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Na^+-DEPENDENT TRANSPORT OF TRICARBOXYLIC ACID CYCLE INTERMEDIATES BY RENAL BRUSH BORDER MEMBRANES

EFFECTS ON FLUORESCENCE OF A POTENTIAL-SENSITIVE CYANINE DYE

STEPHEN H. WRIGHT, SALLY KRASNE, IAN KIPPEN and ERNEST M. WRIGHT

Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90024, and Department of Medicine, 502 Halper Bldg., Cedars-Sinai Medical Center, 8700 Beverly Blvd., Los Angeles, CA 90048 (U.S.A.)

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Summary

The effect of the transport of tricarboxylic acid cycle intermediates on the membrane potential of renal brush border vesicles was studied using fluorescence of the cyanine dye, 3,3'-dipropylthiadicarbocyanine iodide. The behavior of the dye in the preparation was established with valinomycin-induced K^+-diffusion potentials; increases in fluorescence were associated with depolarizing conditions. Addition of 1 mM succinate or citrate to membrane/dye suspensions produced transient increases in fluorescence, indicative of a depolarizing event(s) associated with the transport of these substrates. The transient response in fluorescence was Na^+ dependent, of greater magnitude under Na^+-gradient as compared to Na^+-equilibrium conditions, and was a saturable function of substrate concentration. The specificity of the fluorescence response was identical to that obtained from studies of the competitive inhibition of succinate transport by tricarboxylic acid cycle intermediates and analogs. We conclude that the major tricarboxylic acid cycle intermediates are transported via a common Na^+-dependent transport system in renal brush border membranes.

Abbreviations: diS-C_3-(5), 3,3'-dipropylthiadicarbocyanine iodide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

The renal brush border membrane contains an Na^+-dependent transport system for citrate, succinate and α-ketoglutarate [1,2]. We recently examined the inhibition of succinate transport by structural analogs and found that the receptor of the transport system is specific for the major constituents of the tricarboxylic acid cycle. This study did not, however, demonstrate that the inhibitors of succinate transport were themselves transported. Such demonstrations have required that the substrates be radioactively labeled, an obvious problem when such forms are unavailable or when the study includes large numbers of structural analogs. Recently, it has been shown that the Na^+-dependent transport of organic substrates, such as glucose and amino acids, can produce changes in membrane potential of intact cells [3—5], cytoplasts derived from cells [6], and preparations of isolated renal brush border membranes [7], as evidenced by changes in fluorescence of potential-sensitive cyanine dyes. These observations suggested to us that membrane-permeable dyes could be used to monitor the transport of non-radioactively labeled compounds. The present study describes the response of the cyanine dye, diS-C_3-(5), to renal brush border membranes exposed to tricarboxylic acid cycle intermediates. Evidence is presented indicating that all the major tricarboxylic acid cycle intermediates are transported via a common Na^+-dependent pathway. Evidence is also discussed suggesting that the transport of these substrates results in the depolarization of the brush border membrane.

Materials and Methods

Preparation of isolated brush border membranes. Purified rabbit renal brush border membrane vesicles were prepared using a modification of the Ca^{2+}-precipitation procedure of Schmitz et al. [8] as described by Wright et al. [2]. Membranes prepared using this procedure routinely had an increase in specific activity of trehalase, a brush border membrane marker, of 10-fold over the initial homogenate. ($Na^+ + K^+$)-ATPase and succinate dehydrogenase activities were reduced more than 5-fold [2,9]. Brush border vesicles were pre-loaded with test solutions by washing the final membrane pellet in the desired solution, centrifuging at $50\,000 \times g$ for 30 min, and resuspending in the test solution to a final concentration of from approx. 5 to 8 mg per ml. The final suspensions were kept on ice overnight and used the following day.

Measurements of fluorescence. The fluorescence of the cyanine dye, diS-C_3-(5), was measured with an Aminco spectrophotofluorometer (Model SPF-500). Excitation was at 620 and emission at 669 nm (band widths of 2 nm). Fluorescence signals were recorded with a Houston 2000 *x-y* chart recorder. To minimize adsorption of dye to the cuvette walls during an experiment, cuvettes were incubated overnight in buffer containing 4 μM diS-C_3-(5), thereby saturating the walls of the cuvette with dye. The protocol for a typical experiment was as follows. 10 μl of an ethanolic stock solution of diS-C_3-(5) were added to a quartz cuvette filled with 3.0 ml of test buffer, resulting in a final dye concentration of 2 μM. The solution was quickly mixed, and

the resulting fluorescence signal monitored. After 1 min an aliquot of brush border suspension was added to the cuvette, producing a reaction mixture of concentration from approx. 0.15 to 0.25 mg protein per ml. The concentration was determined empirically to result in the largest fluorescence signals upon addition of test substrates. After an interval of time, an aliquot of a concentrated stock solution of test substrate was added to the reaction mixture, and resulting changes in fluorescence monitored. Details of experimental conditions and variations from this protocol are described in the figure legends. Fluorescence data are expressed in arbitrary units and/or as percent changes in total fluorescence. Because the amount of protein required to produce a maximum fluorescence response did vary between different brush border preparations, absolute values of the fluorescence measurements from separate experiments were not considered comparable.

Measurements of tracer uptake. Measurement of uptake of ^{14}C-labeled substrates was performed using a Millipore filtration procedure described elsewhere [9].

Chemicals. The fluorescent dye, diS-C_3-(5), was generously donated by Dr. Alan Waggoner of Amherst College. Valinomycin was purchased from Sigma. ^{14}C-labeled substrates were purchased from New England Nuclear. All other chemicals were obtained from standard sources and were the highest grade available.

Results

Effect of potential on fluorescence

The magnitude of the fluorescence signal of diS-C_3-(5) in suspensions of brush border vesicles was observed to be a function of the potential difference across the vesicle membrane. Fig. 1 presents the results of a typical experiment examining the fluorescence response to valinomycin-induced K^+-diffusion potentials. The fluorescence was linearly related to the log of the ratio of extra- and intravesicular K^+ concentrations ($[K^+]_o/[K^+]_i$) when vesicles loaded with 15 mM KCl, 135 mM choline chloride were exposed to external solutions containing the K^+ ionophore, valinomycin (2 μM) and KCl concentrations of 15—100 mM (choline chloride was used to replace KCl to maintain constant ionic strength at 150 mM) (Fig. 1a). The fluorescence signal increased as the ratio $[K^+]_o/[K^+]_i$ increased, suggesting that increases in fluorescence are related to depolarizing conditions of the vesicle membrane (i.e., inside positive relative to external condition). Likewise, as shown in Fig. 1b, hyperpolarizing conditions, induced by exposing vesicles loaded with 100 mM KCl to external K^+ concentrations of 10—100 mM and 2 μM valinomycin, resulted in a reduction in fluorescence.

Effect of DiS-C_3-(5) on transport

DiS-C_3-(5) had no marked effect on the transport of 1 mM [^{14}C]succinate into brush border vesicles under Na^+-gradient conditions (100 mM NaCl, 100 mM sorbitol, out: 300 mM sorbitol, in). In the presence of 2 μM diS-C_3-(5), the initial rate of net uptake (determined from 15-s incubations) was 25.1 nmol/mg protein per min, as compared to a control value of 23.5 nmol/mg

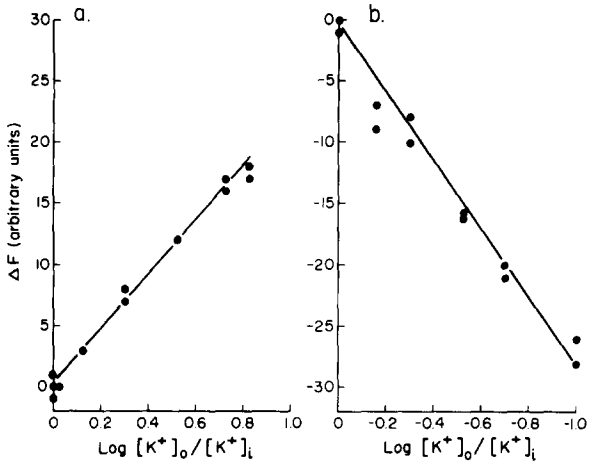

Fig. 1. Effect of valinomycin-induced K^+-diffusion potentials on fluorescence of diS-C_3-(5). (a) Effect of depolarizing conditions on dye fluorescence. Brush border vesicles equilibrated in 15 mM KCl, 135 mM choline chloride, 1 mM Tris-Hepes, pH 7.5, were added to dye solutions containing 15—100 mM KCl (the isosmolarity was maintained with choline chloride), 1 mM Tris-Hepes, pH 7.5, 2 μM diS-C_3-(5), and 2 μM valinomycin. Final membrane protein concentration was 0.19 mg/ml. The resulting fluorescence of the membrane/dye suspension was monitored for 1 min and the 'zero-time' fluorescence of the suspension was estimated by extrapolation of the fluorescence record to time zero. Zero fluorescence was arbitrarily chosen as the fluorescence determined when intra- and extravesicular K^+ concentrations were equal. 10 arbitrary units represent a change in total fluorescence of 10%. (b) Effect of hyperpolarizing conditions on fluorescence. Conditions as in a, except vesicles were pre-equilibrated in 100 mM KCl, 50 mM choline chloride and were placed in solutions with KCl concentrations ranging from 10 to 100 mM. Final membrane protein concentration was 0.17 mg/ml.

protein per min. The peak uptakes (t = 2 min) were 10.6 and 10.5 nmol/mg for the control and dye conditions, respectively. Uptakes at 60 min were 3.0 (control) and 3.5 (dye) nmol/mg. The peak uptake of 1 mM succinate represents an 'overshoot' of the equilibrium value of at least 3-fold.

For most studies examining the fluorescence response of membrane/dye suspensions to various substrates, the test substrates were added to the reaction mixture 1 min after brush border membranes were added to the dye solution. When Na^+-gradient conditions were employed in these studies (i.e., intravesicular $[Na^+] = 0$ at time zero; extravesicular $[Na^+] = 100$ mM), the gradient would have run down in the minute prior to addition of substrate. We examined the effect of this protocol on the uptake of 1 mM succinate (data not shown). In one set of experiments, the rate of uptake (15-s incubations) was reduced approx. 51% compared to control values (8.3 as compared to 16.8 nmol/mg per min); the 1 min preincubation also resulted in a 43% reduction of the peak uptake (4.4 as against 7.7 nmol/mg). The 60 min uptakes were 1.3 (control) and 1.5 (preincubation) nmol/mg. These results indicate that, though the 1 min preincubation of the membranes causes a reduction in the rate of succinate uptake, the remaining Na^+ gradient is adequate to produce net uptake and a peak overshoot indicative of concentrative transport.

Effect of succinate and citrate on fluorescence of DiS-C_3-(5)

The presence of either 1 mM citrate or succinate in suspensions of brush

border membranes incubated with diS-C_3-(5) under Na^+-gradient conditions (100 mM Na^+ out: 0 Na^+ in) results in a transient increase in fluorescence of the reaction mixture. As shown in Fig. 2, an increase in fluorescence, relative to the parallel control situation, was observed whether substrate was present in the reaction mixture at time zero (Fig. 2a and b), or was added after the vesicles had been in the dye medium for 1 min (Fig. 2c and d). While both substrates produced transient increases in fluorescence under each incubation condition, after approx. 1 min citrate caused a decrease in total fluorescence relative to control values (Fig. 2a and c). In contrast, the succinate-induced increase in signal dropped toward the control value (Fig. 2b and d).

When 3 mM D-glucose was added to membrane-dye suspensions under Na^+-gradient conditions identical to those described in Fig. 2c and d, a transient increase in fluorescence was observed, qualitatively similar though smaller in magnitude to that observed for succinate (for example, in one experiment, 3 mM D-glucose caused an increase in signal of 9% as compared to 1 mM succinate, 24%; data not shown). 3 mM L-glucose produced no change in fluorescence.

The magnitude of the change in total fluorescence produced by the addition of a transported substrate was much smaller when the Na^+ gradient was eliminated by pre-equilibrating vesicles in an NaCl buffer. Typically, equilibrium

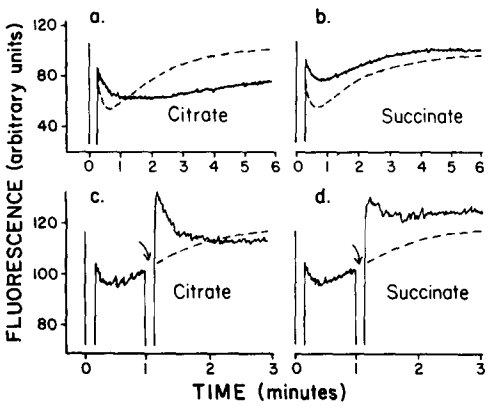

Fig. 2. Effect of citrate and succinate on fluorescence of diS-C_3-(5). (a) (———) Fluorescence record produced when brush border vesicles equilibrated in 300 mM sorbitol, 1 mM Tris-Hepes, pH 7.5, were added to a solution containing 100 mM NaCl, 100 mM sorbitol, 1 mM Tris-Hepes, pH 7.5, 2 μM diS-C_3-(5) and 1 mM citrate. Final membrane protein concentration was 0.10 mg/ml. (- - - - - -) Fluorescence record of the control condition without citrate. The vertical lines on the left of the record resulted from opening the spectrofluorometer to add the membrane suspension. Fluorescence is reported in arbitrary units; zero fluorescence is the value obtained when the emission shutter of the spectrofluorometer was closed. (b) Conditions as in a, except the test solution contained 1 mM succinate. (c) The effect of adding test substrate 1 min after adding brush border membranes to the dye solution. Brush border vesicles equilibrated in 300 mM sorbitol were added to a dye solution identical to that in a, except it contained no citrate. The final membrane protein concentration was 0.25 mg/ml. At t = 1 min, 30 μl of 100 mM sodium citrate were added to the membrane/dye suspension to produce a final citrate concentration of 1 mM. Vertical lines on the record indicate points of addition to the dye solution; the arrow indicates addition of citrate. (- - - - - -) Fluorescence of a control run in which buffer was added to the suspension. (d) Conditions as in c, except the test substrate was succinate. Graphic representations are tracings of actual fluorescence records. All data shown were obtained from a single membrane preparation.

conditions of 150 mM NaCl resulted in an approx. 4% increase in total fluorescence when 3 mM succinate was added to membrane/dye suspensions. Under Na^+-gradient conditions (100 mM NaCl out: 0 NaCl in), there was a transient increase in fluorescence of between 15 and 30%. Likewise, D-glucose produced a larger increase in fluorescence under Na^+-gradient conditions compared to equilibrium conditions (in one experiment, 10% as against 5%, respectively). For the purpose of the present study, Na^+-gradient conditions were employed for most observations.

The magnitude of the fluorescence response is a function of the final concentration of added substrate. Fig. 3 shows the relationship between increases in total fluorescence and increases in the concentration of succinate in the reaction mixture. The relationship is described by an equation identical in form to the Michaelis-Menten equation:

$$\Delta F = \frac{\Delta F_{max}[S]}{K_f + [S]} \quad (1)$$

where ΔF is the increase in fluorescence observed by addition of succinate at concentration [S], ΔF_{max} is the maximal increase in fluorescence, and K_f is the succinate concentration producing a half-maximal fluorescence response. The value for K_f from the experiment in Fig. 3, calculated from a linearization of Eqn. 1 ([S]/ΔF vs. [S]), is 0.10 mM.

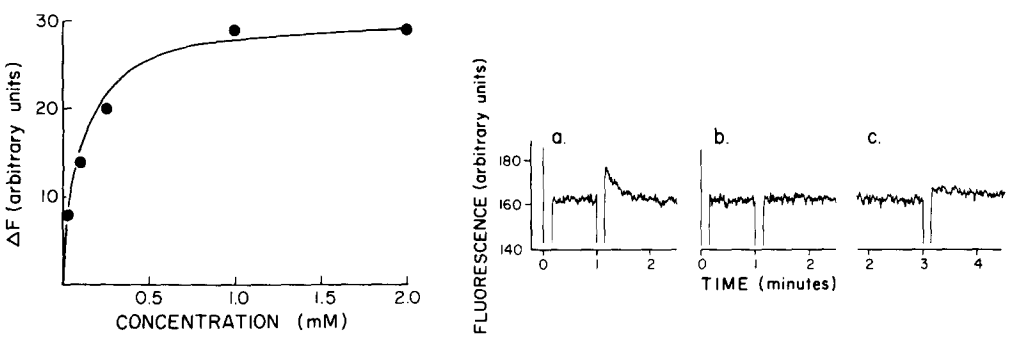

Fig. 3. Effect of substrate concentration on the succinate-induced fluorescence response of diS-C_3-(5). Experimental conditions as in Fig. 2d, except the final membrane protein concentration was 0.27 mg/ml. ΔF values are the maximum increase in total fluorescence of the membrane/dye suspensions compared to the control without added substrate. A ΔF of 10 units represents an increase in total fluorescence of approx. 10%. The line was calculated from Eqn. 1 (see text), using the constants F_{max} = 30.6 units and K_f = 0.10 mM, as derived from fitting the data to a linearization of Eqn. 1. All data shown were obtained from a single membrane preparation.

Fig. 4. Effect of Na^+ on the succinate-induced fluorescence response. (a) Fluorescence record produced when vesicles pre-equilibrated in 150 mM KCl, 1 mM Tris-Hepes, pH 7.5, were added to a solution containing 150 mM NaCl, 1 mM Tris-Hepes, pH 7.5, 2 μM diS-C_3-(5); final membrane protein concentration was 0.17 mg/ml. At t = 1 min, 100 mM sodium succinate was added to produce a final succinate concentration of 1 mM. (b) Experimental conditions identical to those of a, except that 150 mM KCl replaced NaCl in the extravesicular solution. (c) Effect of Na^+-equilibrium conditions on the fluorescence response. Vesicles pre-equilibrated in 150 mM NaCl, 1 mM Tris-Hepes, pH 7.5, 2 μM diS-C_3-(5); final membrane protein concentration was 0.24 mg/ml. At t = 3 min, 3 mM succinate was added to the membrane dye suspension. In all the above cases, fluorescence is expressed in arbitrary units as defined in Fig. 2. All data shown were obtained from a single membrane preparation.

The change in fluorescence associated with the addition of succinate is dependent on the presence of Na^+ in the medium (Fig. 4). When vesicles equilibrated in 150 mM KCl were placed in a solution containing 150 mM NaCl, 2 μM diS-C_3-(5), addition of 1 mM succinate produced a 9% increase in total fluorescence (Fig. 4a). In a parallel experiment, replacement of the NaCl in the external solution with either KCl (Fig. 4b) or choline chloride (data not shown) completely eliminated the fluorescence response. As stated earlier, while the presence of equal Na^+ concentrations in the intra- and extravesicular solutions (i.e., equilibrium conditions) is compatible with induction of the substrate-induced increase in fluorescence (Fig. 4c), the magnitude of the response is much greater under Na^+-gradient conditions (compare Fig. 4c with 4a or Fig. 2d). It should also be noted that even in the presence of a large inwardly directed Na^+ gradient, the magnitude of the fluorescence response is sensitive to the composition of the intravesicular medium. For example, in one experiment, vesicles equilibrated in 300 mM sorbitol showed a 24% increase in total fluorescence when exposed to 150 mM NaCl and 1 mM succinate (substrate added at t = 1 min). In a comparable parallel experiment, vesicles equilibrated in 150 mM KCl produced a 12% increase in fluorescence under identical experimental conditions (data not shown). This effect simply could be due to an increase in membrane conductance in the presence of KCl.

Substrate specificity of the fluorescence response

Addition of 1 mM maleate or isocitrate, structural analogs of succinate and citrate, respectively, produced little or no change in fluorescence of membrane/dye suspensions (Fig. 5). Table I lists the effects on fluorescence of membrane/dye suspensions of a variety of structurally related succinate analogs. All the compounds previously demonstrated to be effective inhibitors of [^{14}C]-succinate transport [2] induced transient increases in total fluorescence similar to that seen with succinate. These compounds included all the tricarboxylic acid cycle intermediates, with the exception of isocitrate. The compounds

TABLE I

FLUORESCENT CHANGES INDUCED BY ORGANIC ACIDS

Test substrates were added to membrane/dye suspensions 1 min after addition of brush border membranes to the dye solution. Final concentration of substrate was 1 mM. The extravesicular solution contained 100 mM NaCl, 100 mM sorbitol, 1 mM Tris-Hepes, pH 7.5, 2 μM diS-C_3-(5). Final protein concentration of the reaction mixture was 0.27 mg/ml. At time zero, the intravesicular composition was 300 mM sorbitol, 1 mM Tris-Hepes, pH 7.5. Fluorescence units are arbitrary; an increase of 10 units represents an increase in total fluorescence of approx. 10%. Errors represent ± 1 S. D., n = 3. Values given without errors represent single experimental determinations. All data shown were obtained from a single membrane preparation.

Compound	ΔF	Compound	ΔF
Citrate	38 ± 1.5	oxaloacetate	26 ± 1.7
Isocitrate	0	α-methylsuccinate	31, 31
α-Ketoglutarate	10 ± 1.2	α,β-dimethylsuccinate	5
Succinate	29 ± 2.2	maleate	3
Fumarate	31 ± 1.5	tricarballylate	17, 15
Malate	34 ± 1.5		

showing little or no effect on fluorescence (isocitrate, maleate, α,β-dimethylsuccinate) have been shown to be relatively poor inhibitors of [^{14}C]succinate transport [2]. Fig. 6 (open circles) demonstrates the relationship between fluorescence and the rate of uptake of succinate in brush border vesicles (uptake calculated from data in Ref. 2). The other data points in Fig. 6 represent the fluorescence responses of the substrates listed in Table I as a function of their predicted rates of uptake by the succinate-citrate pathway, assuming that the inhibitory constants measured by Wright et al. [2] represent the Michaelis constants for transport of these compounds via the same pathway (V values used for these calculations assumed the V value for the tested dicarboxylates was equal to that measured for succinate, while that for the tricar-

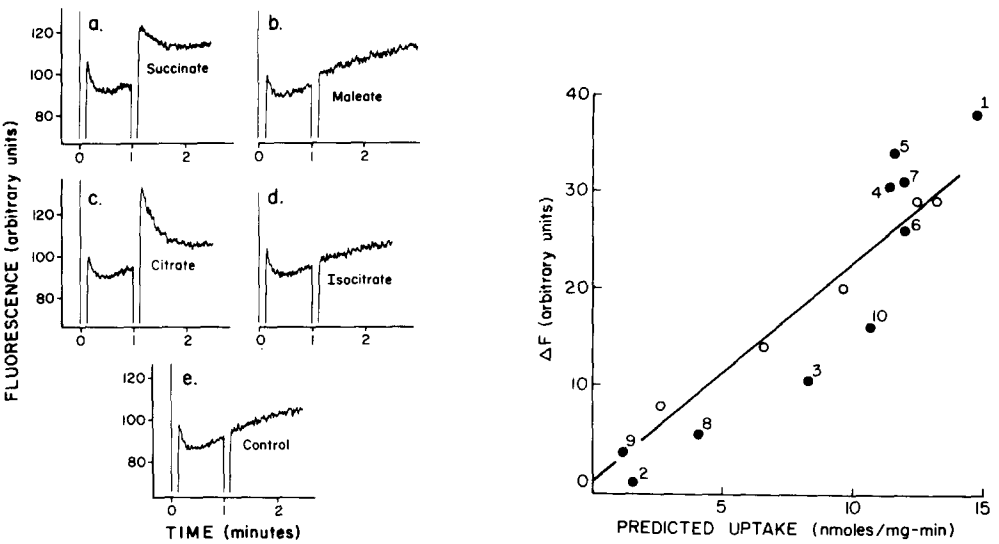

Fig. 5. Specificity of the substrate-induced fluorescence response of diS-C_3-(5). Experimental conditions as in Fig. 2d, except the final membrane protein concentration was 0.27 mg/ml. (a) Effect of 1 mM succinate; (b) 1 mM maleate; (c) 1 mM citrate; (d) 1 mM isocitrate; (e) control condition produced by addition of 30 μl of buffer. All data shown were obtained from a single membrane preparation.

Fig. 6. Relationship between substrate-induced fluorescence response and uptake of succinate (open circles). Experimental conditions as described in Figs. 2d and 3. Values for ΔF produced by succinate are from Fig. 3. Predicted rates of succinate uptake were calculated from kinetic constants for [^{14}C]-succinate transport in renal brush border membranes reported by Wright et al. [2]. The line is a least-squares regression of the succinate data. Closed circles represent the measured values for the substrate-induced fluorescence response produced by the compounds listed in Table I, plotted as a function of their rates of uptake in renal brush borders predicted from the data in Ref. 2. The concentration of each substrate was 1 mM. Estimates of uptake assumed that the dicarboxylates tested had a V value identical to that of succinate (13.9 nmol/mg per min) and had Michaelis constants equal to their apparent inhibitory constants for succinate transport [2]. The tricarboxylates (citrate, isocitrate and tricarballylate) were assumed to have a V value equal to that for citrate (19.0 nmol/mg per min) and Michaelis constants equal to their apparent inhibitory constants for succinate uptake. The exceptions to these assumptions were citrate, for which the V and K_t values reported in Ref. 2 were used, and α-ketoglutarate, for which the V and K_t values reported in Ref. 1 were used. The number code for the solid circles is: 1, citrate; 2, isocitrate; 3, α-ketoglutarate; 4, malate; 5, fumarate; 6, oxaloacetate; 7, α-methylsuccinate; 8, α,β-dimethylsuccinate; 9, maleate; 10, tricarballylate. All data shown were obtained from a single membrane preparation.

boxylates was equal to that measured for citrate). The relationship between ΔF and the predicted rates of uptake of the various tested substrates is adequately described by the relationship for succinate uptake vs. ΔF. These observations provide evidence that the tested substrates are transported by the brush border vesicles via a common pathway.

Effect of altered membrane conductance on fluorescence

As shown in Fig. 1, and as demonstrated in earlier studies [3,6,7,10—12], changes in fluorescence of diS-C_3-(5) are associated with changes in membrane potential, with increases in fluorescence being a usual response to membrane depolarization. The nature of the increase in fluorescence of membrane/dye suspensions caused by tricarboxylic acid cycle intermediates was examined by studying the effect of altered conductance of the brush border membrane, produced by valinomycin in the presence of equal concentrations of intra- and extravesicular KCl. Fig. 7 presents the results of such an experiment in which Na^+ gradients were produced using either 100 mM NaCl or $NaSO_3CH_2CH_2OH$ (sodium isethionate), with a balanced KCl concentration of 10 mM. Fig. 7a is a control recording showing the fluorescence response of a membrane/dye suspension to the addition of 1 mM succinate in the absence of valinomycin. Typical transient increases in total fluorescence were noted. Fig. 7b shows the effect of 3 μM valinomycin under identical experimental conditions. The increased K^+ conductance produced by valinomycin would presumably reduce the fluorescence response if it were caused by the depolarization of the vesicle membrane. The fluorescence transient observed under

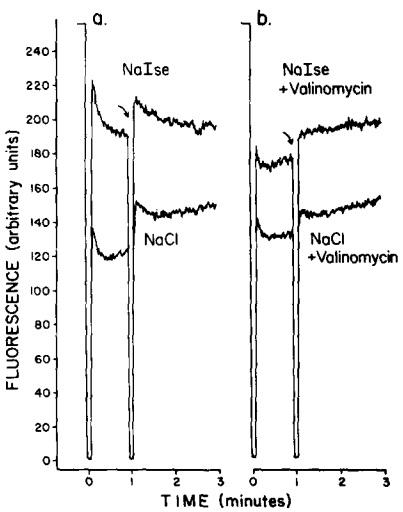

Fig. 7. Effect of valinomycin on the succinate-induced fluorescence response. (a) Fluorescence record produced when vesicles pre-equilibrated in 300 mM sorbitol, 10 mM KCl, 1 mM Tris-Hepes, pH 7.5, were added to a solution containing 10 mM KCl, 100 mM sorbitol, 1 mM Tris-Hepes, pH 7.5, 2 μM diS-C_3-(5), and either 100 mM NaCl (lower trace) or 100 mM sodium isethionate (NaIse) (upper trace); final membrane protein concentration was 0.17 mg/ml. At t = 1 min (arrow) 1 mM succinate was added to the membrane/dye suspension. (b) Experimental conditions identical to those of a, except the solution to which the membranes were added contained 3 μM valinomycin. Fluorescence expressed in arbitrary units. All data shown were obtained from a single membrane preparation.

NaCl-gradient conditions was reduced from 23% ± 1 (S.D., $n = 3$) increase in total fluorescence to 10% ± 2 ($n = 3$) by addition of valinomycin. Valinomycin also reduced the fluorescence response under sodium isethionate-gradient conditions, from a 12 to an 8% increase in total fluorescence. Valinomycin had no effect on the fluorescence response in the absence of K^+ (data not shown). Higher concentrations of KCl, eg., 30 or 75 mM, on top of a 100 mM inwardly directed Na^+ gradient, completely eliminated the fluorescence transient whether or not valinomycin was present, suggesting that under the experimental conditions used in these studies the native K^+ and/or Cl^- conductance was large enough to short-circuit the transient depolarization induced by succinate. In fact, in two preparations studied, 10 mM KCl on both sides of the vesicle membrane was sufficient to eliminate virtually all the succinate-induced fluorescence signal, while two other preparations produced responses comparable to that presented in Fig. 7.

The data presented in Fig. 7 also provide information on the relative permeability of the isolated brush border membrane to the ions, Na^+, Cl^- and isethionate$^-$. Recall that in these experiments, 10 mM KCl was present on both sides of the membranes, thereby permitting us to increase membrane conductance by the addition of valinomycin. When the vesicles were added to the dye solution containing 100 mM NaCl, the fluorescence of the membrane/dye suspension quickly dropped to a minimum value and then increased gradually. This increase in fluorescence indicates a gradual depolarization of the membrane. This observation is explained if it is assumed that the membrane is more permeable to Cl^- than to Na^+; i.e., Cl^- quickly enters the vesicle, resulting in a transient hyperpolarization which gradually decreases as the less permeant Na^+ enters. In the presence of K^+ and valinomycin (Fig. 7b, lower trace), the total fluorescence at the minimum was higher than that noted in the absence of valinomycin, indicating the increase in K^+ conductance produced by valinomycin resulted in a K^+ short-circuit current that reduced the Cl^--supported hyperpolarization. When vesicles were placed in a solution containing 100 mM sodium isethionate (Fig. 7a, upper trace), total fluorescence was greater than in the comparable NaCl conditions (Fig. 7a, lower trace), and showed no transient minimum. Addition of 3 μM valinomycin to the sodium isethionate solution produced a drop in total fluorescence compared to the control situation (upper traces, Fig. 7b vs. 7a, respectively). The latter observation suggests that the brush border membrane is more permeable to Na^+ than to isethionate$^-$. The apparent sequence of ion permeabilities is $Cl^- > Na^+ >$ isethionate$^-$.

Discussion

The transport of succinate and citrate in renal brush border membrane vesicles results in a transient increase in fluorescence of membrane suspensions incubated with the potential-sensitive dye, diS-C_3-(5). The fluorescence response is dependent on Na^+, occurs under either Na^+-gradient or equilibrium Na^+ conditions, and is a saturable function of substrate concentration. The structural analogs of succinate that produce a similar fluorescence response coincide with the structural specificity of the succinate-citrate transport receptor [2]. The observations presented here demonstrate the presence of

a transport pathway in the renal brush border membrane which is specific for the major intermediates of the tricarboxylic acid cycle.

Three observations indicate that the substrate-induced fluorescence response of membrane/dye suspensions is caused by the transport of the added substrate. First, the fluorescence response is Na^+ dependent, and the magnitude of the increase in total fluorescence is greater under Na^+-gradient conditions compared to Na^+-equilibrium conditions. Likewise, the transport of ^{14}C-labeled citrate, succinate and α-ketoglutarate is also Na^+ dependent [1,2], and the rate of transport is stimulated 4—10-fold by Na^+-gradient as compared to equilibrium conditions [1]. Second, the kinetics of the fluorescence response are similar to the kinetics of transport of succinate and citrate. The Michaelis constant for succinate transport in brush border vesicles is 0.11 mM [2], which corresponds quite closely to the concentration of succinate that produced a half-maximal fluorescence response, 0.10 mM. Third, the substrate specificity of the fluorescence response agrees well with the reported structural specificity of the transport receptor. Wright et al. [2] reported that the most effective inhibitors of succinate transport contain a four-carbon dicarboxylate chain in the *trans* configuration, with a limited number of R groups permitted as substituents on the α-carbon of the 'succinate backbone'. Di-substitution of both the α- and β-carbons greatly reduces inhibitory potential. In the present study, the compounds that fullfilled these structural criteria, such as the major tricarboxylic acid cycle intermediates, produced fluorescence responses similar to those observed for succinate and citrate. Substrates such as maleate, isocitrate and α,β-dimethylsuccinate, which are poor inhibitors of succinate transport, also produced little or no fluorescence response. The correlation between fluorescence and predicted uptake (Fig. 6) would not be expected if the tested compounds were not transported by the succinate-citrate transport system.

The transport of sugars and amino acids has also been shown to produce changes in fluorescence of diS-C_3-(5) in certain systems, and these changes have been interpreted as indicating that the transport systems studied are electrogenic [13]. Beck and Sacktor [7], using a preparation of renal brush border membranes similar to that used in the present study, reported that D-glucose transport causes a transient increase in dye fluorescence, and that the response is eliminated by addition of ionophores under appropriate experimental conditions. They explained their results as demonstrating that Na^+ is cotransported electrogenically with glucose across the brush border membrane, and supported this suggestion by demonstrating that D-glucose stimulates $^{22}Na^+$ flux into brush boder vesicles. Laris et al. [3] demonstrated that diS-C_3-(5) provides a reasonable estimate of the membrane potential of Ehrlich ascites tumor cells. They went on to show that the addition of several amino acids produces a rapid depolarization of the cells (i.e., increase in total fluorescence of the suspension).

Three observations suggest that the increase in fluorescence of diS-C_3-(5) in suspensions of renal brush border membranes induced by tricarboxylic acid cycle intermediates is due to a depolarization of the plasma membrane. First, increases in fluorescence are also produced by inwardly directed K^+-diffusion potentials in the presence of the ionophore valinomycin (Fig. 1); second, K^+ + valinomycin reduces changes in fluorescence produced by suc-

cinate (Fig. 7); and, finally, similar fluorescence transients produced by D-glucose [7] are consistent with direct demonstrations of the depolarizing nature of glucose transport in intact cells and tissues (for a review see Ref. 14).

It has not escaped our attention that it is difficult to reconcile the apparent depolarization of the brush border membrane with current views about the mechanism of Na^+-dependent transport of the tricarboxylic acid cycle intermediates. In the case of sugars and amino acids, changes in membrane potential are accounted for by the cotransport of the neutral organic solute with Na^+. The tricarboxylic acid cycle intermediates, which are polyvalent anions (Z = −3 for citrate, −2 for succinate), are also transported by an Na^+-cotransport system [1,2] in which about two Na^+ are transported with each citrate molecule [15]. Thus, even with the cotransport of two Na^+, citrate transport should by hyperpolarizing, while succinate transport should be electroneutral. Small differences between the fluorescence transients produced by citrate and succinate have been noted (Fig. 2), and these may be a reflection of the difference in net charge between the two solutes; nevertheless, each solute produces an initial depolarization (Fig. 2). Currently, we are investigating further the difference in the fluorescence responses between citrate and succinate, and we are attempting to discriminate between depolarization caused directly by transport processes and depolarizing event(s) secondary to transport.

In conclusion, the potential-sensitive dye, diS-C_3-(5) provides a valuable assay for demonstrating the presence of a brush border transport system specific for tricarboxylic acid cycle intermediates. Further work will be required to determine if the apparent depolarization of the brush border membrane is a direct result of the transport process or is secondarily related.

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