

Wrote or contributed to the writing of the manuscript: Zwart, Peeva, Rong, Sher.

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