Structure, Function, and Expression Pattern of a Novel Sodium-coupled Citrate Transporter (NaCT) Cloned from Mammalian Brain*

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Citrate plays a pivotal role not only in the generation of metabolic energy but also in the synthesis of fatty acids, isoprenoids, and cholesterol in mammalian cells. Plasma levels of citrate are the highest (−135 μM) among the intermediates of the tricarboxylic acid cycle. Here we report on the cloning and functional characterization of a plasma membrane transporter (NaCT for Na⁺-coupled citrate transporter) from rat brain that mediates uphill cellular uptake of citrate coupled to an electrochemical Na⁺ gradient. NaCT consists of 572 amino acids and exhibits structural similarity to the members of the Na⁺-dicarboxylate cotransporter/Na⁺-sulfate cotransporter (NaDC/NaSi) gene family including the recently identified Drosophila Indy. In rat, the expression of NaCT is restricted to liver, testis, and brain. When expressed heterologously in mammalian cells, rat NaCT mediates the transport of citrate with high affinity (Michaelis-Menten constant, −20 μM) and with a Na⁺:citrate stoichiometry of 4:1. The transporter does interact with other dicarboxylates and tricarboxylates but with considerably lower affinity. In mouse brain, the expression of NaCT mRNA is evident in the cerebral cortex, cerebellum, hippocampus, and olfactory bulb. NaCT represents the first transporter to be identified in mammalian cells that shows preference for citrate over dicarboxylates. This transporter is likely to play an important role in the cellular utilization of citrate in blood for the synthesis of fatty acids and cholesterol (the precursors for the generation of energy (liver and brain)). NaCT thus constitutes a potential therapeutic target for the control of body weight, cholesterol levels, and energy homeostasis.

Mammalian tissues express two different sodium-coupled transporters for succinate and other dicarboxylate intermediates of citric acid cycle (1). These transporters, known as NaDC1 (sodium-dicarboxylate cotransporter) and NaDC3, can be distinguished from one another primarily based on their substrate affinity. NaDC1 is a low affinity transporter with a Michaelis-Menten constant (Kᵢ) for succinate in the millimolar range whereas NaDC3 is a high affinity transporter with a Kᵢ for succinate in the micromolar range. NaDC2, identified in Xenopus laevis intestine, is functionally and structurally related to the mammalian NaDCs, but may represent a species-specific ortholog of NaDC1 (2). The mammalian NaDCs have been studied from different species and their functional characteristics have been elucidated in different heterologous expression systems (3–10). NaDC1 is expressed primarily in the intestine and kidney whereas NaDC3 is expressed not only in the intestine and kidney but also in the brain, liver, and placenta (1). A unique feature of both of these transporters is that they interact with dicarboxylates with greater preference than with citrate, a tricarboxylate at physiological pH. Furthermore, even though NaDC1 and NaDC3 are able to transport citrate to some extent, only the dianionic form of citrate is recognized as the substrate by these transporters. Thus, NaDC1 and NaDC3 are specific for dicarboxylates. NaDCs are structurally related to the Na⁺-coupled sulfate transporters NaSi and SUT1 (1). Together, these transporters constitute the NaDC/NaSi gene family.

Recently, Rogina et al. (11) reported on the identification of a gene in Drosophila melanogaster which, when mutated, confers life span extension to the organism. Interestingly, the predicted protein product of this gene, known as Indy (for I’m Not Dead Yet), shows significant homology to mammalian NaDCs. It was therefore suggested that Indy is the Drosophila ortholog of either NaDC1 or NaDC3 and that disruption of its function may restrict the cellular entry of dicarboxylates, leading to decreased energy production, a metabolic condition similar to caloric restriction. This seemed logical because caloric restriction in rats and non-human primates has been shown to be associated with life span extension (12, 13). However, even though it was assumed, based on the structural similarity, that Drosophila Indy is a sodium-coupled transporter for dicarboxylates similar to mammalian NaDCs, its transport identity has not been established. We have recently cloned Drosophila Indy and investigated its transport function. These studies have predicted unexpected results. Drosophila Indy does possess the ability to transport succinate as do mammalian NaDCs but the transport is Na⁺-independent. Furthermore, unlike mammalian NaDCs, Drosophila Indy transports citrate very effectively. The affinity for citrate is severalfold greater in the case of Drosophila Indy than in the case of mammalian NaDCs. These findings suggested that neither NaDC1 nor NaDC3 is the mammalian ortholog of Drosophila Indy. Therefore, we searched the GenBank™ data base to see if there are additional transporters in mammals that are structurally related to NaDC1 and NaDC3. This search led to the identification of a novel mammalian transporter that is structurally similar to

Received for publication, July 15, 2002
Published, JBC Papers in Press, August 11, 2002, DOI 10.1074/jbc.M207072200

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The abbreviations used are: NaDC, sodium-dicarboxylate cotransporter; NaCT, sodium-coupled citrate transporter; NaSi, sodium-coupled sulfate transporter; SUT, sulfate transporter; Indy, I’m not dead yet; NMDG, N-methyl-D-glucamine; Mes, 4-morpholineethanesulfonic acid; EST, expressed sequence tag.

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K. Inoue and V. Ganapathy, unpublished observations.
mammalian NaDCs as well as to Drosophila Indy. Interestingly, this new transporter transports citrate much more effectively than succinate, a characteristic more similar to that of Drosophila Indy than to that of mammalian NaDCs. However, the transport process is coupled to Na\(^+\), a feature distinct from that of Drosophila Indy but similar to that of mammalian NaDCs. Based on these functional characteristics, we have designated this novel transporter as NaCT (for Na+-coupled citrate transporter). This represents the first sodium-coupled transporter in mammals to be identified that shows preference for citrate as a substrate.

**EXPERIMENTAL PROCEDURES**

**Materials**—\((^{14}\text{C})\text{Citrate}\) (specific radioactivity, 55 mCi/mmol), \(^{[1]}\text{H}\)succinate (specific radioactivity, 40 Ci/mmol), and \(^{[3]}\text{P}\)Clivuvrate (specific radioactivity, 15 mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA). The human retinal pigment epithelial (HRPE) cell line, used routinely in our laboratory for heterologous expression of cloned transporters, was originally provided by M. A. Del Monte (W. K. Kellogg Eye Center, Dept. of Ophthalmology, Ann Arbor, MI). The cell line was maintained in Dulbecco’s minimum essential medium/F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Lipofectin was purchased from Invitrogen, and transcription enzymes were purchased from Promega Biosciences (Madison, WI). Magna nylon transfer membranes used in library screening were purchased from Micron Separations (Westboro, MA). Unlabeled monocarboxylates, dicarboxylates, and tricarboxylates were obtained from Sigma.

**Cloning of NaCT from Rat Brain**—A search of the GenBank\(\text{TM}\) database for murine ESTs (established sequence tags) with the amino acid sequence of Drosophila Indy as a query identified several ESTs whose predicted amino acid sequences showed significant similarity to that of mammalian sodium-coupled dicarboxylate transporters NaDC1 and NaDC3. Many of these ESTs were identical to murine NaDC1 and NaDC3, which have been already cloned and functionally characterized (9, 10). There were three ESTs (GenBank\(\text{TM}\) accession nos. BB261903, BB393630, and BB641100) with overlapping nucleotide sequences, and the predicted amino acid sequence from these ESTs did not correspond to that of murine NaDC1 or NaDC3. However, there was significant similarity between this sequence and that of NaDC1, NaDC3, and Drosophila Indy. There is no information in the literature on the functional identity of this putative mammalian transporter. The sequence similarity however suggested that it might represent a new member of the sodium/dicarboxylate cotransporter gene family. We used the overlapping nucleotide sequences of two of these ESTs to design primers for RT-PCR to obtain a cDNA probe that is specific for this transporter. The forward primer was 5’-TCTTTTCTCCACACCTGCTCT-3’. The predicted size of the RT-PCR product, based on the positions of the primers, was 943 bp. Since both of these ESTs were identified from a mouse cerebral cortex cDNA library, we used poly(A) RNA from mouse brain as the template for RT-PCR to obtain the probe. This yielded a cDNA product of expected size. The product was subcloned in pGEM-T vector, and the cDNA-specific activity. In experiments in which the cation and anion dependence of the uptake process was investigated, NaCl was replaced isoosmotically with LiCl, KCl, N-methyl-D-glucamine (NMDG) chloride, sodium gluconate, or mannitol. When the influence of pH on the uptake process was investigated, uptake buffers of different pH values were prepared by appropriately altering the concentrations of Tris, Mes, and Hepes. Traces of citrate and 1 mM mercaptoethanol were added to each buffer. These time periods were chosen from time course studies with respective substrates to represent linear uptake conditions. Endogenous uptake was always determined in parallel using the cells transfected with pSPORT1 vector alone. The uptake activity in cDNA-transfected cells was adjusted for the endogenous uptake activity to calculate the cDNA-specific activity. In experiments in which the cation and anion dependence of the uptake process was investigated, NaCl was replaced isoosmotically with LiCl, KCl, N-methyl-D-glucamine (NMDG) chloride, sodium gluconate, or mannitol. 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Mammalian Sodium-coupled Citrate Transporter

**RESULTS**

**Structural Features of Rat NaCT**—The cloned rat NaCT cDNA is 3254 bp long with a poly(A) tail and an open reading frame (24–1742 bp) coding for a protein of 572 amino acids (GenBank™ accession number AF522186). The cloned transporter is new and has not been reported previously in the literature. Based on the amino acid sequence, rat NaCT belongs to the NaDC/NaSi (sodium-dicarboxylate cotransporter/sodium-sulfate cotransporter) gene family which, to date, consists of three sodium-coupled dicarboxylate transporters (NaDC1, NaDC2, and NaDC3) and two sodium-coupled sulfate transporters (NaSi and SUT1) (1). However, the rat NaCT is structurally more closely related to sodium-coupled dicarboxylate transporters than to sodium-coupled sulfate transporters. It shows a 44–50% sequence identity with rat NaDCs (Fig. 1). It also shows significant structural similarity to *Drosophila* Indy (34% identity). Hydropathy analysis suggests that rat NaCT possesses eleven putative transmembrane domains, similar to the previously known sodium-coupled dicarboxylate transporters. A search of GenBank™ data base using the rat NaCT amino acid sequence as a query has revealed that a region of sequence similarity to the sodium-coupled dicarboxylate transporters, which is very interesting because the previously described NaDCs possess much lower ability to transport citrate than to transport succinate (1). Since rat NaCT was able to transport citrate preferentially, we used this tricarboxylate as a substrate for subsequent functional characterization of the transporter. The cDNA-mediated uptake of citrate was linear even up to 30 min (Fig. 3B). The uptake process operated maximally at pH 7 (Fig. 3C).

The involvement of Na⁺ in the uptake process mediated by rat NaCT was evaluated by monitoring the uptake of citrate in vector-transfected cells and in rat NaCT cDNA-transfected cells in the presence and absence of Na⁺. This was done by isosmotically replacing NaCl in the uptake medium with NMDG chloride, KCl, LiCl, or mannitol (Table I). The cDNA-specific uptake was almost completely abolished when Na⁺ was removed from the medium. The uptake process was however not dependent on Cl⁻ because the replacement of Cl⁻ with gluconate had only a small effect on the cDNA-specific citrate uptake.

The substrate specificity of rat NaCT was examined by competition studies in which we assessed the ability of various unlabeled compounds (2.5 mM) to compete with [¹⁴C]citrate (7 μM) for uptake via rat NaCT (Table II). Unlabeled citrate was the most potent inhibitor of [¹⁴C]citrate uptake mediated by rat NaCT. Among the dicarboxylates, succinate and malate were very potent inhibitors. Fumarate and α-ketoglutarate were comparatively less effective. Maleate, the cis isomer of fumarate, was one of the least potent inhibitors, showing stereoselectivity of the transporter. The monocarboxylates pyruvate and lactate were not effective in inhibiting [¹⁴C]citrate uptake.

**Functional Features of Rat NaCT**—We assessed the transport function of rat NaCT in a heterologous expression system in a mammalian cell line (HRPE) using the vaccinia virus expression technique. Since rat NaCT shows high structural similarity to the sodium-coupled dicarboxylate transporters, we first tested the ability of rat NaCT to transport succinate in the presence of a Na⁺ gradient (Fig. 3A). The uptake of succinate (80 nM) in cells transfected with rat NaCT cDNA was about 7-fold higher than in cells transfected with vector alone, indicating that the cloned transporter does possess the ability to mediate the uptake of this dicarboxylate. We then tested whether pyruvate (a monocarboxylate) and citrate (a tricarboxylate) are recognized as transport substrates by rat NaCT. Surprisingly, the uptake of both of these compounds (pyruvate, 135 μM; citrate, 35 μM) was higher in cDNA-transfected cells than in vector-transfected cells. The cDNA-induced increase in pyruvate uptake was marginal (~3-fold) whereas the increase was much more marked in the case of citrate (~90-fold). This was very interesting because the previously described NaDCs possess much lower ability to transport citrate than to transport succinate (1). Since rat NaCT was able to transport citrate preferentially, we used this tricarboxylate as a substrate for subsequent functional characterization of the transporter. The cDNA-mediated uptake of citrate was linear even up to 30 min (Fig. 3B). The uptake process operated maximally at pH 7 (Fig. 3C).

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**Kinetic Features of Rat NaCT**—Citrate uptake mediated by rat NaCT was saturable with a *K₉* of 18 ± 4 μM (Fig. 4A). Kinetic analysis of Na⁺ dependence of citrate uptake via rat NaCT showed that the relationship between the uptake and...


The inhibitory potencies of other compounds were in the following order: succinate > fumarate = cis-aconitate > α-ketoglutarate. While citrate was the most potent inhibitor, isocitrate had no detectable inhibitory effect. The IC$_{50}$ values (i.e., concentrations of inhibitors causing 50% inhibition) calculated for citrate and succinate, the two most potent inhibitors, from these dose-response curves are 28 ± 4 and 172 ± 24 μM, respectively. The IC$_{50}$ for other compounds are severalfold higher than these values. Using the IC$_{50}$ values for citrate and succinate, we calculated the corresponding inhibition constants by the method of Cheng & Prusoff (26). The inhibition constants for these two compounds are 16 ± 2 and 98 ± 14 μM, respectively. These data show that, among the compounds tested, rat NaCT possesses the highest affinity for citrate. Thus, the affinity of rat NaCT for succinate is 6-fold lower than that for citrate. The affinities for other dicarboxylates and tricarboxylates are even lower. Thus, citrate is relatively a specific substrate for rat NaCT. For comparison, we analyzed the relative affinities of rat NaDC3 for these compounds. This was done in a similar way by assessing the potencies of these compounds to inhibit the uptake of $^3$H]succinate (80 nM) mediated by rat NaDC3 cloned from placenta using the same heterologous expression system (Fig. 5B). The results show that rat NaDC3 interacts with dicarboxylates (succinate, fumarate, and α-ketoglutarate) much more preferentially than with tricarboxylates (citrate, isocitrate, and cis-aconitate). The inhibition constants for the three dicarboxylates are in the range of 15–60 μM whereas the corresponding values for the three tricarboxylates are in the range of 2–4 mM. Thus, rat NaDC3 recognizes a number of dicarboxylates as preferential substrates and has very low affinity for tricarboxylates. This qualifies rat NaDC3 to be recognized as a sodium-coupled dicarboxylate transporter. On the contrary, rat NaCT transports citrate but interacts with other tricarboxylates very poorly. It has to be noted that this transporter does recognize succinate as a substrate, but its affinity for succinate is severalfold lower than for citrate. Furthermore, other dicarboxylates interact with the transporter very poorly. Based on these data, we identify the newly cloned transporter as a sodium-coupled citrate transporter.

We then assessed the physiological relevance of the differential substrate specificity of rat NaCT and rat NaDC3. Plasma contains significant levels of citrate (−25 mg/liter) and succinate (−5 mg/liter) (27–30). α-Ketoglutarate, oxaloacetate, fumarate, and malate are also present in the plasma but at much lower levels. Normal fasting levels of citrate and succinate in the plasma are −135 and 40 μM, respectively. The plasma levels of citrate increase by 25–30% above the fasting levels after meal (29). To investigate the physiological relevance of the substrate specificity of NaCT and NaDC3, we assessed the ability of these two transporters to mediate the transport of citrate and succinate in the presence of a Na$^+$ gradient when the medium contained physiological concentrations of these two compounds (i.e., 135 μM citrate and 40 μM succinate). Under these conditions, the values for succinate uptake mediated by rat NaCT and rat NaDC3 were 0.005 ± 0.001 and 0.48 ± 0.01 nmol/10$^6$ cells/min, respectively. In contrast, the values for citrate uptake mediated by these two transporters were 0.24 ± 0.01 and 0.04 ± 0.01 nmol/10$^6$ cells/min, respectively. These data show that, at physiological concentrations of succinate and citrate found in plasma, NaDC3 functions primarily to transport succinate whereas NaCT functions primarily to transport citrate.

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Fig. 3. Uptake of citrate, succinate, and pyruvate by rat NaCT.
A, uptake of $^3$H]succinate (80 nM), $^1$C]pyruvate (135 μM), and $^{14}$C]citrate (35 μM) in vector-transfected (V) and rat NaCT cDNA-transfected (NaCT) HRPE cells. B, time course of $^{14}$C]citrate (18 μM) uptake in vector-transfected (C) and rat NaCT cDNA-transfected (●) HRPE cells. C, influence of extracellular pH on the uptake of $^{14}$C]citrate (7 μM) that was mediated specifically via rat NaCT.

Na$^+$ concentration was sigmoidal, indicating the involvement of multiple Na$^+$ ions per transport cycle (Fig. 4B). The data were analyzed by Hill equation to determine the Na$^+$:citrate stoichiometry. This analysis gave a value between 3 and 4 (3.3 ± 0.3) for the Hill coefficient (n$^H$). Since citrate exists predominantly as a trivalent anion under the experimental conditions (i.e., pH 7.5), cotransport of citrate with 3 Na$^+$ ions will render the transport process electrically silent whereas cotransport with 4 Na$^+$ ions will render the process electrogenic. Therefore, we investigated whether or not the rat NaCT-mediated citrate uptake in the presence of Na$^+$ is influenced by membrane potential. For this purpose, we compared the cDNA-specific citrate uptake between control conditions and membrane depolarizing conditions (i.e., high extracellular K$^+$) (Fig. 4C). The uptake was inhibited significantly (43 ± 2%) when membrane was depolarized, indicating that the uptake process is influenced by membrane potential. Since depolarization inhibits the uptake, we conclude that the uptake process mediated by rat NaCT is electrogenic associated with a net transfer of positive charge into the cells. This would then suggest that at least 4 Na$^+$ are cotransported with one citrate in the transport process.

We then compared the affinities of rat NaCT for different tricarboxylates (citrate, isocitrate, and cis-aconitate) and dicarboxylates (succinate, fumarate, and α-ketoglutarate) by monitoring the potencies of these compounds to inhibit the uptake of $^{14}$C]citrate (14 μM) mediated by rat NaCT (Fig. 5A). Among these compounds, citrate was the most potent inhibitor of rat NaCT-mediated $^{14}$C]citrate uptake. The inhibitory potencies of other compounds were in the following order: succinate > fumarate = cis-aconitate > α-ketoglutarate. While citrate was the most potent inhibitor, isocitrate had no detectable inhibitory effect. The IC$_{50}$ values (i.e., concentrations of inhibitors causing 50% inhibition) calculated for citrate and succinate, the two most potent inhibitors, from these dose-response curves are 28 ± 4 and 172 ± 24 μM, respectively. The IC$_{50}$ for other compounds are severalfold higher than these values. Using the IC$_{50}$ values for citrate and succinate, we calculated the corresponding inhibition constants by the method of Cheng & Prusoff (26). The inhibition constants for these two compounds are 16 ± 2 and 98 ± 14 μM, respectively. These data show that, among the compounds tested, rat NaCT possesses the highest affinity for citrate. Thus, the affinity of rat NaCT for succinate is 6-fold lower than that for citrate. The affinities for other dicarboxylates and tricarboxylates are even lower. Thus, citrate is relatively a specific substrate for rat NaCT. For comparison, we analyzed the relative affinities of rat NaDC3 for these compounds. This was done in a similar way by assessing the potencies of these compounds to inhibit the uptake of $^3$H]succinate (80 nM) mediated by rat NaDC3 cloned from placenta using the same heterologous expression system (Fig. 5B). The results show that rat NaDC3 interacts with dicarboxylates (succinate, fumarate, and α-ketoglutarate) much more preferentially than with tricarboxylates (citrate, isocitrate, and cis-aconitate). The inhibition constants for the three dicarboxylates are in the range of 15–60 μM whereas the corresponding values for the three tricarboxylates are in the range of 2–4 mM. Thus, rat NaDC3 recognizes a number of dicarboxylates as preferential substrates and has very low affinity for tricarboxylates. This qualifies rat NaDC3 to be recognized as a sodium-coupled dicarboxylate transporter. On the contrary, rat NaCT transports citrate but interacts with other tricarboxylates very poorly. It has to be noted that this transporter does recognize succinate as a substrate, but its affinity for succinate is severalfold lower than for citrate. Furthermore, other dicarboxylates interact with the transporter very poorly. Based on these data, we identify the newly cloned transporter as a sodium-coupled citrate transporter.

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NaSi gene family. Structurally and functionally, NaCT is
NaDC3. To determine the expression pattern of NaCT mRNA in mouse brain, we subjected sagittal sections of mouse brain to in situ hybridization with digoxigenin-labeled riboprobes specific for mouse NaCT. Hybridization with antisense probe has revealed that the NaCT mRNA is expressed widely in the mouse brain, primarily in the neurons of the cerebral cortex, hippocampal formation, cerebellum, and olfactory bulb (Fig. 6A). The signals are specific as evidenced from markedly reduced signals with sense probe (Fig. 6B). In the cerebellum, the hybridization signals with antisense probe are most intense in the Purkinje cell bodies, followed by the neurons in the granular layer (Fig. 6, C and D). Expression is also evident in the interneurons of the molecular layer, namely the stellate cells located in the outer portion of the molecular layer and the basket cells located closest to the border between the molecular and Purkinje layers. While the white matter is negative for hybridization signal, the deep cerebellar nuclei are intensely positive for expression. In the hippocampal formation (Fig. 6, E and F), the pyramidal cells in the cornus ammonis regions CA3, CA2, and CA1 are highly positive for mRNA expression, followed by neurons in the subiculum. Granule cells, which are the projection neurons of the dentate gyrus, are intensely positive for the hybridization signal. Similarly, the interneurons of the polymorphic layer in the dentate gyrus are also positive for expression. The molecular layer that has very few cell bodies is largely negative. In the cerebral cortex, the expression is widespread. Based on the expression pattern in the hippocampal formation and cerebellum, the observed expression in the cerebral cortex is likely to be restricted primarily to neurons.

**DISCUSSION**

**Functional Differences Between NaCT and NaDC1/NaDC3**—NaCT represents the newest member of the NaDC/NaSi gene family. Structurally and functionally, NaCT is closely related to NaDC1 and NaDC3. But, there are significant differences among these three transporters in terms of substrate specificity, substrate affinity, and tissue expression pattern. NaCT is Na⁺-coupled and exhibits much higher affinity for citrate than for succinate. This is in contrast to NaDC1 and NaDC3 which, though Na⁺-coupled as NaCT, exhibit much higher affinity for succinate than for citrate. It has to be pointed out here that NaDC1 and NaDC3 do transport citrate, but only the divalent form of citrate is recognized as a substrate.

| Salt          | Citrate Uptake |
|---------------|---------------|
|               | Vector | cDNA | cDNA-specific | % |
| NaCl          | 0.84 ± 0.14 | 38.8 ± 3.3 | 38.0 ± 3.3 | 100 |
| NMDC chloride | 1.22 ± 0.22 | 1.6 ± 0.3 | 0.4 ± 0.3 | 1 |
| KCl           | 0.49 ± 0.03 | 0.6 ± 0.1 | 0.1 ± 0.1 | 0 |
| LiCl          | 0.51 ± 0.02 | 1.6 ± 0.1 | 1.1 ± 0.1 | 3 |
| Sodium gluconate | 0.48 ± 0.03 | 29.6 ± 2.3 | 29.1 ± 2.3 | 77 |
| Mannitol      | 0.59 ± 0.03 | 0.7 ± 0.1 | 0.1 ± 0.1 | 0 |

**Table I**

Ion dependence of citrate uptake via rat NaCT

Uptake of [14C]citrate (7 μM) was measured in vector-transfected HRPE cells and in rat NaCT cDNA-transfected HRPE cells at pH 7.5 in the presence of various inorganic salts or mannitol. Values represent means ± S.E.

| Salt          | Citrate Uptake |
|---------------|---------------|
|               | pmol/10⁶ cells/min | % |
| None          | 108.3 ± 5.5 | 100 |
| Citrate       | 1.5 ± 0.1 | 1 |
| Succinate     | 5.7 ± 0.2 | 5 |
| Malate        | 7.8 ± 0.1 | 7 |
| Fumarate      | 17.7 ± 1.4 | 16 |
| α-Ketoglutarate | 36.5 ± 2.1 | 34 |
| Maleate       | 78.8 ± 1.5 | 73 |
| Pyruvate      | 89.4 ± 7.2 | 83 |
| Lactate       | 89.6 ± 2.0 | 83 |

**Table II**

Substrate specificity of rat NaCT

Uptake of [14C]citrate (14 μM) was measured in vector-transfected HRPE cells and in rat NaCT cDNA-transfected HRPE cells in the absence or presence of various monocarboxylates, dicarboxylates, or tricarboxylates (2.5 mM). Data (means ± S.E.) represent only cDNA-specific uptake.

Analysis of Expression Pattern of NaCT mRNA in Mouse Brain by In Situ Hybridization—To determine the expression pattern of NaCT mRNA in mouse brain, we subjected sagittal sections of mouse brain to in situ hybridization with digoxigenin-labeled riboprobes specific for mouse NaCT. Hybridization with antisense probe has revealed that the NaCT mRNA is expressed widely in the mouse brain, primarily in the neurons of the cerebral cortex, hippocampal formation, cerebellum, and olfactory bulb (Fig. 6A). The signals are specific as evidenced from markedly reduced signals with sense probe (Fig. 6B). In the cerebellum, the hybridization signals with antisense probe are most intense in the Purkinje cell bodies, followed by the neurons in the granular layer (Fig. 6, C and D). Expression is also evident in the interneurons of the molecular layer, namely the stellate cells located in the outer portion of the molecular layer and the basket cells located closest to the border between the molecular and Purkinje layers. While the white matter is negative for hybridization signal, the deep cerebellar nuclei are intensely positive for expression. In the hippocampal formation (Fig. 6, E and F), the pyramidal cells in the cornus ammonis regions CA3, CA2, and CA1 are highly positive for mRNA expression, followed by neurons in the subiculum. Granule cells, which are the projection neurons of the dentate gyrus, are intensely positive for the hybridization signal. Similarly, the interneurons of the polymorphic layer in the dentate gyrus are also positive for expression. The molecular layer that has very few cell bodies is largely negative. In the cerebral cortex, the expression is widespread. Based on the expression pattern in the hippocampal formation and cerebellum, the observed expression in the cerebral cortex is likely to be restricted primarily to neurons.
forms of NaDC is in substrate selectivity. NaCT is relatively very specific for citrate. Even though it recognizes the tricarboxylate citrate as its substrate, it interacts very poorly with other structurally related tricarboxylates such as isocitrate and cis-aconitate. NaCT does transport succinate, but its affinity for this dicarboxylate is 6-fold less than for citrate. In contrast, NaDC3 exhibits comparable affinity for several structurally related dicarboxylates such as succinate, malate, fumarate, oxaloacetate, and \( \alpha \)-ketoglutarate. Furthermore, NaDC3 does not differentiate among the tricarboxylates citrate, isocitrate, and cis-aconitate though the affinity of the transporter for these tricarboxylates is severalfold lower than for the dicarboxylates. This indicates that NaDC3 has broad substrate selectivity among the dicarboxylates and the divalent forms of tricarboxylates. The same is true with NaDC1 (1). This is not the case with NaCT. The substrate selectivity of NaCT is comparatively more restricted. This is particularly evident from the lack of interaction between NaCT and isocitrate. Citrate differs from isocitrate only in the position of the hydroxyl group. Thus, among the tricarboxylates tested, citrate has the highest affinity for NaCT whereas isocitrate has no detectable affinity for the transporter. These data suggest that NaCT is primarily a citrate transporter. This is especially appreciable when the abilities of NaCT and NaDC3 to transport succinate or citrate are compared under physiological conditions with plasma concentration of citrate far exceeding the combined concentrations of other potential substrates. Under these conditions, NaCT transports primarily citrate whereas NaDC3 transports primarily succinate. Therefore, NaCT does not possess broad specificity toward structurally related tricarboxylates. Its substrate selectivity is essentially restricted to cit-
rate. On the other hand, NaDC1 and NaDC3 are Na\(^+\)-coupled transporters with broad specificity toward structurally related dicarboxylates.

**Differences in Tissue Expression Pattern Between NaCT and NaDC1/NaDC3**—In the rat, NaCT mRNA is expressed primarily in the liver, testis, and brain. In contrast, NaDC1 mRNA is expressed mostly in the small intestine and kidney whereas NaDC3 mRNA is expressed primarily in the kidney, small intestine, liver, placenta, and brain (5, 6). Thus, these three transporters differ significantly in the expression pattern in rat tissues. Even though NaCT mRNA and NaDC3 mRNA are expressed in rodent brain, the distribution pattern is very different (Ref. 18 and present study). NaCT mRNA is detectable very widely in the brain. The expression is most abundant in the hippocampal formation, cerebellum, cerebral cortex, and olfactory bulb. On the other hand, NaDC3 mRNA is expressed primarily in the meningeal layers of supporting tissue that surround the brain. Its expression is evident but weak in the cerebral cortex, hippocampus, and cerebellum. Furthermore, NaCT mRNA expression is found mostly in neurons whereas NaDC3 mRNA expression occurs mostly in glial cells.

**Physiologic and Therapeutic Significance of NaCT as a Citrate Transporter**—NaCT is the first plasma membrane transporter described in mammals that functions primarily in the cellular uptake of citrate. The plasma concentration of citrate is 135 \( \mu \text{M} \). Since the \( K_c \) for the transport of citrate via NaCT is \(~20 \ \mu \text{M} \), the transporter is likely to play an efficient role in the cellular entry of citrate under physiological conditions. Citrate occupies a pivotal position in cellular metabolism. It is not only an intermediate in the citric acid cycle that is the primary site of biological energy production in most cells, but also is a source of cytosolic acetyl CoA for the synthesis of fatty acids, isoprenoids, and cholesterol and for the elongation of fatty acids. Acetyl CoA present in the cytoplasm originates from citrate produced within mitochondria. A tricarboxylate transporter located in the inner mitochondrial membrane mediates the electroneutral efflux of citrate from the mitochondrial matrix in exchange for cytosolic malate or succinate (31). Following the entry into the cytoplasm, citrate is cleaved by ATP-citrate lyase to generate acetyl CoA. The identification of NaCT as a plasma membrane citrate transporter suggests that citrate in the circulation may also serve as an important source of cytoplasmic citrate. NaCT is a highly concentrative transporter with a Na\(^+\)-citrate stoichiometry of 4:1. It is electrogenic and thus the cellular entry of citrate via NaCT is energized not only by a Na\(^+\) gradient but also by the membrane potential. Citrate that enters the cells via NaCT may either serve as a precursor for the biosynthesis of fatty acids and cholesterol or enter the mitochondrial matrix to serve as an intermediate in the citric acid cycle. The choice between these two pathways will depend on the hormonal milieu and metabolic state of the cell. The expression of NaCT in the liver is of physiologic importance in this respect because this organ plays a vital role in the synthesis of fatty acids, isoprenoids, and cholesterol. Therefore, NaCT may prove to be a potential therapeutic target for controlling hepatic production of fatty acids and cholesterol. A selective blocker or inhibitor of this transporter may prevent hepatic utilization of citrate in blood in these biosynthetic processes.

Since NaCT is expressed in the brain mostly in neurons, we speculate that this transporter may play an important role in supplying citrate for these cells as a metabolic precursor for production of ATP via citric acid cycle. Interestingly, even though the mitochondrial tricarboxylate transporter (32, 33) and the plasma membrane NaCT transport citrate, there is no structural similarity between these two transporters. The mitochondrial tricarboxylate transporter consists of 298 amino acids and possesses six putative transmembrane domains (33). The membrane topology of this transporter is similar to that of several other transporters in the inner mitochondrial membrane (32). In contrast, the plasma membrane NaCT is a much larger protein and its membrane topology, with a predicted eleven transmembrane domains, is different from that of the mitochondrial tricarboxylate transporter. Furthermore, these two transporters also differ in energetics. The mitochondrial tricarboxylate transporter is an electroneutral exchanger and there is no role for a Na\(^+\) gradient in the transport process. In contrast, NaCT is driven by an electrochemical Na\(^+\) gradient. Some microbial organisms possess a sodium-coupled citrate transporter (34), but there is no significant structural homology between this transporter and mammalian NaCT.

**Is NaCT the Mammalian Ortholog of Drosophila Indy?**—Indy is a gene in Drosophila which, when made dysfunctional, leads to an extension of the average life span of the organism (11). The putative protein product of this gene is structurally similar to mammalian NaDC1 and NaDC3 and therefore it was suggested that Drosophila Indy is most likely the species-specific ortholog of either NaDC1 or NaDC3 (11). Recently, we isolated a functional clone of Drosophila Indy and characterized its transport function.\(^2\) Drosophila Indy functions as a Na\(^+\)–independent, electroneutral transporter for a variety of citric acid cycle intermediates. The functional characteristics of Drosophila Indy are different from those of NaDC1 and NaDC3. Therefore, neither NaDC1 nor NaDC3 represents the mammalian ortholog of Drosophila Indy. The newly identified NaCT is the newest member of the NaDC/NaSi gene family in mammals. Then the question arises as to whether NaCT represents the mammalian ortholog of Drosophila Indy. We do not have a definitive answer to this intriguing question. Drosophila Indy transports citrate much more effectively than it does succinate. In this respect, the NaCT resembles Drosophila Indy. Furthermore, there is also significant similarity between the tissue expression pattern of NaCT in mammals and that of Indy in Drosophila. NaCT is expressed abundantly in mammalian liver, a highly metabolic organ involved in fatty acid and cholesterol biosynthesis and energy storage. Similarly, in Drosophila, Indy is expressed abundantly in the fat body, an organ of metabolic function compared with that of liver in mammals. In Indy, the two transporters differ in their protein structure. While Drosophila Indy is a Na\(^+\)-independent transporter for citrate, NaCT is a Na\(^+\)-coupled transporter for citrate. Furthermore, Drosophila Indy is electroneutral with no role for membrane potential in the transport process mediated by the transporter. In contrast, NaCT is electrionic with its transport function associated with membrane depolarization. Thus, the two transporters are similar in substrate selectivity and tissue expression pattern but are different in their transport mechanism. Therefore, it is not known at this time whether or not NaCT is the mammalian ortholog of Drosophila Indy. Future studies directed toward the elucidation of potential influence of targeted disruption of the gene coding for NaCT on metabolic energy production and life span extension in mammals will most likely answer this question.

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