Mechanistic Insights and Functional Determinants of the Transport Cycle of the Ascorbic Acid Transporter SVCT2

ACTIVATION BY SODIUM AND ABSOLUTE DEPENDENCE ON BIVALENT CATIONS

We characterized the human Na⁺:ascorbic acid transporter SVCT2 and developed a basic model for the transport cycle that challenges the current view that it functions as a Na⁺-dependent transporter. The properties of SVCT2 are modulated by Ca²⁺/Mg²⁺ and a reciprocal functional interaction between Na⁺ and ascorbic acid that defines the substrate binding order and the transport stoichiometry. Na⁺ increased the ascorbic acid transport rate in a cooperative manner, decreasing the transport Km without affecting the Vmax, thus converting a low affinity form of the transporter into a high affinity transporter. Inversely, ascorbic acid affected in a bimodal and concentration-dependent manner the Na⁺ cooperativity, with absence of cooperativity at low and high ascorbic acid concentrations. Our data are consistent with a transport cycle characterized by a Na⁺:ascorbic acid stoichiometry of 2:1 and a substrate binding order of the type Na⁺:ascorbic acid:Na⁺. However, SVCT2 is not electrogenic. SVCT2 showed an absolute requirement for Ca²⁺/Mg²⁺ for function, with both cations switching the transporter from an inactive into an active conformation by increasing the transport Vmax without affecting the transport Km or the Na⁺ cooperativity. Our data indicate that SVCT2 may switch between a number of states with characteristic properties, including an inactive conformation in the absence of Ca²⁺/Mg²⁺. At least three active states can be envisioned, including a low affinity conformation at Na⁺ concentrations below 20 mM and two high affinity conformations at elevated Na⁺ concentrations whose Na⁺ cooperativity is modulated by ascorbic acid. Thus, SVCT2 is a Ca²⁺/Mg²⁺-dependent transporter.

Vitamin C exists in two chemically distinct forms: the reduced ionizable form (ascorbic acid) and the oxidized non-ionic form (dehydroascorbic acid) (1). Human cells acquire vitamin C using two different transporter systems that differ in structural as well as functional terms. One transporter system is a bidirectional, low affinity, high capacity system that includes several members of the facilitative glucose transporter (GLUT) family, shows absolute specificity for oxidized vitamin C, and transports dehydroascorbic acid down a substrate concentration gradient. Fourteen glucose transporter isoforms (GLUT1–GLUT14) have been molecularly characterized (2, 3), and there is evidence obtained from expression studies in Xenopus laevis oocytes indicating that GLUT1, GLUT3, and GLUT4 (4–9) are dehydroascorbic acid transporters. The evidence regarding GLUT2 is still controversial, and there are no data regarding the capacity of GLUT8, GLUT10, and GLUT12–GLUT14 to transport dehydroascorbic acid. GLUT5 is a fructose transporter unable to transport dehydroascorbic acid, which is probably also the situation with GLUT7, GLUT9, and GLUT11, although these last transporters have been less studied from a functional perspective.

A second vitamin C transport system is a high affinity, low capacity system, the sodium-ascorbic acid co-transporters (SVCTs), composed of two members that show absolute specificity for ascorbic acid and transport the substrate down the electrochemical sodium gradient (10, 11). SVCT1 and SVCT2 have been expressed in heterologous systems, but only SVCT1 has been functionally characterized in detail. SVCT1 has an apparent transport Km in the range of 50–200 mM, is electrogenic, and shows a Hill coefficient (nH) for Na⁺ near 2 (11). SVCT2 has been difficult to express in heterologous systems at high efficiency, and the data from these studies do not show a high degree of reproducibility (11–14). As a result of these limitations, there is no clear information regarding the values of basic functional parameters such as the transport Km (from 6 to 200 mM), the nH (from 1 to 2), the Na⁺:ascorbic acid stoichiometry, the substrate binding order, or the mechanism by which bivalent cations affect transport.

We present here a detailed characterization of the ascorbic acid transporter SVCT2 of human melanoma cells and propose...
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a basic model for the transport cycle. Our data from conventional and quantitative PCR immunocytochemistry and immunoblotting, cloning and expression in HEK-293 cells, and transport and electrochemical studies indicate that SVCT2 is a high affinity transporter of Na\(^+\) and ascorbic acid that shows positive cooperativity for Na\(^+\) and has an absolute requirement for Ca\(^2+\)/Mg\(^2+\) for activity. The transport cycle is characterized by a 2:1 Na\(^+\):ascorbic acid transport stoichiometry and a substrate binding order of the type Na\(^+\):ascorbic acid:Na\(^+\).

EXPERIMENTAL PROCEDURES

Cell Culture—Human melanoma cells (SK-MEL-131) were grown in RPMI 1640–25 mM Hepes (pH 7.4) with 10% (v/v) fetal bovine serum and penicillin/streptomycin (100 units/ml) (15).

RT-PCR—mRNA was prepared using the Micro poly(A) Pure kit (Ambion Inc.), and reverse transcription was performed using the rapid RT-PCR kit (Clontech Inc.) (9). Approximately 10 ng of cDNA synthesis product was used as template in a reaction mix containing 40 pmol of the primer pair (13), with the program: 2 min at 94°C; 35 cycles of 2 min each at 94°C, 1 min at 55°C, and 2 min at 72°C; and 7 min at 72°C. The PCR products were separated by electrophoresis on 2% agarose gel and visualized with ethidium bromide.

Immunolocalization—The cells were fixed in 4% paraformaldehyde, washed, and blocked in phosphate-buffered (pH 7.6) saline, 1% bovine serum albumin, incubated for 18 h at room temperature in the same buffer containing anti-SVCT or goat preimmunum serum (Santa Cruz Biotechnology, 1:1000), washed, and incubated with anti-goat IgG-horseradish peroxidase (DAKO, 1:100), and the reaction revealed with H\(_2\)O\(_2\) and 3,3-diaminobenzidine (16, 17).

Uptake Assays—The uptake experiments were carried out as previously described using 3-day-old cell monolayers in 12-well tissue culture plates (8, 15). The transport medium contained 15 mM Hepes buffer (pH 7.6), 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgCl\(_2\), and 0.1 mM dithiothreitol. For experiments in Na\(^+\)-free buffer, NaCl was replaced with choline chloride. Monolayers were washed with transport medium, and uptake experiments were initiated by replacing the medium with prewarmed transport medium containing 0.1 µCi of L-\[^{14}\text{C}\]ascorbic acid (PerkinElmer Life Sciences) and cold ascorbate at the concentrations indicated in the figure legends. Uptake was terminated by adding ice-cold stopping solution (15 mM Hepes buffer, pH 7.6, 135 mM NaCl, 5 mM KCl, 0.8 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 0.2 mM MgCl\(_2\)). The monolayers were washed and lysed in 10 mM Tris/HC1 (pH 8.0), 0.2% SDS, and the incorporated radioactivity was determined by scintillation spectrometry. The data are presented as the average standard deviation and correspond to a minimum of three assays performed independently in triplicate. Kinetic parameters were determined using the Michaelis-Menten equation and by using the linear transformation of Eadie-Hofstee.

 Stoichiometry Determination—We performed parallel time course uptake assays of \(^{22}\text{Na}\) and radiolabeled ascorbic acid for determination of the sodium/ascorbic acid uptake ratio. The transport medium contained a mixture of sodium transport inhibitors (1 mM ouabain, 0.1 mM amiloride, and 0.1 mM bumetanide) and 0.25 µCi of \(^{22}\text{sodium}\) (PerkinElmer Life Sciences) or radiolabeled ascorbic acid.

Cloning of SVCT2 and Expression in HEK-293 Cells—The full-length SVCT1 and SVCT2 cDNA were obtained by RT-PCR using oligonucleotide primer pairs that flank the start and stop codons of SVCT1 and SVCT2, respectively (12). PCR amplification was done using 0.4 µl of cDNA template, 0.5 µM of each primer, and the following set of reactions: 2 min at 94°C; 36 cycles of 30 s at 94°C, 30 s at 65°C, and 2 min at 72°C; and 7 min at 72°C. The PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, extracted and purified from agarose gels with the QIAx kit (Qiagen), cloned in the pEGFP vector (Invitrogen), sequenced, and analyzed by BLAST at the NCBI server at www.ncbi.nlm.nih.gov/. SVCT1-GFP and SVCT2-GFP were constructed using standard molecular biology techniques, cloned in the pEGFP vector, and sequenced. Transfection experiments in HEK-293 cells were performed using Lipofectamine (Invitrogen) following the manufacturer’s instructions.

Patch Clamp—Patch clamp in the whole cell configuration was performed 18–24 h post-transfection using an Axopatch-1 (Axon Instrument) apparatus. Culture medium was replaced with an external solution containing 10 mM Hepes (pH 7.4), 150 mM NaCl, 5.4 mM KCl, 1.0 mM MgCl\(_2\), 2.0 mM CaCl\(_2\), and 10 mM glucose, and the cells were stabilized at room temperature for 30 min. Transfected cells were selected under fluorescence microscopy and the whole cell configuration was established with a holding potential of −60 mV. The currents were filtered at 2 kHz and digitalized (Digidata 1200, Axotape and Pclamp 9.0; Axon Instruments) for further nonlinear analysis.

Statistics—The transport data were processed for nonlinear analysis with the program IgorPro (WaveMetrics). Patch clamp data were analyzed with the program Origin (Microcal Inc.).

RESULTS AND DISCUSSION

Melanoma Cells Express a Single Ascorbic Acid Transporter That Corresponds to SVCT2—RT-PCR experiments using cDNAs prepared from RNA obtained from melanoma cells and primers specific for SVCT1 or SVCT2 generated amplification products of the size expected for SVCT2 (370 bp) but not for SVCT1 (Fig. 1A, lanes 1 and 2). The specificity of the amplification reaction was verified by using human liver RNA that was positive for SVCT1 and negative for SVCT2 and total human brain RNA that was positive for SVCT2 and negative for SVCT1 (Fig. 1A, lanes 3 and 4, and data not shown). These results are in agreement with studies of the presence of SVCT1 and SVCT2-specific transcripts in mammalian tissues and cells (9, 11, 12). Expression of SVCT2 at the protein level was confirmed by immunoblotting (Fig. 1B) and immunocytochemistry with antibodies anti-SVCT1 or -SVCT2 (Fig. 1, B–D). The immunoblotting experiments using anti-SVCT2 and total membranes prepared from SK-MEL cells showed one reactive band with an estimated molecular mass of 50,000 daltons (Fig. 1B, lane 2), which is consistent with the size of SVCT2 predicted from the coding region of the cDNA, but there was no immunoreactive band with anti-SVCT1 (Fig. 1B, lane 1). Control experiments revealed an immunoreactive band in membranes prepared from renal Madin-Darby canine kidney cells
that express SVCT1 (data not shown). The immunolocalization experiments confirmed expression of SVCT2 in the melanoma cells, but were negative for SVCT1 (Fig. 1, C–E). Proper reactivity of the antibodies was confirmed by using cells and tissues known to express the different transporters. Human renal epithelial cells were positive for SVCT1 and SVCT2, hepatocytes were positive for SVCT1 and negative for SVCT2, and adrenal cells were positive for SVCT2 and negative for SVCT1 (data not shown), which is consistent with previous data on the differential expression of SVCT1 and SVCT2 in different tissues and cells (11, 18).

Transport studies confirmed that the melanoma cells expressed an ascorbic acid transporter with functional properties similar to those of SVCT2 (Fig. 2, A–E). Time course analysis of ascorbic acid uptake revealed that the melanoma cells take up ascorbic acid at a constant rate of 130 pmol/min/million cells for at least 10 min (data not shown). Dose-response experiments showed that transport approached saturation at 100 μM ascorbic acid (Fig. 2A), and analysis of the transport data with the Eadie-Hofstee method generated a straight line that is indicative of the presence of a single functional component, for which we calculated an apparent \( \text{K}_m \) of 17 ± 2 μM (n = 8) and a \( V_{\text{max}} \) of 150 ± 4 pmol/min/million cells (Fig. 2B).

Expression studies of cloned SVCT1 and SVCT2 have rendered results indicating that they may show different \( \text{K}_m \) values depending on the expression system used and also on the cell, tissue, or species from where the transporters were cloned. Rat SVCT1 and SVCT2 expressed in human CaCO-2 cells have transport \( \text{K}_m \) values of 113 and 15 μM, respectively (9). The reported differences may be attributable to aspects of the experimental procedures. Thus, two different transport \( \text{K}_m \) values have been determined for the same transporter (cloned from the same tissue and expressed in the same system) by two different laboratories (13, 14). We have examined the kinetic properties of SVCT2 expressed in 10 different primary cells and cell lines and found that in all cases the transport \( \text{K}_m \) is close to 20 μM (data not shown). In this context, the functional properties of the ascorbic acid transporter expressed in the melanoma cells are fully compatible with its molecular identification as SVCT2.

**Na⁺ Cooperativity and the Na⁺:Ascorbic Acid Stoichiometry**—A fundamental property of the ascorbic acid transporters of the SVCT type is their potent activation by sodium ions. Uptake of ascorbic acid by the melanoma cells required the presence of sodium ions, as shown by a greater than 95% decrease in the rate of uptake (from 130 to less than 7 pmol/min/million cells) when...
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FIGURE 2. Na⁺ cooperativity and transport stoichiometry of SVCT2. A, a saturation curve for the transport of ascorbic acid as a function of graded ascorbic acid (AA) concentrations from 2 to 100 μM. Conditions: 3-min uptake assay, 135 mM Na⁺. B, Eadie-Hofstee linearization of the data in A (n = 6), C, effect of graded Na⁺ concentrations, from 5 to 135 mM, on the transport of ascorbic acid. Conditions: 3-min uptake assay, 100 μM ascorbic acid. D, Hill plot of the data in C; E, effect of monovalent cations on ascorbic acid transport. Conditions: 3-min uptake assay in the presence of 135 mM Na⁺, choline, Li⁺, Cs⁺, or K⁺ in medium containing 100 mM ascorbic acid. F, time course of ascorbic acid-coupled Na⁺ transport. Conditions: 3-min uptake assay in the absence (○) or in the presence (●) of 100 μM ascorbic acid, in medium containing 135 mM Na⁺ and traces of 22Na⁺. G, time course of Na⁺-coupled ascorbic acid transport. Conditions: 3-min uptake assay of 100 μM 14C-ascorbic acid in the presence of 135 mM Na⁺ (●) or choline (○). H, Na⁺/ascorbic acid transport stoichiometry from data such as in F and G (n = 4). Except for B (n = 6) and H (n = 4), the data correspond to the mean ± the standard deviation of one experiment of two performed in quadruplicate.

The Na⁺ in the incubation buffer was replaced with choline⁺, Cs⁺, K⁺, or Li⁺ (Fig. 2E). To further analyze this issue, transport of 100 μM ascorbic acid, a concentration at which SVCT2 is near saturation, was measured in the presence of graded extracellular concentrations (0–135 mM) of Na⁺. A clear increase in the ascorbic acid transport rate as a function of increasing Na⁺ concentration was observed (Fig. 2C), with the rate increasing at least 20-fold when the concentration of extracellular Na⁺ went from 0 to 135 mM, a process that was characterized by a Km of 19 mM (the Na⁺ concentration that increased the transport rate to 50% of the maximal effect) of 35 mM and a sigmoidal relationship between uptake rate and Na⁺ concentration, suggesting that the effect of Na⁺ on the transport rate was cooperative (Fig. 2C). Confirming this interpretation, when the data were fitted to the Hill equation, the line obtained showed an nH of 1.9 (Fig. 2D), which is consistent with the presence of at least two sodium sites showing positive cooperativity.

An nH of 1.9 can be interpreted as indicating that the binding of Na⁺ to the first Na⁺-binding site increases the affinity of the second Na⁺ site and does not provide information on the Na⁺: ascorbic acid stoichiometry (20, 21). However, previous results obtained in expression studies of cloned SVCT2, which indicated a cooperative Na⁺ effect with an nH near 2, have been erroneously interpreted as indicating a 2:1 Na⁺:ascorbic acid stoichiometry (12, 18). We directly addressed this issue by measuring the transport of 22Na in the presence and in the absence of 100 μM ascorbic acid and comparing it with the transport of ascorbic acid in the presence and in the absence of 135 mM NaCl (Fig. 2, F–H). Under these conditions, the ascorbic acid-coupled net 22Na transport rate was 0.36 nmol/10⁶ cells/min (Fig. 2F), and the Na⁺-coupled net ascorbic acid transport rate was 0.20 nmol/10⁶ cells/min (Fig. 2G). Thus, at 100 μM ascorbic acid and 135 mM NaCl, the transport of one molecule of ascorbic acid was coupled to the transport of two Na⁺ molecules, resulting in a 2:1 sodium:ascorbic acid stoichiometry (Fig. 2H).

Na⁺ Activates Transport by Decreasing the Ascorbic Acid Transport Km—We examined the mechanism by which Na⁺ increases the rate of ascorbic acid transport. At 5 and 15 mM Na⁺, the saturation curves were hyperbolic, and the transporter was still not saturated at 5 mM ascorbic acid, indicating that SVCT2 was fully capable of transporting ascorbic acid at low Na⁺ concentrations, but with a transport Km in the millimolar range (Fig. 3, A and B). A detailed analysis of the transport kinetics using graded extracellular Na⁺ concentrations (from 5 to 35 mM) revealed that Na⁺ increased the transport rate by decreasing the transport Km for ascorbic acid in a complex, nonlinear, and dose-dependent manner (Fig. 3C). Thus, there was an almost linear decrease in the ascorbic acid transport Km from 2 mM to 120 μM as Na⁺ increased from 5 to 20 mM, followed by a second, slower phase in which the transport Km decreased from 120 to 17 μM with a change in Na⁺ from 20 to 135 mM (Fig. 3C). Overall, there was more than a 100-fold decrease in the value of the Km for ascorbic acid when Na⁺ was increased from 5 to 135 mM. In contrast, there was no appreciable change in the transport Vmax, which remained at ~150 pmol/10⁶ cells/min when Na⁺ was increased from 5 to 135 mM (Fig. 3C).

Our data showing a biphasic effect of Na⁺ on the Km of SVCT2 for ascorbic acid could be interpreted as indicating the occurrence of two successive conformational rearrangements in SVCT2, the first occurring at low concentrations of Na⁺ followed by a second at higher Na⁺ concentrations. Moreover, the kinetic data indicating that the ascorbic acid transport Km changes as a function of the Na⁺ concentration supports a substrate binding order of the type Na⁺-ascorbic acid-Na⁺. The kinetic analysis does not provide enough data to identify the specific step in the overall transport cycle that is affected by the presence of Na⁺. Data obtained with the Na⁺-glucose and the Na⁺-glutamate co-transporters was interpreted as indicating that the Na⁺ effect on the transport Km is associated with conformational transitions that expose the substrate binding site (22, 23). However, recent data with the Lac permease, a transporter of lactose whose transport activity is driven by the
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These results, indicating that the transport of ascorbic acid is strongly influenced by the simultaneous presence of Na\(^+\) and that the interaction of Na\(^+\) with the transporter is affected by the presence of ascorbic acid, are fully consistent with the function of SVCT2 as a Na\(^+\)-ascorbic acid co-transporter. The effect of ascorbic acid on the Na\(^+\) cooperativity can be rationalized if we include in the analysis the evidence indicating that the Na\(^+\)-ascorbic acid stoichiometry is 2:1 and that the substrate binding order is Na\(^+\)-ascorbic acid-Na\(^+\). Thus, the data demonstrating low Na\(^+\) cooperativity at low ascorbic acid concentrations can be interpreted as evidence indicating that ascorbic acid does not participate in the binding of the first Na\(^+\) but affects the binding of the second Na\(^+\), an interpretation that is consistent with a binding order of the type Na\(^+\)-ascorbic acid-Na\(^+\) and the Na\(^+\) cooperativity observed at concentrations of ascorbic acid near or above the

electrochemical H\(^+\) gradient, indicated that the H\(^+\) effect is not related to changes in the transporter affinity for the substrate (24). Thus, our present data cannot be interpreted as indicating the occurrence of Na\(^+\)-driven changes in the substrate binding site(s) leading to increased affinity for ascorbic acid, as opposed to conformational changes that directly affect the substrate translocation steps (see Fig. 6A).

Ascorbic Acid Affects the Na\(^+\) Cooperativity and the Na\(^+\)50—Because SVCT2 is a co-transporter of Na\(^+\) and ascorbic acid, and Na\(^+\) affects the transport of ascorbic acid, we asked whether ascorbic acid affects the Na\(^+\) cooperativity. For this, we selected a wide range of ascorbic acid concentrations, from 5 to 500 \(\mu\)M, and measured the rate of transport of ascorbic acid at increasing Na\(^+\) concentrations. These experiments revealed that the Na\(^+\) cooperativity was moderate at low ascorbic acid concentrations (5 \(\mu\)M) and was lost at high (>200 \(\mu\)M) ascorbic acid (Fig. 3D). Thus, although at 5 \(\mu\)M ascorbic acid the ascorbic acid uptake curve was sigmoidal, with an \(n_1\) of less than 1.4 (Fig. 3, D and E), the corresponding curve at 500 \(\mu\)M ascorbic acid was clearly hyperbolic, with an \(n_1\) of 1.0 (Fig. 3, D and E). A detailed analysis revealed that the \(n_1\) for Na\(^+\) varied in a complex and bimodal manner as a function of the ascorbic acid concentration; it increased from 1.4 at low ascorbic acid concentrations to a maximum value of 2 at 50–100 \(\mu\)M ascorbic acid and decreased to ~1.0 at ascorbic acid concentrations >200 \(\mu\)M (Fig. 3F). On the other hand, the Na\(^+\)50 decreased in a unimodal, nonlinear manner with increasing concentrations of ascorbic acid, from 80 \(\mu\)M at 5 \(\mu\)M ascorbic acid to 35 \(\mu\)M at 50 \(\mu\)M ascorbic acid, and reached a lowest value of 20 \(\mu\)M at 500 \(\mu\)M ascorbic acid (Fig. 3F).

FIGURE 3. Reciprocal functional interactions between Na\(^+\) and ascorbic acid in SVCT2. A–C, effect of Na\(^+\) on the transport \(K_m\) for ascorbic acid. A, saturation curve for the transport of ascorbic acid at 5 ( ), 15 ( ), and 135 ( \(\mu\)M) Na\(^+\). B, Eadie-Hofstee analysis of the data in A, C, effect of graded Na\(^+\) concentrations, from 5 to 135 mM, on the \(K_m\) ( ), and \(V_{max}\) ( ) for the transport of ascorbic acid. D–F, effect of ascorbic acid on the Na\(^+\) cooperativity. D, effect of graded concentrations of Na\(^+\), from 5 to 135 mM, on the transport rate of 5 ( ), 100 ( ), and 500 ( \(\mu\)M) ascorbic acid. E, Hill plot of the data in D, F, effect of graded ascorbic acid concentrations, from 5 to 500 \(\mu\)M, on the \(n_1\)Na\(^+\) ( ) and the Na\(^+\)50 ( ) for the transport of ascorbic acid. The data correspond to the means ± the standard deviation of one experiment of two performed in quadruplicate.

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Ca\(^2+\) and Mg\(^2+\) Are Essential for SVCT2 Function—Next, we analyzed the effect of Ca\(^2+\) and Mg\(^2+\) on ascorbic acid transport. These experiments revealed that, in the presence of extracellular Na\(^+\), the transporter was fully functional in the absence of either Ca\(^2+\) or Mg\(^2+\), but there was no transport in the simultaneous absence of both cations (Fig. 4A). In the absence of extracellular Na\(^+\), there was no transport irrespective of the presence or the absence of Ca\(^2+\) or Mg\(^2+\) (Fig. 4A). Dose-response experiments showed that the rate of ascorbic acid transport increased as a function of increasing concentrations of extracellular Ca\(^2+\) and approached saturation at 1 mM Ca\(^2+\), with the presence of a single functional component with an apparent \(K_m\) of 50 \(\mu\)M and a \(V_{max}\) of 120 pmol/10⁶ cells/min (Fig. 4, B and C). Similar to the effect of Ca\(^2+\), the rate of ascor-
The effect of Ca\(^{2+}\) and Mg\(^{2+}\) on transport can be interpreted as indicating that SVCT2 possesses binding sites for Ca\(^{2+}\) and Mg\(^{2+}\) and that the binding of the bivalent cations induces a conformational transition in the transporter from a non-active to a fully active conformation. Thus, Ca\(^{2+}\)/Mg\(^{2+}\) are essential for the function of SVCT2, which therefore can be defined as a bivalent cation-dependent transporter. On the other hand, the binding of Na\(^{+}\) induces a transition in the transporter from a low to a high affinity conformation, which indicates that SVCT2 is activated by Na\(^{+}\), but it is not a Na\(^{+}\)-dependent transporter.

Is SVCT2 Electrogegenic?—Because of the charge ratio for a 2:1 Na\(^{+}\)-ascorbic acid transport stoichiometry (two positive charges and one negative), we asked whether SVCT2 is electrogenic. SVCT2 is expressed at low levels in the plasma membrane of cells after transfection of the corresponding cDNA in mammalian cells or injection of the cRNA in X. laevis oocytes, making more difficult the quantification of putative currents associated with the transport of ascorbic acid (11–14). We failed in several attempts to detect currents associated with the transport of ascorbic acid in the melanoma cells, an observation probably caused by the low levels of endogenous SVCT2 expression in these cells and, similarly, were unable to detect expression in X. laevis oocytes injected with in vitro synthesized SVCT2 RNA (data not shown). There are few examples of laboratories measuring substrate-induced currents in cells endogenously expressing low levels of the Na\(^{+}\)-glucose or the Na\(^{+}\)-glutamate co-transporters, which is in line with our failed efforts in the melanoma cells (21).

In a further attempt to resolve this issue and to obtain additional evidence that SVCT2 is the ascorbic acid transporter expressed in the melanoma cells, we cloned a full-length SVCT2 cDNA from SK-MEL cells RNA by PCR using oligonucleotide primer pairs specific for the 5'- and 3'-ends of human SVCT2 coding region. The ~2-kb amplification product was submitted to automated sequencing, and the data were analyzed by BLAST at the NCBI server at www.ncbi.nlm.nih.gov/. The BLAST analysis revealed that the full-length clone isolated from the SK-MEL cells had 100% identity with the published human SVCT2 sequence, therefore confirming that the ascorbic acid transport increased as a function of increasing extracellular Mg\(^{2+}\) concentrations and approached saturation at 1 mM Mg\(^{2+}\), with the presence of a single functional component with an apparent $K_m$ of 100 $\mu$M and a $V_{max}$ of 120 pmol/10$^6$ cells/min (Fig. 4, B and C). Initial experiments performed using 25 $\mu$M CaCl$_2$ revealed a decrease in the $V_{max}$ of transport without changes in the transport $K_m$ (Fig. 4D). Further experiments performed using CaCl$_2$ concentrations from 10 $\mu$M to 2 mM confirmed that the decreased transport rate observed at low Ca\(^{2+}\) concentrations is due to a decrease in the transport $V_{max}$ without changes in the transport $K_m$ (Fig. 4E). Moreover, neither the Na\(^{+}\) cooperativity or the Na\(^{+}\)$_{50}$ were affected at low Ca\(^{2+}\) concentrations (Fig. 4, F and G). Similarly, the decreased transport rate observed at low Mg\(^{2+}\) concentrations was also related to a decreased transport $V_{max}$ without changes in the transport $K_m$, the Na\(^{+}\) cooperativity, or the Na\(^{+}\)$_{50}$ (data not shown).

![FIGURE 4. Essential role of Ca\(^{2+}\)/Mg\(^{2+}\) on SVCT2 function. A, effect of Ca\(^{2+}\) and Mg\(^{2+}\) on SVCT2-mediated ascorbic acid (AA) transport. B, saturation curve for the transport of ascorbic acid as a function of graded Ca\(^{2+}\) (○) or Mg\(^{2+}\) (●) concentrations from 5 $\mu$M to 2 mM. C, Eadie-Hofstee analysis of the effect of Ca\(^{2+}\) (○) or Mg\(^{2+}\) (●) on the ascorbic acid transport rate. D, saturation curve for the transport of ascorbic acid as a function of graded ascorbic acid concentrations from 2 to 100 $\mu$M in the presence of 25 (○) or 250 $\mu$M Ca\(^{2+}\) (●). E, $K_m$ (○) and $V_{max}$ (●) for the transport of ascorbic acid in the presence of graded Ca\(^{2+}\) concentrations. F, effect of graded Na\(^{+}\) concentrations, from 5 to 135 mM, on the transport of ascorbic acid in the presence of 25 (○) or 250 $\mu$M Ca\(^{2+}\) (●), G, $n_{max}$ (○) and Na\(^{+}\)$_{50}$ (●) for the transport of ascorbic acid in the presence of graded Ca\(^{2+}\) concentrations. The data correspond to the means ± the standard deviation of one experiment of two performed in quadruplicate.](image-url)
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generated a straight line from which we calculated an apparent $K_m$ of 13 ± 1 µM ($n = 3$). The $V_{max}$ of transport in the SVCT2-GFP-expressing cells was 486 ± 11 pmol/min/million cells, compared with a value of 36 ± 3 pmol/min/million cells in GFP-expressing cells (Fig. 5E). Further studies revealed that transport of ascorbic acid by SVCT2-GFP-expressing cells was highly sensitive to the presence of $\text{Na}^+$ and $\text{Ca}^{2+}/\text{Mg}^{2+}$. We observed a greater than 90% decrease in transport in the absence of extracellular $\text{Na}^+$ in the incubation buffer, and no transport was measurable, even in the presence of $\text{Na}^+$, during the simultaneous absence of $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ (data not shown). At 100 µM ascorbic acid, the effect of sodium on transport was cooperative, as shown by an $n_H$ of 1.9 ± 0.1 and a $\text{Na}_\text{app}$ of 32 ± 2 mM (Fig. 5F). Similar to the effect observed for SVCT2 in the melanoma cells, the $\text{Na}^+$ cooperativity was lost at high (500 µM) ascorbic acid concentrations (data not shown).

The above data indicated that cloned SVCT2, expressed at high levels as the chimera SVCT2-GFP in HEK-293 cells, maintained the properties of SVCT2 from melanoma cells, including an ascorbic acid transport $K_m = \sim 15$ µM, activation by $\text{Na}^+$ in a cooperative manner, lack of $\text{Na}^+$ cooperativity at high ascorbic acid concentrations, and absolute dependence on $\text{Ca}^{2+}/\text{Mg}^{2+}$ for function. Therefore, considering the high level of expression observed in the transfected HEK-293 cells, we directly assayed the possible electrogenic properties of SVCT2 by measuring $\text{Na}^+$ currents associated with the transport of ascorbic acid in the transfected cells (Fig. 5, G and H). Transfected cells, selected under fluorescent illumination and subjected to patch clamp analysis with graded concentrations of ascorbic acid from 20 µM to 3 mM, showed no evidence of ascorbic acid-induced $\text{Na}^+$ currents (Fig. 5G and H). These results are consistent with previous results indicating no measurable ascorbic acid $\text{Na}^+$-induced currents in X. laevis oocytes expressing SVCT2 (11). We cannot, however, discard the possibility that our experimental setting is not sensitive enough to detect a current of low amplitude with these results alone. Therefore, to validate these results, we performed a number of control experiments measuring ascorbic acid-induced $\text{Na}^+$ currents in HEK-293 cells transfected with the chimera.

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**FIGURE 5. Expression of SVCT2-GFP in HEK-293 cells.** A, fluorescent microscopy of HEK-293 cells expressing SVCT1-GFP. B, fluorescent microscopy of HEK-293 cells expressing SVCT2-GFP. C, fluorescent microscopy of HEK-293 cells expressing GFP. D, time course of the uptake of ascorbic acid (AA) in HEK-293 cells expressing SVCT2-GFP (●) or GFP (○). E, Eadie-Hofstee analysis of the saturation curve for the transport of ascorbic acid as a function of graded ascorbic acid concentrations from 2 to 100 µM in control HEK-293 cells (●) and in cells expressing SVCT2-GFP (●). Conditions: 3-min uptake assay, 135 mM Na+. F, effect of graded Na+ concentrations, from 5 to 135 mM, on the transport of ascorbic acid in HEK-293 cells expressing SVCT2-GFP. G, ascorbic acid-elicited currents in HEK-293 cells expressing SVCT1-GFP and SVCT2-GFP. The indicated concentrations of ascorbic acid (in mM) were added to the external solution, and the currents were measured as indicated under “Experimental Procedures.” H, dose-response analysis of ascorbic acid-elicited currents in HEK-293 cells expressing SVCT1-GFP (●) and SVCT2-GFP (●). For D–F, the data correspond to the means ± the standard deviation of one experiment of two performed in quadruplicate. Patch clamp data were obtained from individual cells that were analyzed several times under the different experimental conditions; each cell analyzed was considered a different experiment. In H, the data represent the averages ± the standard error of the mean of three independent determinations, each performed at least in triplicate.

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Acidic acid transporter expressed by the melanoma cells is SVCT2 (data not shown). We next examined the functional properties of SVCT2 overexpressed in HEK-293 cells transfected with the chimeric cDNA SVCT2-GFP (Fig. 5). Observation of the transfected cells by fluorescence microscopy revealed abundant expression of SVCT2-GFP at the level of the cell membrane (Fig. 5B), whereas control cells expressing GFP alone showed an intracellular pattern of fluorescence, without labeling of the plasma membrane (Fig. 5C). Time course analysis of ascorbic acid uptake revealed a 20-fold increase in the rate of transport of ascorbic acid in the SVCT2-GFP-expressing cells, compared with control GFP-expressing cells (Fig. 5D). Dose-response experiments showed that, in SVCT2-GFP-expressing cells, transport approached saturation at 100 µM ascorbic acid and analysis of the transport data with the Eadie-Hofstee method...
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SVCT1-GFP. Because expression studies in X. laevis oocytes have revealed that SVCT1 is electrogenic (11), these experiments served, as controls for both the sensitivity of the experimental set up and a possible negative role of GFP in the chimera SVCT2-GFP. Observation of the transfected cells by fluorescence microscopy revealed abundant expression of SVCT1-GFP at the level of the cell membrane (Fig. 5A), which is similar to the results observed in cells expressing SVCT2-GFP. Time course analysis of ascorbic acid uptake by SVCT1-GFP-expressing cells revealed a marked increase in the ascorbic acid transport rate compared with control GFP-expressing cells, transport that was activated by Na\(^+\) and proceeded with an apparent \(K_m\) of 75 \(\mu\)M and a \(V_{max}\) that was similar to that in SVCT2-GFP-expressing cells (data not shown). Transfected cells, selected under fluorescent illumination and subjected to patch clamp analysis with graded concentrations of ascorbic acid from 20 \(\mu\)M to 3 mM, showed a dose-dependent ascorbic acid-induced current (Fig. 5, G and H). Thus, SVCT1 expressed at high levels as the chimera SVCT1-GFP in HEK-293 cells maintained the functional properties expected for native SVCT1, including its electrogenicity, therefore validating the results obtained in cells expressing the chimera SVCT2-GFP, indicating that SVCT2 is not electrogenic.

How can we explain the apparent paradox that a transporter that transports Na\(^+\) and ascorbic acid with a 2:1 stoichiometry is not electrogenic? SVCT2 is a member of the major facilitator family of transporters that couples the free energy released from the downhill translocation of Na\(^+\) in response to an electrochemical Na\(^+\) gradient to drive the energetically uphill accumulation of ascorbic acid. Based on what is currently known about the transport mechanism of the major facilitator family transporters (25, 26) and our present data, the mechanism of Na\(^+\)-ascorbic acid influx can be explained by a kinetic scheme consisting of at least eight steps (Fig. 6A): (i) binding of the first Na\(^+\) to the outward facing conformation of SVCT2, (ii) binding of ascorbic acid, (iii) binding of the second Na\(^+\), (iv) a conformational change that results in the inward facing conformation, (v–vii) release of Na\(^+\)/ascorbic acid/Na\(^+\) (unknown order), and (viii) return to the outward conformation. Because expression studies in X. laevis oocytes at high levels as the chimera SVCT1-GFP in HEK-293 cells lacking Na\(^+\) show an absolute dependence on Ca\(^2+\) and Mg\(^2+\) (extracellular concentrations > 1 mM), shows Na\(^+\) cooperativity (at least two Na\(^+\)-binding sites) and transports Na\(^+\) and ascorbic acid with a 2:1 stoichiometry down the electrochemical Na\(^+\) gradient. Thus, plasma membrane SVCT2 is a Na\(^+\)-ascorbic acid co-transporter that is activated by Na\(^+\) and shows an absolute dependence on Ca\(^2+\) or Mg\(^2+\) in intracellular SVCT2, which is exposed to high ascorbic acid and low Na\(^+\) and Ca\(^2+\)/Mg\(^2+\) concentrations, is a low affinity transporter lacking Na\(^+\) cooperativity.

FIGURE 6. Proposed structural-functional model of SVCT2 for the transport of ascorbic acid. A, the mechanism of Na\(^+\)-ascorbic acid (AA) influx can be explained by a kinetic scheme consisting of eight steps: (i) binding of the first Na\(^+\) to the outward facing conformation of SVCT2, (ii) binding of ascorbic acid, (iii) binding of the second Na\(^+\), (iv) a conformational change that results in the inward facing conformation, (v–vii) release of Na\(^+\)/ascorbic acid/Na\(^+\) (unknown order), and (viii) return to the outward conformation. B, mono- and bivalent cations play fundamental roles in defining the transport capacity of SVCT2, and therefore intracellular and plasma membrane transporters that are exposed to different ionic conditions will express different functional properties. The fully active plasma membrane transporter (extracellular Na\(^+\) concentration > 100 mM) is a high affinity transporter (\(K_m\) < 20 \(\mu\)M) that binds Ca\(^2+\) and/or Mg\(^2+\) (extracellular concentrations > 1 mM), shows Na\(^+\) cooperativity (at least two Na\(^+\)-binding sites) and transports Na\(^+\) and ascorbic acid with a 2:1 stoichiometry down the electrochemical Na\(^+\) gradient. Thus, plasma membrane SVCT2 is a Na\(^+\)-ascorbic acid co-transporter that is activated by Na\(^+\) and shows an absolute dependence on Ca\(^2+\) or Mg\(^2+\). Intracellular SVCT2, which is exposed to high ascorbic acid and low Na\(^+\) and Ca\(^2+\)/Mg\(^2+\) concentrations, is a low affinity transporter lacking Na\(^+\) cooperativity.

SVCT2 Conformational-Functional Transitions—We can envision three different forms of the transporter, defined by two successive conformational transitions that occur as a function of the availability of bi- and monovalent ions: (i) an inactive form that lacks the capacity of transporting ascorbic acid and would exist only in the total absence of bivalent cations, (ii) an active, low affinity form with bound bivalent cations that transports ascorbic acid with very low affinity in the presence of Na\(^+\) concentrations below 20 mM, and (iii) a fully
active, high affinity form with bound Ca$^{2+}$ and/or Mg$^{2+}$ that interacts with Na$^+$ in a cooperative manner, is capable of transporting both Na$^+$ and ascorbic acid, and transports ascorbic acid with a $K_m$ of $\sim 15 \mu M$.

Our immunolocalization data indicate that, in melanoma cells, SVCT2 is localized at the level of the plasma membrane as well as intracellularly. We have observed a similar distribution pattern for SVCT2 in a number of primary cells and cell lines cultured in the laboratory and also in situ in samples of human tissue (data not shown). There is no current data indicating whether intracellular SVCT2 is capable of transporting ascorbic acid and what would be its role in the intracellular compartmentalization of ascorbic acid (27–29). The intracellular concentration of Na$^+$ is $\sim 15 \mu M$, a concentration at which SVCT2 is active but shows a transport $K_m$ for ascorbic acid $> 0.5 \mu M$. On the other hand, although free Mg$^{2+}$ is present intracellularly at micromolar concentrations, the intracellular concentration of free Ca$^{2+}$ can vary from very low levels (in the nanomolar range) to micromolar concentrations depending on the cell type and the specific functional state of the cells (30). We therefore conclude that, under physiological conditions, SVCT2 may exist in two functional states defined by their respective subcellular localizations. The plasma membrane transporters, containing bound Ca$^{2+}$ and Mg$^{2+}$ and exposed to an extracellular medium containing 135 mM Na$^+$, would function as high affinity transporters of ascorbic acid (Fig. 6B). On the other hand, the intracellular transporters, containing bound Mg$^{2+}$ and exposed to a medium low in Na$^+$, would function as low affinity ascorbic acid transporters whose activity may be modulated by fluctuations in the free cytoplasmic Ca$^{2+}$ concentrations. At the plasma membrane, these properties define a transport cycle that favors the binding and dissociation of ascorbic acid at the exo- and endofacial surface of the transporter, respectively, with the result that ascorbic acid is preferentially transported into the cell down the electrochemical Na$^+$ gradient.

Conclusions—We developed a partial model for the transport cycle of SVCT2 that challenges the current view indicating that SVCT2 is a Na$^+$-dependent transporter (Fig. 6B). Our data indicated that Na$^+$ increases the rate of ascorbic acid transport in a cooperative manner, decreasing the transport $K_m$ without affecting the transport $V_{\text{max}}$. Thus, the net effect of Na$^+$ is to convert a low affinity form of SVCT2 into a high affinity transporter. Interestingly, ascorbic acid affected in a bimodal and concentration-dependent manner the Na$^+$ cooperativity, with an absence of cooperativity at high ($\geq 200 \mu M$) ascorbic acid concentrations, indicating a reciprocal functional interaction between the two transported substrates, ascorbic acid and Na$^+$. This interaction defines the substrate binding order and the transport stoichiometry. All together, our data are consistent with a half-transport cycle characterized by a 2:1 Na$^+$:ascorbic acid stoichiometry and a substrate binding order of the type Na$^+$:ascorbic acid:Na$^+$. However, SVCT2 is not electrogenic. SVCT2 showed an absolute requirement for Ca$^{2+}$/Mg$^{2+}$ for function, with both cations switching SVCT2 from an inactive into an active conformation by increasing the transport $V_{\text{max}}$ without affecting the transport $K_m$, the Na$^+$ cooperativity, or the $Na_{50}$. We conclude that SVCT2 is a Ca$^{2+}$/Mg$^{2+}$-dependent co-transporter of Na$^+$ and ascorbic acid. Several active states can be envisioned, including a low affinity conformation at Na$^+$ concentrations below 20 mM and a high affinity conformation at elevated Na$^+$ concentrations whose Na$^+$ cooperativity is modulated by ascorbic acid.

We believe that the functional properties of SVCT2 from melanoma cells are generally applicable to SVCT2 from other cells and tissues. We used cultured melanoma cells to develop our studies because PCR, immunocytochemical, and functional data indicated that they express SVCT2 and show no evidence of SVCT1 expression. It has been suggested that SVCT1 is expressed mainly in epithelial cells, whereas SVCT2 is expressed in a wide number of cells and tissues (10, 11). However, when we analyzed SVCT1 and SVCT2 expression in a number of cell lines and primary cells, we found that both transporters are simultaneously expressed in most cells, including prostate cancer cells (LNCaP, PC-3, and DU-145), colon cancer cells (CaCo-2), immortalized human brain endothelial cells (human brain microvascular endothelial cells and human cerebral endothelial cells), rat and human hepatoma cells (H4 and HepG2), renal cell lines from dog (Madin-Darby canine kidney cells), and opossum (opossum kidney endothelial cells), and primary cultures of endothelial cells from human umbilical vessels (HUVEC), and human tonsils (HUTEC). On the other hand, only rat hepatocytes expressed SVCT1 in isolation, and cultured melanoma cells showed expression of SVCT2 alone. Additionally, we have identified two additional cell lines that express SVCT2 in isolation and show no evidence of SVCT1 expression, the endothelial-type cell line ECV-304, and the human embryonic renal cell line HEK-293, and in both cell lines SVCT2 shows sodium activation and absolute dependence on Ca$^{2+}$/Mg$^{2+}$ for function (data not shown). We recently cloned and expressed in HEK-293 cells, full-length SVCT2 clones isolated from colon cancer cells (CaCo-2), human brain microvascular endothelial cell lines, and human breast cancer cells (ZRB-75) and found that they show activation by sodium and Ca$^{2+}$/Mg$^{2+}$ dependence (data not shown). We therefore conclude that the proposed mechanism of SVCT2 function, indicating activation by sodium and bivalent cation dependence, is not melanoma cellspecific and applies to SVCT2 expressed under different cellular contexts.

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