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Ion dependence of cystine and lysine uptake by rat renal brush-border membrane vesicles

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The shared transport system for uptake of L-cystine and L-lysine was examined in isolated rat renal brush-border membrane vesicles for the ionic requirements for activation of the system. No requirement for sodium was seen for either cystine or lysine influx. However, the efflux of lysine from the vesicle was stimulated by Na^+. Therefore, the transport system appears to be asymmetric in its requirement for sodium. Two different divalent cations were used in the membrane isolations which resulted in different responses of cystine uptake to the electrogenic movement of K^+ out of the vesicle. Membranes prepared by Mg-aggregation showed no stimulation of cystine influx by the imposition of a transient interior negative potential while vesicles prepared by Ca-aggregation did respond to electrogenic stimulation by an outwardly directed K-diffusion potential in the presence of valinomycin. Lysine influx was stimulated by electrogenic potassium efflux in both Mg-prepared and Ca-prepared membranes. No difference in sodium requirement for cystine influx was seen between the vesicles isolated by different cation-aggregation methods.

Introduction

Classical cystinuria is an hereditary disorder of amino acid transport involving what is generally accepted to be a shared carrier system for the reabsorption of cystine and dibasic amino acids from the kidney. The disease is expressed in both the kidney and intestine, presumably at the brush-border membrane. Bannai [1] has pointed out that the relationship of cystine and lysine transport to sodium ion, which is an important activator in the transport process of many other substances, is still unclear. In renal cortical slices and tubules, L-[^{35}S]cystine uptake, as measured by ^{35}S accumulation, is lower in the absence of sodium than when sodium is present, while lysine uptake is not affected [4,7,8,18]. Experiments using rat renal brush-border membrane vesicles have indicated that there are two systems for cystine uptake, a high-affinity one shared with lysine and a low-affinity one which is unshared [11,17]. Cystine uptake by rat renal brush-border vesicles is higher in the presence of an inwardly directed sodium gradient than when sodium is equilibrated across the membrane. However, no overshoot in the time course of uptake of cystine is seen. On the other hand, the uptake of L-lysine by the same vesicles shows a distinct overshoot under sodium gradient conditions; yet, lysine influx is the same in the presence or absence of sodium [12]. Cystine and lysine are seen to mutually inhibit the uptake of the other and also participate in exchange diffusion both under sodium gradient and sodium equilibrated conditions [11,12,17]. The differences in handling of the two amino acids as evidenced by the parameters measured during transport studies using brush-border vesicles, as well as the difference in sodium requirements measured in rat renal cortical slices, has led to the desire to determine the relationship between sodium and cystine uptake. The results of these studies are presented in this report.

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Materials and Methods

Membrane isolation

Rat renal brush-border vesicles were prepared from the kidneys of adult normal Sprague-Dawley rats fed

ad libitum on Purina Rat Chow. The rats, weighing 250–350 grams, were killed by decapitation. Kidneys were removed, decapsulated and placed in 0.9% NaCl solution at 4°C. Rat cortical tissue was removed by using a Stadie-Riggs microtome. Rat renal brush-border membrane vesicles were isolated as previously described [11] using a slight modification of the divalent cation-aggregation technique described by Booth and Kenny [3]. Our previous reports have used $MgCl_2$ to aggregate membranes. In this report, either $MgCl_2$ or $CaCl_2$ was used as specified in the text. The final vesicle preparation was suspended in either THM buffer (100 mM mannitol + 2 mM Hepes adjusted to pH 7.4 with Tris) or MM buffer (100 mM mannitol in 2 mM Mes adjusted to pH 5.8 with Tris). The protein concentration in the final brush-border preparations ranged from 2 to 6 mg/ml as determined by Bio-Rad assay using γ-globulin as the standard.

Transport incubations

Standard incubations for uptake of 0.02 mM L-[^{35}S]cystine and 0.1 mM D[^{14}C]glucose consisted of a test tube containing 0.475 ml buffer A (60 mM mannitol, 20 mM Hepes, 20 mM Tris), 25 μl 2M NaCl, and labeled substrate of the desired concentration in 10 μl of 0.25 M HCl. For inwardly directed salt gradients and for no salt conditions, timing was initiated by the addition of 0.05 ml brush-border vesicles in THM buffer (100 mM mannitol in 2 mM Hepes adjusted to pH 7.5 with Tris). To examine uptake under conditions where salts were equilibrated across the membrane, the vesicles were suspended in THM containing 100 mM of the specified salt. The final incubation volume was 0.560 ml and final pH of the mixture was 7.5. Standard incubations for uptake of 0.02 mM L-[^{14}C]lysine and 0.02 mM L-[^{14}C]proline consisted of a test tube containing 0.475 ml THM buffer, 25 μl 2M NaCl, and 10 μl labeled substrate of the desired concentration in water. Uptake was initiated by the addition of 0.05 ml membrane vesicles in THM buffer resulting in a final volume of 0.560 ml and pH = 7.5. At the designated time, the incubations were stopped by rapid filtration on Sartorius nitrocellulose filters (No. 11306, 0.45 μm) and washed with 10 ml 0.9% NaCl in 2 mM Hepes adjusted to pH 7.4 with Tris. These methods have been described previously [12,17]. Uptake or accumulation is expressed as nmoles of substrate taken up per mg of membrane protein. In each experiment quadruplicate replicates of each incubation were made for each data point. All incubations contained L-[^{3}H]glucose in order to monitor vesicle size and simple diffusion. Specific conditions for each type of experiment are given in the figure legends.

To examine the role of Na^+ in uptake incubations, 100 mM NaCl in the standard incubation medium was replaced by other salts (KCl, LiCl, or choline chloride) or by 100 mM mannitol as indicated in the text. Stepwise replacement of NaCl by choline chloride was used in determining apparent affinities of the system for Na^+. In all cases, the final incubation had a volume of 0.560 ml and a final pH = 7.5.

Efflux of lysine from vesicles was assessed by preloading vesicle suspensions in THM for 10 min with 0.02 mM L-[^{14}C]lysine and 100 mM of the desired salt. Efflux was initiated by adding 0.05 ml of preloaded vesicle suspension (containing 0.2–0.3 mg protein) to 1 ml of THM buffer at time zero. After the designated incubation period, the amount of lysine retained within the vesicles was determined by rapid filtration of the vesicles as described above. No stop solutions are ever used as they cannot totally prevent efflux. Zero time of efflux was determined by co-filtration of the membrane vesicles and the 1 ml of THM buffer without prior mixing.

To assess the effect of a transient interior negative membrane potential on the rate of uptake, 1 μl valinomycin in ethanol (1.5 mg/ml) was added to the standard incubation mixture in the experimental incubations and 1 μl absolute ethanol was added to the control incubations. Uptake was initiated by the addition of 0.05 ml membrane vesicles in THM buffer containing 100 mM KCl resulting in a final volume of 0.561 ml and pH = 7.5. Inward Na^+ gradients consisted either of 100 mM NaCl or 50 mM Na_2SO_4.

To determine the influence of an outwardly directed proton gradient on uptake, aliquots of 0.05 ml brush-border vesicles suspended in MM buffer were added to standard incubation mixtures described above where NaCl was replaced by Na gluconate and either 1 μl of ethanol or FCCP in ethanol (5 mM) was added to control or experimental incubations, respectively. The final volume of the incubations were 0.561 ml and the final pH was 7.5.

Materials

The radioactive material used in these experiments was purchased from several companies: L-[^{35}S]cystine from Amersham Corporation, L-[^{14}C]lysine from ICN Radiochemicals; and L-[^{14}C]proline, L-[^{3}H]glucose, and D-[^{14}C]glucose from DuPont NEN Research Products. A strict criteria of cystine purity as described by Schafer and Watkins [16] was used in which quantitative reduction to cysteine by dithiothreitol was determined by high-voltage electrophoresis and thin-layer chromatography [13,19] for each batch of labeled cystine. Only batches which showed greater than 93% reduction to cysteine were used. Cystine solubilized in 0.5 M HCl and diluted with buffer is utilized within 5 min for transport studies. Unlabeled amino acids, salts, and buffer components were of the highest purity commercially available.

Results

We have previously shown that accumulation of L-cystine is time dependent, reaching a steady-state after 90 min of incubation. However, time dependent cystine binding to the intravesicular surface of the membrane has also been demonstrated [11]. This binding is not specifically related to the transport system and reflects the absence of an intravesicular reducing system in the membrane vesicle. In whole cells, cystine is reduced in the cytoplasm to cysteine which is transported by a different carrier from cystine. Binding of cystine to the intravesicular surface of the membrane vesicle is negligible during the first 15 s of uptake and represents transport into an intravesicular space.

Fig. 1 shows the time curve of 0.02 mM cystine accumulation under inwardly directed gradients of 100 mM NaCl, 100 mM LiCl, and 100 mM KCl which were imposed at time zero ($t = 0$). No evidence of an overshoot is seen in any of the curves when the salts formed inwardly directed gradients. Stepwise replacement of sodium by choline in the 100 mM salt gradient resulted in no change in 15-s uptake, indicating no apparent affinity of the cystine uptake system for Na^+ (Fig. 2). Table I shows that no difference in the rate of cystine influx (0 to 15 s) is seen in the presence or absence of extravesicular sodium. Only at longer time points (> 0.25 min) is there any significant difference in accumulation of [^{35}S]cystine within the vesicle. Thus, cystine influx shows no sodium dependence.

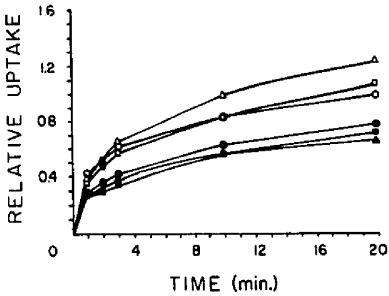

Fig. 1. Time curve of uptake of 0.02 mM L[^{35}S]cystine by rat renal brush-border membrane vesicles isolated by Mg-aggregation. Standard incubations were performed as in Methods. Uptake was measured under inwardly directed gradients of 100 mM NaCl (○), KCl (△), or LiCl (□) and when there was 100 mM NaCl (●), KCl (▲), or LiCl (■) equilibrated across the membrane and expressed as relative uptake. Relative uptake is the uptake of cystine in nmol/mg membrane protein at any point divided by the average uptake of cystine after 20 min under a NaCl gradient. Standard error bars have been omitted from the drawing for clarity, but no significant differences between corresponding time points for gradient conditions or for equilibrated conditions prior to any 10-min points were seen. The symbols represent the mean of 8–48 determinations for each point.

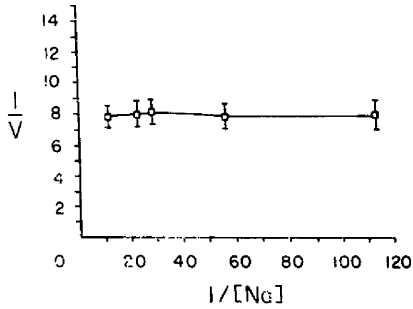

Fig. 2. Effect of [Na^+] on 15-s uptake of 0.02 mM [^{35}S]cystine in Mg-prepared brush-border membrane vesicles. Vesicles were incubated for 15 s as indicated in Methods under gradient conditions where 100 mM NaCl was replaced stepwise by choline chloride in the standard incubation medium. The symbols represent the mean ± the standard error of 10–12 determinations for each sodium concentration. Standard errors are represented by vertical bars. V is in nmol/mg membrane protein per 15 s and [Na^+] is in M.

When an inward Na^+-gradient is imposed, time dependent cystine accumulation is higher than when intravesicular Na^+ is present at $t = 0$ and uptake is assessed with sodium equilibrated across the membrane. Cations other than Na^+, i.e., Li^+ and K^+, also show the phenomenon of higher cystine accumulation under gradient conditions. Prior to 20 min of incubation, no significant difference between the uptake values measured under Na^+, Li^+, or K^+ are seen. Only at 20 min is the uptake in the presence of an initial KCl gradient significantly higher than that measured in the presence of Na^+ or Li^+. Under cation equilibration, no difference in the level of cystine accumulation is observed with Na^+, Li^+, or K^+. No differences in vesicle size, as measured by L-[^{3}H]glucose diffusion, or in the percent of cystine binding are observed between any incubation conditions being compared (different salts

TABLE I

Uptake of 0.02 mM [^{35}S]cystine by Mg-prepared renal brush-border vesicles in the presence or absence of extravesicular NaCl

Incubations were performed as described in Methods for the uptake of 0.02 mM [^{35}S]cystine in the presence of a 100 mM NaCl gradient or when no salt was present in the incubation buffer. Values are means ± S.E. of 12 determinations per point and are expressed as the fraction of uptake relative to the uptake measured at 20 min in the presence of a 100 mM NaCl gradient.

Time of incubation	NaCl gradient	No salt
6 s	0.1611 ± 0.0176	0.1432 ± 0.0131
15 s	0.2745 ± 0.0322	0.2185 ± 0.0248
1 min	0.4829 ± 0.0375	0.3216 ± 0.0218 [a]
20 min	1.0000 ± 0.0108	1.1200 ± 0.0277 [a]

[a] Indicates the value is different from that obtained in the presence of NaCl with $P < 0.001$ as determined by the Student's t-test.

used or whether the salts were equilibrated across the vesicle).

The higher level of accumulation observed when a salt gradient is imposed at $t = 0$ does not appear to be due to the anion present. Results of measuring cystine accumulation in the presence of inwardly directed gradients of 100 mM NaSCN or 50 mM Na_2SO_4 were identical and levels retained were higher than when these salts were equilibrated across the membrane prior to initiation of uptake (unpublished results).

The degree of non-transport related cystine binding to brush-border vesicles incubated for longer time periods [11] makes the evaluation of the differences in cystine accumulation problematic. Therefore, the uptake of lysine, which is also a substrate for the cystine carrier, was examined in the vesicles. Similar to the situation with cystine, no sodium requirement for lysine entry into renal brush-border membrane vesicles can be detected, but accumulation at early time points is higher under a sodium gradient than when sodium is equilibrated across the membrane at $t = 0$ [12]. This is due to the intravesicular presence of the sodium ion. The substitution of anions did not change this phenomenon of higher accumulation at early time points in the absence of intravesicular salts (unpublished results). When a non-penetrating or slowly penetrating cation, *N*-methyl-D-glucamine, replaced sodium as the chloride salt in standard incubations, the uptake curves were identical to those measured in the absence of any extravesicular salt up to 30 min. When vesicles were loaded with *N*-methyl-D-glucamine chloride, no difference in the accumulation at early time (< 5 min) points was seen whether the extravesicular buffer contained NaCl or no salt. This indicates that when the extravesicular cation is unable to penetrate into the vesicle, accumulated substrate does not efflux from the vesicle.

To confirm this hypothesis, vesicles were allowed to take up lysine in THM buffer without NaCl. NaCl or

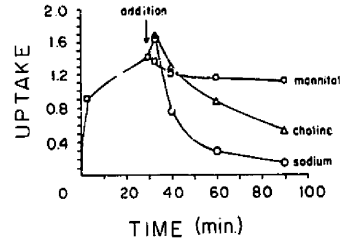

Fig. 3. Effect of sodium and choline on accumulation of 0.02 mM [^{14}C]lysine by Mg-prepared brush-border membrane vesicles. Membrane vesicles in THM buffer were incubated for 30 min in the absence of NaCl. After 30 min, NaCl (○), choline chloride (△), or mannitol (□) was added to a final concentration of 100 mM and accumulation assessed at the time points indicated. Symbols used represent the mean of four determinations per data point. Standard errors are included within the size of the symbol used to designate the mean. Each curve was duplicated in two or more separate experiments. Uptake is in nmol/mg membrane protein.

the more slowly penetrating choline chloride were then added to the incubation (25 μl 2M salt to a final volume of 0.560 μl). The addition of Na or choline chloride to the lysine-loaded vesicles caused a small transient increase in lysine accumulation followed by a progressive loss of lysine from the vesicle as shown in Fig. 3. The initial rise in uptake after the addition of either NaCl or choline chloride might be due to the known electrogenic nature of lysine accumulation [12]. Accumulated lysine was lost more rapidly from vesicles which were taking up Na^+ than those taking up choline. After 60 min, the amount of lysine remaining in the vesicles was remarkably similar to the levels reached under equilibration under these same salts. Other experiments indicate that under equilibrated conditions for NaCl, choline chloride or buffered mannitol alone, lysine accumulation is lowest with NaCl, slightly higher with choline chloride, but highest in mannitol without salts (Fig. 4a). The same relationship was seen when

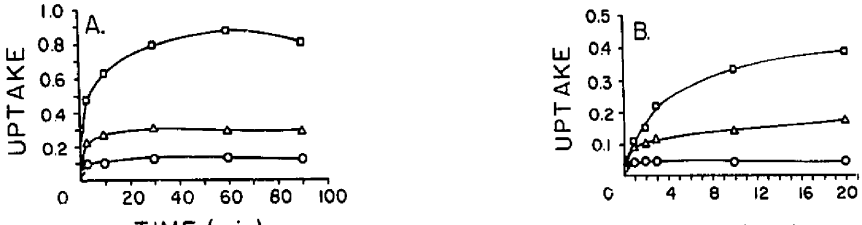

Fig. 4. Equilibrium accumulation of lysine in the presence or absence of NaCl and choline chloride. Uptake of 0.02 mM [^{14}C]lysine was measured under equilibrated salt conditions as detailed in Methods in the presence of no salt (□), 100 mM NaCl (○), or 100 mM choline chloride (△). Panel A shows accumulation under standard incubation conditions where the vesicles were suspended in THM containing either no salt (□), 100 mM NaCl (○), or 100 mM choline chloride (△). Panel B shows the same conditions with the addition of 10 mM KCl to both intravesicular and extravesicular buffers. Vesicles were added to the incubation buffer along with 2 μl/ml valinomycin (1.5 mg/ml) in ethanol and incubated at 22°C for 10 min. Uptake was initiated by the addition of [^{14}C]lysine. The symbols used represent the mean of four determinations per data point. Standard errors are included within the size of the symbol used to designate the mean. Each curve was duplicated in two or more separate experiments. Uptake is in nmol/mg membrane protein.

the vesicles were potential clamped by K^+ and valinomycin (Fig. 4b) except 15 s uptake in choline chloride was the same as with mannitol buffer. This suggests that an asymmetry exists in sodium requirement between the influx and efflux processes for lysine in isolated renal brush-borders. When lysine efflux from rat renal brush-border vesicles into buffer containing no salt was measured directly, cis-stimulation of lysine efflux by NaCl is seen when compared to lysine loaded vesicles with choline chloride. After 15 s of efflux, only $63.7 \pm 2.26\%$ of the lysine remained in vesicles loaded with NaCl and lysine while $84.5 \pm 0.31\%$ of the lysine remained in vesicles loaded with choline chloride and lysine. It is possible that the higher cystine accumulation which occurs in the presence of a sodium gradient may be a reflection of the same asymmetric requirement for sodium as appears for lysine movement.

In rat renal brush-border vesicles isolated by Mg-aggregation, no evidence for electrogenic transport of cystine was found previously [10]. We have used a number of methods to explore the possibility of electrogenic stimulation of cystine uptake in brush-border vesicles isolated by Mg-aggregation. These included attempts at measuring "overshoots" in the time course of uptake under inwardly directed Na-gradients, imposing interior electronegative membrane potentials by establishing large potassium diffusion potentials (using K_2SO_4 and KCl) across the membrane in the presence of valinomycin, and testing for stimulation of cystine uptake in the presence of FCCP by vesicles possessing an outwardly directed proton gradient. None of these manipulations resulted in stimulated cystine accumulation. The results are somewhat different in vesicles isolated by Ca-aggregation. Fig. 5 shows the uptake of 0.02 mM [^{35}S]cystine under an inwardly directed 100 mM NaCl gradient by Mg-aggregated and by Ca-aggregated membranes, both of which were loaded with 100 mM KCl. The time curve of cystine uptake by Ca-aggregated vesicles was lower than that by Mg-prepared brush-borders membrane vesicles. When valinomycin was added at the start of the incubation, a stimulation in the uptake of cystine occurred in the vesicles isolated by Ca-aggregation but not in those isolated by Mg-aggregation. The role of Na^+ in cystine influx into Ca-aggregated membranes did not appear to differ from that in Mg-aggregated membranes. Fig. 6 shows the uptake of 0.02 mM [^{35}S]cystine in the Ca-aggregated membranes. No difference in influx and no significant difference in accumulation level of cystine was seen under NaCl or KCl inwardly directed gradients or when no salt was added.

We therefore examined the response of vesicles prepared by Ca-aggregation to the ionophores valinomycin and FCCP. Valinomycin is used in the presence of a potassium gradient to enhance membrane potential through the electrogenic movement of K, creating a

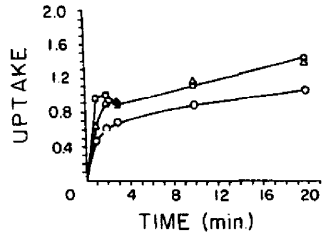

Fig. 5. Effect of electrogenic potassium efflux on the uptake of 0.02 mM L-[^{35}S]cystine by rat renal brush-border membrane vesicles isolated by divalent cation aggregation techniques using Mg (△) or Ca (○, □). Vesicles were suspended in THM buffer containing 100 mM KCl and transport was assessed as described in Methods. Electrogenic potassium efflux was initiated by the addition of valinomycin. For Mg-prepared membranes the same symbol is used for both control and experimental (valinomycin added) conditions. For Ca-prepared membranes, control incubations (○) contained 1 μl absolute ethanol and experimental incubations (□) contained 1 μl valinomycin in ethanol (1.5 mg/ml). The symbols represent the mean of four determinations. Standard errors are included within the size of the symbol used to designate the mean. Uptake is in nmol/mg membrane protein.

transient interior negative potential in the vesicles. The effect of the imposition of a K-diffusion potential in the presence of valinomycin on cystine, lysine, glucose, and proline uptake after 6, 15, and 60 s of incubation can be seen in Table II. Values given are averages of uptake in the presence of valinomycin expressed as a percent of control uptake. Glucose and proline, two substrates whose transport is known to be electrogenic in brush-border vesicles, were used as standards to monitor the experimental conditions. For all three early time periods examined, the imposed K-diffusion potential stimulated glucose and proline uptake dramatically in the presence of valinomycin, while uptake

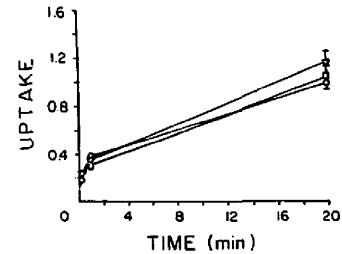

Fig. 6. Time curve of 0.02 mM [^{35}S]cystine uptake by Ca-prepared brush-border membrane vesicles. Uptake was measured under standard incubations conditions for salt gradients. Vesicles were in THM and the extravesicular medium contained either 100 mM NaCl (○), 100 mM KCl (▽), or no salt (□). Symbols represent the means ± the standard errors of 8–12 determinations per point. Standard errors are represented by vertical bars. Where no bar appears, the S.E. is contained within the size of the symbol used to denote the mean. Uptake is in nmol/mg membrane protein.

TABLE II

Effect of K-diffusion potential (inside negative) in the presence of valinomycin on uptake by Ca-prepared renal brush-border vesicles

Incubations were performed as described in Methods. Values given are the means ± S.E. of the number of determinations given in parenthesis for experimental points. An equal number of control incubations (without ionophor) was performed at the same time. Each uptake value was expressed as a percent of the average uptake in corresponding control incubations performed in parallel. Control incubations were taken as 100%.

Incubation time	Substrate			
	cystine (% control)	lysine (% control)	glucose (% control)	proline (% control)
6 s	133 ± 19.0 (32)	119 ± 17.2 (16)	319 ± 38.2 (16) [b]	164 ± 5.3 (8) [b]
15 s	147 ± 16.0 (40) [a]	177 ± 36.3 (56) [a]	303 ± 11.5 (16) [b]	162 ± 6.7 (8) [b]
1 min	125 ± 17.4 (32)	173 ± 22.7 (16) [a]	306 ± 28.0 (16) [b]	181 ± 10.6 (8) [b]

[a] Indicates a significant difference from the control value of 100% with $P < 0.05$.
[b] Indicates significance with $P < 0.001$.

of lysine and cystine was stimulated significantly only after 15 s.

FCCP, a protonophore, was used to promote electrogenic H^+ movement. Table II shows the response in Ca-prepared brush-border vesicles of the transport of cystine, lysine, proline, and D-glucose to an outwardly directed proton gradient in the presence of an inwardly directed sodium gluconate gradient. When FCCP was added to promote electrogenic movement of protons, the overshoots for glucose and lysine uptake were stimulated at the earliest time points. Proline uptake was also stimulated after 6 s of incubation in the presence of FCCP. Response of proline uptake to the control incubation conditions (inward Na gluconate gradient and vesicles with intravesicular pH = 5.8) was to eliminate the dramatic overshoot formation in the first minute of uptake which is seen under standard incubation conditions (inward NaCl gradient and intravesicular pH = 7.1). Whether it is the presence of the gluconate ion, absence of the chloride ion, or intravesicular pH which causes the overshoot to be lost is not known; however, electrogenic proton efflux in the presence of FCCP does result in appearance of a small overshoot in proline uptake. No stimulation of cystine uptake was seen during the first minute of incubation. However, cystine accumulation was higher after 20 min of incubation in the presence of FCCP than when FCCP was omitted from the incubation.

Discussion

A number of differences between cystine and lysine transport characteristics have been observed. At physiological pH, lysine carries a net positive charge, whereas cystine is present in two ionic species (70% neutral + 30% anionic) resulting in a net negative charge [1]. Cystine transport in cortical slices is sodium dependent [18], whereas lysine entry is not dependent on sodium [8,18]. Lysine is transported against a concentration gradient and is accumulated within the cell [8,18]. In intact cells, cystine is not transported against a concentration gradient because it is normally reduced

TABLE III

Effect of an outwardly directed proton gradient in the presence of FCCP on uptake by Ca-prepared renal brush-border vesicles

Incubations were performed as described in Methods. Values given are the means ± S.E. of the number of determinations given in parenthesis for experimental points. An equal number of control incubations (without ionophor) was performed at the same time. Each uptake value was expressed as a percent of the average uptake in corresponding control incubations performed in parallel. Control incubations were taken as 100%.

Incubation time	Substrate			
	cystine (% control)	lysine (% control)	glucose (% control)	proline (% control)
6 s	130 ± 19.6 (8)	173 ± 10.6 (12) [c]	319 ± 34.7 (12) [c]	174 ± 17.1 (14) [a]
15 s	130 ± 14.7 (16)	139 ± 9.0 (28) [b]	246 ± 24.8 (8) [c]	143 ± 17.6 (16)
1 min	128 ± 9.1 (12)	150 ± 13.2 (12) [b]	137 ± 20.4 (12)	94 ± 9.6 (16)

[a] Indicates a significant difference from corresponding control value of 100% with $P < 0.05$.
[b] Indicates significance with $P < 0.01$.
[c] Indicates significance with $P < 0.001$.

a concentration gradient because it is normally reduced to cysteine after it enters the cell [5,6]. Reduction, however, is not required for cystine transport as shown by the studies in isolated brush-border vesicles [2,11,17]. In the absence of a reducing system, cystine binds to intravesicular protein [11], but no significant binding on the luminal surface of the brush-border membrane has been detected [2,11]. In brush-border vesicles, binding of lysine occurs both internally and at the luminal surface, but intravesicular free lysine still accumulates to form distribution ratios greater than unity [12]. In isolated brush-border vesicles, [^{35}S]cystine accumulation (where binding to intravesicular protein accounts for most of the radioactivity) is increased by the presence of a sodium gradient while [^{14}C]lysine accumulation is higher in the absence of sodium [11,12]. Accumulation of lysine in vesicles appears to be proportional to the Donnan potential, whereas accumulation of cystine does not [2,12], perhaps because of the intravesicular binding. On the other hand, both mutual inhibition and heteroexchange diffusion between cystine and lysine occur. Lysine uptake by renal brush-border membranes occurs via a single sodium independent system which is inhibited by cystine [12]. Thus, the evidence remains strong that cystine and lysine share a common transport carrier system in spite of the differences in the transport characteristics of these two amino acids.

The data reported here show there is no obligatory role for sodium in cystine influx. Other cations are equally effective, or ineffective, in altering the rate of cystine influx. Indeed, the absence of any monovalent cation does not diminish the influx rate. A similar result has been reported by States and Segal in the opossum kidney cell line, OK [20]. Furlong and Posen have noted that cystine uptake by brush-border membrane vesicles isolated from human kidney is also relatively sodium independent [9]. In fact, we have found that oxidation products of cystine (cystine-S-S-dioxide) are very sensitive to sodium stimulation (unpublished data). Contamination of even 1% of the radioactivity with these products contributes significant distortion to the uptake characteristics for L-[^{35}S]cystine. Therefore, we monitor the cystine purity in every experiment. Our experiments with cystine and lysine uptake by rat renal brush-border vesicles have shown that systems which may demonstrate increased accumulation under conditions of a sodium gradient as opposed to when sodium is equilibrated across the membrane do not necessarily have an obligatory requirement for sodium as an activator for influx. Asymmetry of the carrier with regard to sodium requirement seems likely since the presence or absence of sodium in the cis position has no effect on initial rate of uptake for lysine [9,16] and for cystine (this report), but intravesicular cations do stimulate lysine efflux. The overshoot for lysine observed under a sodium gradient was not a stimulation of lysine influx produced by the inward flux of the sodium ion but a stimulation of lysine efflux produced by the intravesicular presence of a monovalent cation. Sodium was the most efficient stimulator of lysine efflux but KCl and choline chloride were also able to induce lysine efflux [12]. The non-specific binding of cystine to intravesicular proteins negates the possibility of performing cystine efflux studies corresponding to those done with lysine.

In renal brush-border membranes isolated by Mg-aggregation methods [3,11], no overshoot in the time curve of uptake of cystine [11,17] is seen, but there is an overshoot in the time curve of lysine uptake [12]. The overshoot for lysine uptake was enhanced by the addition of valinomycin to membrane vesicles which had an outwardly directed KCl gradient. The effect of valinomycin, however, was not significant at very early time points (< 15 s). Similar use of valinomycin with K-loaded vesicles had no effect on the time curve for cystine uptake (this report) nor did examining the effects of membrane potential on cystine uptake by using different ratios of potassium inside and outside the vesicle in the presence of valinomycin [10]. The method of membrane preparation has a significant effect on the nature of some transport systems measured in the isolated membrane. For the Na-H exchanger, the substitution of calcium for magnesium as the divalent cation used to aggregate brush-border membranes resulted in detection of an electrogenic component of this system [15]. Similarly, the isolation method used may have preserved the stimulatory response in cystine uptake with the addition of valinomycin to K-loaded vesicles reported by Biber et al. [2]. In this report, we also noted a change in the response of the cystine transport system in Ca-prepared membranes to increased intravesicular electronegativity produced by a K-diffusion potential in the presence of valinomycin. However, compared to electrogenic Na-cotransport systems for D-glucose and L-proline already studied in renal brush-border vesicles, the effects were quite different for cystine and lysine. A slight but significant increase in cystine uptake occurred in Ca-prepared membranes 15 s after valinomycin was added to K-loaded vesicles. Both cystine and lysine uptake by Ca-prepared brush-border membrane vesicles showed slower responses to the imposition of the transient interior negative potential.

The effect of electrogenic proton efflux from the vesicle (in the presence of FCCP) on lysine, proline, and glucose uptake was to stimulate uptake at very early time points to a greater extent than later time points. The uptake of cystine does not appear to respond to the membrane potential changes induced by electrogenic proton efflux while lysine uptake does. Why two substrates which share a transport system do

not act in a similar manner to perturbations of the incubation medium is not clear. The transient increase in intravesicular negativity produced by an outwardly directed K-diffusion system in the presence of valinomycin appears to stimulate influx on the combined cystine-lysine transport system. However, the response to increased intravesicular negativity resulting from rapid H^+ efflux involved only lysine uptake. The H^+ gradient itself (in the absence of FCCP) caused no stimulation in uptake. Intravesicular pH may determine the significance of the studies with FCCP since we have shown elsewhere [14] that, when intravesicular pH is low, lysine influx increases with an increase in extravesicular pH while cystine influx does not. Under the conditions used in the FCCP studies, lysine uptake is the same as under standard incubation conditions, while cystine uptake is depressed.

The shared transport system for influx of cystine and lysine in isolated renal brush-border membrane vesicles is sodium independent and stimulated by a transient increase in intravesicular electronegativity, indicating that influx may be energized by the membrane potential. However, the complexities of the transport system, as reflected in studies employing whole cells, must be considered when seeking an explanation of the clinical picture seen in the inherited defect in cystinuria. The possibility of different efflux systems as well as the complications of substrate reduction in whole cells continue to obscure efforts at delineating the transport process. The answer to the riddle of cystinuria remains unclear and the method of approach to this question must be reconsidered. Isolation of the carrier protein is necessary for any further evaluation of the transport system.

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