Biochimica et Biophysica Acta, 775 (1984) 17–21
Elsevier

BBA 72135

ASYMMETRY OF THE Na^+-SUCCINATE COTRANSPORTER IN RABBIT RENAL BRUSH-BORDER MEMBRANES

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(Received March 19th, 1984)

Key words: Brush-border vesicle; Cotransport kinetics; Krebs-cycle intermediate

We have investigated the symmetry of Na^+-succinate cotransport in rabbit renal brush-border membrane vesicles. Succinate influx and efflux kinetics were measured under voltage-clamped conditions using [^{14}C]succinate and a rapid filtration procedure. Both influx and efflux were Na^+-dependent, saturable, temperature-sensitive, and influenced by the *trans* Na^+ and succinate concentrations. The system was judged to be asymmetric, since the maximal velocity for influx was 3-fold higher than that for efflux, and *trans* Na^+ inhibited influx more than efflux. This may be due to the asymmetrical insertion of the transporter in the brush-border membrane, which leads to differences in either the forward and backward translocation rates of the fully loaded carrier or the Na^+ and succinate binding constants at the inner and outer faces of the membrane.

Introduction

The brush-border membrane of the proximal tubule contains a specific Na^+-dependent cotransport system for Krebs-cycle intermediates [1–6]. Previous work by this group has characterized the transport of succinate into renal brush-border vesicles, and suggested a model for the cotransporter [7–11]. We propose that succinate, and other Krebs-cycle intermediates, are transported as divalent anions along with three sodium ions. The sodium ions bind first to the transporter to increase its affinity for succinate. All of this earlier work so far has been based on solute influx into brush-border vesicles; we have now explored the symmetry of the transporter by measuring succinate efflux from the vesicles. The results indicate that the Na^+-succinate cotransporter is asymmetrical.

Materials and Methods

Brush-border membrane vesicles were prepared from rabbit kidney cortex by a Ca differential centrifugation procedure [2]. The final intravesicular ion and buffer concentrations were adjusted by suspension of the vesicles in the appropriate medium prior to final centrifugation. Unless otherwise specified, for uptake measurements the vesicles were preloaded with 200 mM choline chloride/ 100 mM KCl/45 mM Tris/84 mM Hepes (pH 7.5). For efflux studies, the vesicles were preloaded with 200 mM NaCl/100 mM KCl/45 mM Tris/84 mM Hepes at pH 7.5. This Na^+ concentration was chosen to ensure that the intravesicular Na^+ concentration would not be rate-limiting during the initial succinate efflux. Previous experiments (Ref. 9, Fig. 3) have demonstrated that succinate influx was saturated at 100

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone.

mM Na^+. All experiments were carried out on membranes stored in liquid nitrogen. The kinetic experiments were performed using membranes prepared on the day of the experiment.

Influx experiments. Uptakes of [^{14}C]succinate were studied using the rapid mixing and filtration technique described previously [9]. Initial rates of influx were obtained from 1 s uptakes in membranes voltage-clamped at zero potential with equimolar intra- and extravesicular KCl (100 mM) and 25 μg/ml valinomycin + 75 μM FCCP [10]. The quench solution contained 100 mM KCl, 1 mM Tris-Hepes (pH 7.5) and sufficient mannitol to maintain isoosmolarity with the uptake buffer.

Efflux experiments. The vesicles were preloaded with [^{14}C]succinate and carrier succinate by diluting an aliquot of concentrated vesicles into an equal volume of buffer containing twice the final desired succinate concentration, [^{14}C]succinate, 200 mM NaCl, 100 mM KCl, Tris-Hepes buffer at pH 7.5 and sufficient mannitol to maintain isoosmolarity. The vesicles were incubated for 30–45 min at 22°C and were then briefly frozen in liquid nitrogen. We have shown previously (Ref. 1, Fig. 1) that [^{14}C]succinate uptake approaches a steady state under these conditions. Each aliquot of vesicles was thawed at 37°C just until the ice melted and was held for 15 min at 0°C before beginning the experiment.

Efflux was measured by a rapid mixing and filtration method. Preloaded vesicles were placed as a 5 μl droplet on the side of a 16 × 100 mm plastic test-tube in close proximity to a 500 μl aliquot of medium. Efflux was initiated by rapid mixing of the two drops on a vortex mixer. After the appropriate time interval, the reaction was quenched by the rapid addition of 4 ml ice-cold stop solution (100 mM KCl/1 mM Tris-Hepes (pH 7.5) and sufficient mannitol to maintain the isoosmolarity with the efflux medium). 4 ml of the mixture was rapidly filtered through a 0.45 μm filter (Millipore HAWP) and the filter was washed with an additional 4 ml of cold stop solution. Both the filter and filtrate were collected and assayed by liquid-scintillation spectroscopy. The sum of the filter and filtrate radioactivity served to confirm the volume of the 5 μl aliquot of membranes. As for influx, all solutions except quench/wash contained 25 μg/ml valinomycin and 75 μM FCCP.

The intravesicular succinate concentration was varied between 0.05 and 16 mM. The actual intravesicular concentration was calculated in each experiment from the [^{14}C]succinate content of the vesicles (mol/mg protein) and the equilibrium L-[^{14}C]glucose space (μl/mg protein) determined for each preparation of vesicles. Glucose and succinate equilibrium uptakes were identical [12,6].

All chemicals were obtained from Sigma (St. Louis, MO), and [^{14}C]succinate and L-[^{14}C]glucose were from Amersham Corporation (Arlington Heights, IL) In the transport experiments, each time point was assayed in triplicate and is presented as the mean ± S.D.

Results

Two experiments illustrating the full time-course of succinate efflux from brush-border vesicles are shown in Fig. 1. The vesicles were loaded with 0.9

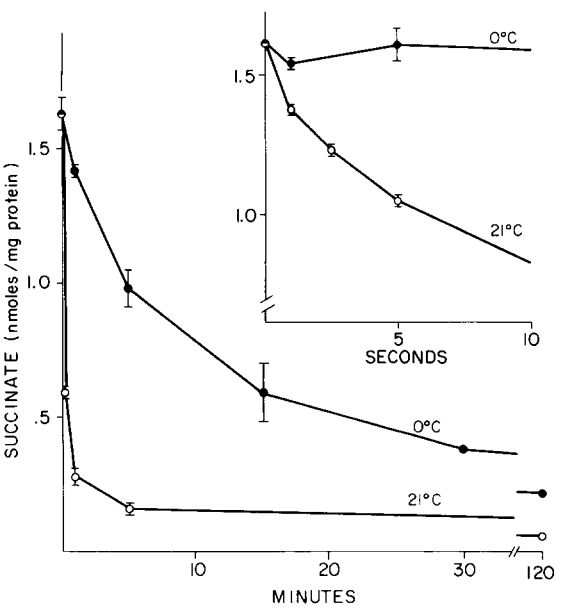

Fig. 1. The time-course of succinate efflux from renal brush-border vesicles at 21 and 0°C. Vesicles were preloaded with 200 mM NaCl, 100 mM KCl, 45 mM Tris/84 mM Hepes (pH 7.5), 0.9 mM succinate, 75 μM FCCP and 25 μg/ml valinomycin. At time zero, the membranes were diluted 100-fold into 200 mM choline chloride/100 mM KCl/45 mM Tris-84 mM Hepes (pH 7.5)/25 μg valinomycin/75 μM FCCP. The initial fluxes were 6.0 ± 0.8 nmol/mg per min at 21°C, and 0.2 ± 0.04 nmol/mg per min at 0°C. Datum points are presented as the mean ± S.D. of triplicate assays at each time point.

mM succinate and 200 mM NaCl, and efflux was initiated by diluting the vesicles 100-fold with 200 mM choline chloride buffer at either 21 or 0 °C. In both experiments, the succinate in the vesicles fell rapidly from the initial 1.6 ± 0.1 nmol/mg protein towards the equilibrium value of 0.016 nmol/mg protein. The efflux curves could not be fitted to a single exponential decay curve which probably reflects the heterogeneous size and membrane properties in the vesicle population (see Refs. 13 and 14). The half-times for efflux were about 15 s at 21 °C and 8 min at 0 °C.

To obtain quantitative information about the kinetics of succinate efflux we studied the first few seconds of the washout curve (Fig. 1 inset, and Fig. 2). The efflux was approximately linear with time as the internal succinate concentration fell from 100 to 75% of the initial concentration. Initial rates were estimated by linear-regression analysis of the linear portion of the efflux curves. In Fig. 1, the initial rate of efflux decreased from 6 ± 0.8 to 0.2 ± 0.04 nmol/mg per min when the temperature was reduced from 21 to 0 °C. This yields an activation energy for succinate efflux of 27 kcal/mol, which agrees with that observed for succinate uptake [11].

Similar experiments to those in Fig. 1 were performed to determine the sodium-dependence of succinate efflux. These were difficult experiments owing to problems in loading vesicles with succinate in the absence of sodium. In one series, we were successful in loading vesicles with 0.06 mM succinate in the presence of 5 and 100 mM NaCl (in these experiments, 95 mM choline chloride replaced 95 mM NaCl). Efflux was initiated by diluting the vesicles with 100 mM choline chloride. Increasing the internal sodium from 5 to 100 mM increased the efflux rate from 72 ± 24 to 378 ± 48pmol/mg per min.

The effects of *trans* Na and succinate on succinate influx and efflux are shown in Table I. The *cis* Na concentration was 200 mM and that of *cis* succinate 0.5 mM. Increasing the *trans* Na to 200 mM inhibited influx 98% and efflux 50%. This *trans* Na inhibition was relieved, partially in the case of influx, by increasing the *trans* succinate from 0.2 to 5 mM. With *trans* succinate alone, there was a 25% inhibition of influx and no significant change in the efflux rate. In previous experiments at a *cis* NaCl concentration of 100 mM, *trans* succinate produced a small stimulation of succinate influx [11].

TABLE I

THE EFFECT OF *trans* Na AND SUCCINATE ON SUCCINATE FLUX

Both efflux and influx data is expressed as a percentage of the control value. The influxes were obtained from triplicate 1 s uptakes. The external media contained 200 mM NaCl, 100 mM KCl, 0.5 mM succinate, Tris-Hepes buffer at pH 7.5, 25 μg/ml valinomycin, 75 μM FCCP and sufficient mannitol to maintain the solution isoosmotic with the intravesicular solution. The loading solution (*trans* for uptake) contained 200 mM choline chloride (control) or NaCl, zero or 5 mM succinate, and was otherwise identical to the uptake solution. The control value for influx was 27 ± 0.4 nmol/mg per min. The same batch of membranes was used to measure efflux as described in Fig. 1. The loading solution was identical to the influx uptake buffer, and the membranes were diluted 100-fold into buffers identical to the loading buffer in the influx experiments. The initial rate of succinate efflux in the control membranes was 5.5 ± 0.6 nmol/mg per min. Note that the control influx was 5-fold higher than the control efflux for mirrored experimental conditions.

Influx	Uptake (% control)
Control	100 ± 2
trans Na	1 ± 2
trans Na + succinate	23 ± 5
trans succinate	78 ± 3
Efflux	
Control	100 ± 10
trans Na	46 ± 20
trans Na + succinate	94 ± 12
trans succinate	109 ± 11

The succinate efflux rate saturated as the intravesicular succinate was increased (Fig. 2) from 0.5 to 7.9 mM. The rates were 4.1 ± 0.3 nmol/mg per min at 0.5 mM, 11 ± 0.5 nmol/mg per min at 2.1 mM and 14 ± 1 nmol/mg per min at 7.9 mM, i.e., efflux increased only 3.5-fold as the concentration increased 16-fold.

A comparison of the kinetics of succinate influx and efflux on one batch of membranes in a representative experiment is given in Fig. 3. The data are plotted as rate J(nmol/mg per min) vs. J/[succinate]. The K_t values were very similar for both processes, 1.2–1.3 mM, but the J_{max} for efflux was only one-third of that for influx. In three efflux kinetic experiments, the average K_t was 1.6 ± 0.3 mM and the J_{max} 23 ± 6 nmol/mg per min. The

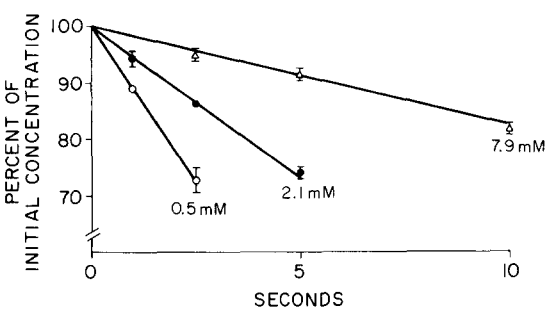

Fig. 2. Concentration-dependence of succinate efflux. The experimental conditions were identical to those in Fig. 1, except that the loading succinate concentration was varied between 0.5 and 7.9 mM. Initial rates of efflux were estimated by linear-regression analysis of the linear phase of the efflux curve. These were 4.1 ± 0.3 nmol/mg per min at 0.5 mM, 11 ± 0.5 nmol/mg per min at 2.1 mM and 14 ± 1.0 nmol/mg per min at 7.9 mM. All experiments were conducted on the same membrane preparation.

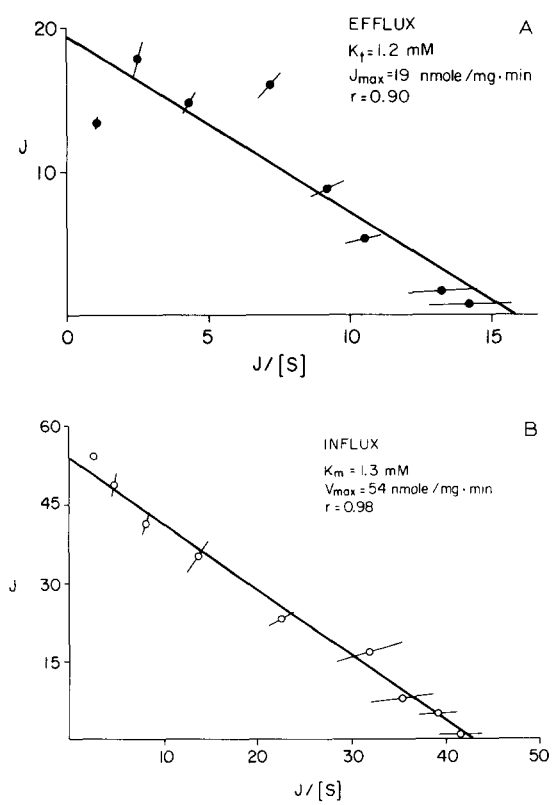

Fig. 3. Woolf-Augustinsson-Hofstee plots of the kinetics of succinate influx and efflux on one membrane preparation. Experiments were conducted as described in Figs. 1 and 2.

influx kinetic parameters, $K_t = 0.9 \pm 0.2$ mM and $J_{max} = 65 \pm 10$ nmol/mg per min, agree with those obtained earlier at a *cis* Na concentration of 100 mM [11].

Discussion

While there is considerable documentation about the mechanism and kinetics of solute uptake into renal brush-border vesicles (e.g., Refs. 15 and 16), there is little information about the symmetry of the transporters. The major difficulty in studying efflux via Na-cotransport in vesicles stems from their small volume (approx. $1 \cdot 10^{-14}$ cm^3/vesicle) and the relatively poor control of the driving forces (internal solute and Na concentrations, and membrane potential). In this study, we have attempted to minimize these problems by measuring initial rates of efflux, using internal sodium concentrations much higher than required to produce saturation, and short-circuiting the membrane potential with K^+ and valinomycin. This ensures that succinate efflux is measured under conditions approximating the initial driving forces. Uncertainties do, however, exist about the actual free solute concentrations in the intravesicular space and large errors arise in calculating efflux rates from small differences in radioactivity with large amounts of isotope in the vesicle.

Nevertheless, these experiments demonstrate qualitatively the reversibility of Na-succinate cotransport. Succinate efflux was shown to be: (1) Na-dependent – increasing the internal Na^+ from 5 to 100 mM increased the efflux 5-fold; (2) saturable, with a K_t of 1.6 mM and a J_{max} of 23 nmol/mg per min; (3) highly temperature-sensitive, with an activation energy of 27 kcal/mol; (4) inhibited by *trans* Na, which was relieved by *trans* succinate. However, there were quantitative differences between Na – dependent succinate influxes and effluxes: (1) the J_{max} value for influx was about 3-times higher than that for efflux (65 vs. 23 nmol/mg per min), and (2) *trans* Na inhibition was much more pronounced with influx experiments than with efflux experiments. *Trans* Na inhibited influx greater than 95% but only inhibited efflux 50%. These two observations indicate a functional asymmetry in the succinate carrier.

There are two relevant reports on the symmetry of Na-cotransport systems. The first is on glycine transport in pigeon red-cell ghosts [17]. In this case, the J_{max} value for influx was about half that for efflux. This may be due to differences in rate constants for the inward and outward movement of the glycine-Na-carrier complex, but there could also be differences in the Na and/or glycine binding constants between the inner and outer faces of the membrane. Since brush borders retain their normal orientation in vesicle preparations [18], the asymmetry of the succinate kinetic constants, while similar in magnitude, is the reverse of glycine in the pigeon red cell.

The second comparison is with glucose transport in intestinal brush-border vesicles [14]. In this study, there was marked asymmetry in the *trans* effects of Na and glucose. *Trans* Na inhibited glucose influx and this was converted into stimulation by the addition of *trans* glucose. On the other hand there, was virtually no effect of *trans* substrates in efflux experiments. Likewise, glucose efflux was less sensitive to membrane potential than influx. This asymmetry in glucose flux mirrored earlier observations by Semenza's group (see Ref. 14) on the susceptibility of the carrier to inactivation by proteinases and thiol reagents. These effects have been incorporated into an asymmetrical gated channel model.

As with glucose and glycine, the succinate carrier is asymmetrical, but the precise origin of this behavior is unclear. In each system, transport across the membrane in each direction is a complex function of multiple rate constants and so asymmetry may arise at either the binding and/or translocation step(s). It will require ingenuity to determine the origin of the vesicular asymmetry, but experiments with either 'scrambled' or inside-out vesicles should be able to establish whether the function asymmetry in the plane of the membrane is related to the asymmetrical insertion of the transport proteins or whether it is simply due to technical limitations of efflux experiments.

Acknowledgements

We wish to thank Randi Levine for technical assistance with this study and V. Harms and H. Ross for comments on the manuscript. Supported by grants from the USPHS (AM 19567 and NS 09666).

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