Transport System ASC for Neutral Amino Acids

AN ELECTRONEUTRAL SODIUM/AMINO ACID COTRANSFER SENSITIVE TO THE MEMBRANE POTENTIAL*

(Received for publication, September 25, 1991)

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The influx of L-threonine through system ASC does not influence the membrane potential in cultured human fibroblasts although comparable fluxes of amino acids through another Na+-dependent agency, system A, effectively depolarize the cells. The membrane potential, however, stimulates the influx of amino acids through system ASC with a maximal effect at -50 mV. The sensitivity of amino acid influx through system ASC to the membrane potential is not constant, but rather, is dependent on intracellular and extracellular concentrations of the substrates, Na+ and amino acids, of the system. Conditions which favor the loading of the ASC carrier at the external surface reduce the sensitivity of ASC-mediated amino acid influx to the membrane potential; in contrast, the sensitivity of this amino acid influx increases under conditions which favor loading of the carrier at the internal surface. Trans-stimulation, a well known characteristic of system ASC, also varies with the concentrations of the substrates of the system and, in fact, this characteristic is not observed when external Na+ is low. These data may be accommodated by a model in which an electrically silent mode of operation of the transporter is dominant. The influence of the membrane potential on the transport system is dependent on the extent to which a charge-translocating step in the cycling of the carrier is rate limiting (relative rate limitance).

Two major systems, A and ASC, mediate the Na+-dependent transport of neutral amino acids in vertebrate cells (1). A number of differences in the operational features of these two systems are well known. System A accepts N-methylated substrates (2), is inhibited by amino acids in the trans compartment (3), is adaptively regulated (4), and is sensitive to changes in extracellular pH (5). On the other hand, system ASC does not accept N-methylated substrates (2), is trans-stimulated (3, 6), is unaffected by adaptive regulation (7), and is relatively unaffected by changes in extracellular pH (8).

A detailed knowledge of the bioenergetic features of the transport process has been achieved only for system A which is a secondary active transport mechanism dependent on the Na+ electrochemical potential gradient existing across the plasma membrane (9). Only limited and incomplete information has been obtained on the bioenergetics of system ASC although this agency is present in a number of cells (10) and, in fact, constitutes the major route for neutral amino acids in some mammalian models (11, 12). For example, it is known that Na+ is cotransported with amino acids through the system, but the stoichiometry of the cotransport is reported to be variable (13). A model for the system proposed by Christensen (13) describes the operation of the system as an obligatory Na+-amino acid exchange with no net flux of either substrate. This model would imply that the system is electro-neutral, but no direct assessment of the rheogenicity of system ASC has been reported thus far. The membrane potential, however, is reported to influence the transport activity of system ASC in cultured fetal human fibroblasts (14) but not in Ehrlich ascites tumor cells or human erythrocytes (15).

In view of the uncertainties about the bioenergetics of system ASC, it was decided to reinvestigate the question of the influence of the membrane potential on the system and to extend the investigation so as to include a study of the rheogenicity of the system. Advantage was taken of the recent finding that at a concentration of 50 μM L-threonine behaves as a nearly specific substrate of system ASC in cultured human fibroblasts (16). Therefore, in these cells the influx of 50 μM L-threonine could be used as a reliable transport probe of system ASC even in the absence of inhibitors of other amino acid transport systems (16).

As a result of the investigations reported here, we have found that the operation of system ASC is not rheogenic under the conditions studied and that it is influenced by membrane potential. A model for the operation of the system has been developed that follows the general scheme of Heinz et al. (17). According to this model the influence of the membrane potential on a transport system is dependent on the extent to which a charge-translocating step in the cycling of the carrier is rate limiting for the entire transport process (relative rate limitance).

EXPERIMENTAL PROCEDURES

Cell Culture—Human foreskin fibroblasts were obtained from a 15-year-old donor and routinely grown in 10-cm diameter dishes (Falcon) in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS). The conditions of culture were as follows: pH 7.4, atmosphere 5% CO2 in air, temperature 37 °C. All experiments were made on fibroblast subcultures resulting from 4 × 105 cells seeded onto 2-cm2 wells of disposable 24-well trays (Nunc) and incubated for 3 days in 1 ml of growth medium. The culture medium was always renewed 48 h before the experiment. Cells were used at a...
density of \(35 \pm 5 \, \mu g\) of protein/cm\(^2\).

Incubations—Cell monolayers were washed twice in a modified Eagle's balanced salt solution (EBSS), containing 116 mM NaCl, 20 mM HEPES, 5.4 mM KCl, 1 mM NaH\(_2\)PO\(_4\), 0.8 mM MgSO\(_4\), and 5.5 mM glucose and buffered at pH 7.4 with 1 mM Tris. Monolayers were then incubated at 37°C for the desired time in EBSS modified as required by the experimental design. In these media, the sum of \([Na^+]\), \([K^+]\), and N-methyl-D-glucamine or choline was kept constant at 140 mM.

Cells described in the text below as replete were kept in Dulbecco's modified Eagle's medium until immediately before the flux measurement. Cells described as depleted were incubated in EBSS for specified periods of time before the flux measurement. Cells referred to as threonine-loaded were first depleted of amino acids for a given period of time and then incubated with high concentrations of L-threonine for 10 min prior to the flux measurement.

All the assays of amino acid transport were performed using the cluster-tray method (18) with which it is possible to treat the 24 wells of the multidish simultaneously. Incubations were terminated by rapidly rinsing the cell monolayers with ice-cold 0.1 M MgCl\(_2\). The cells were then fixed in place by the addition of ice-cold ethanol (0.1 ml). Ethanol was allowed to dry, and the soluble pool was extracted in 2 ml of 10 mM CsCl in water for measurements of radioactivity and/or Na\(^+\). Cell monolayers were then dissolved with 0.5% sodium deoxycholate in 1 mM NaOH and assayed for protein content directly in the well using a modified Lowry procedure (18).

Fluorometric Assessment of Qualitative Changes in the Membrane Potential—Changes in membrane potential were detected as changes in the fluorescence intensity of the dye bis-1,3,5-diethylthiobarbiturate trimethineoxonol (bis-oxonol), monitored with a LS-50 Perkin-Elmer spectrophotometer. For these measurements cells were grown on a coverslip which was put into a cuvette containing EBSS at 37°C with constant stirring. Dyebas was added from an ethanol stock solution to a final concentration of 100 nM. Fluorescence was recorded with excitation and emission at 530 nm (5-nm slit width) and 570 nm (7.5-nm slit width), respectively. Since the baseline fluorescence varied with each coverslip, the data are reported as percent of the total change from the baseline after the addition of \(1 \mu M\) gramicidin D employed to achieve complete cell depolarization. This percentage change is calculated from the expression:

\[
\Delta F\% = \frac{F_2 - F_1}{F_2 - F_0} \times 100
\]

where \(F_1\) is the fluorescence measured after the addition of the amino acid to be tested, \(F_2\) is the fluorescence at baseline, and \(F_0\) is the fluorescence after the addition of gramicidin D. \(\Delta F\%\) is hereafter defined as "relative change in fluorescence." In the example shown in Fig. 1, the change in fluorescence detected upon the addition of L-proline at a concentration of 5 mM is of 32 arbitrary units (A.U.), while the change detected after the addition of gramicidin is of 126 A.U., for a \(\Delta F\%\) of 25%. A calibration curve in which membrane potential was measured with 0.5% sodium deoxycholate at 37°C with constant stirring was employed to establish the relationship between percent change in fluorescence and the membrane potential.

Calculations—The data of amino acid fluxes were expressed as micromoles or nanomoles per ml of intracellular water per min.

Materials—Dulbecco’s modified Eagle’s medium was purchased from GIBCO. Bis-oxonol was obtained from Molecular Probes (Eugene, OR). 3-O-Methyl-D-[\(U-^{14}C\)]glucose (300 Ci/mmol) was obtained from Du Pont, Bad Homburg, Germany. L-[\(5-^{3}H\)]Proline (43 Ci/mmol) and L-[\(5-^{3}H\)]threonine (19 Ci/mmol) were purchased from Amersham International, Little Chalfont, England. Ethanol was obtained from Carlo Erba-Farmitalia, Milan, Italy; 2-methylaminoisobutyric acid from Aldrich Chemical Co. Sigma was the source of all other chemicals.

RESULTS

Electroneutrality of ASC-mediated Influx of L-Threonine—Simultaneous measurements of the entry of L-threonine and of the influence of this influx on the membrane potential were made on parallel cultures of cultured human fibroblasts. Since L-threonine can enter human fibroblasts through both systems A and ASC, it is necessary to distinguish between the two entry routes in evaluating the effect of threonine influx through system ASC on the membrane potential. In order to make this distinction, two sets of cells were employed in which the relative contribution of the two transport systems was varied. One set consisted of replete cells in which the activity of system A was low due both to adaptive regulation (4) and trans inhibition (3). A second set of cells was depleted by preincubation for 3 h in EBSS so as to enhance the activity of system A through both the reduction of trans inhibition (3) and the triggering of the adaptive increase (4). Two concentrations of L-threonine, which roughly correspond to the \(K_c\) of the amino acid for the two transport agencies (16), were employed in each condition. To determine the contribution of each system to threonine influx, parallel measurements of threonine influx were made in the absence or in the presence of a saturating concentration of MeAIB, the characterizing analogue of system A.

As seen in Table I influx of 0.05 and 2 mM threonine in replete cells and with 0.05 mM in depleted cells was not inhibited by 10 mM MeAIB indicating that under these conditions influx occurred mostly through system ASC and not through system A. In these three conditions, L-threonine influx was not associated with significant changes in membrane electrical potential. On the contrary, in depleted cells the influx with 2 mM threonine caused a marked depolarization and was appreciably MeAIB-sensitive, indicating that under this condition some of the influx of the amino acid occurred through system A. It should be emphasized that although the influx of L-threonine at 2 mM in replete cells was 5-fold larger than that with depleted cells, no depolarization or MeAIB sensitivity was observed in the replete cells.

![Graph showing changes in bi-oxonol fluorescence upon addition of L-proline and gramicidin D](http://www.jbc.org/fig.jpg)
Experimental Procedures; after a stable signal had been attained, L-
performed using 0.1 mM L-proline as a substrate for system
through systems A or ASC on the membrane potential was
fluorescent indicator bis-oxonol was added as described under “Ex-
cally neutral process.

threonine influx should have been readily observable since

These measurements, therefore, indicate that the entrance of
L-threonine through system ASC is predominantly an electri-

A direct comparison of the influence of amino acid fluxes
through systems A or ASC on the membrane potential was
performed using 0.1 mM L-proline as a substrate for system
A (3) and 0.05 mM L-threonine as a substrate for system ASC
(16). The results, shown in Fig. 2, indicate that the addition
of the substrate of system A produced a ΔF% of 4% while no
significant change in fluorescence, i.e. in membrane potential,
was detected after addition of L-threonine. Net influx, meas-
ured in parallel cultures, was 95 nmol/ml/min for L-proline
and 135 nmol/ml/min for L-threonine. If the operation of
system ASC were comparable, in terms of electrogenicity, to
that of system A, changes in the membrane potential with L-
threonine influx should have been readily observable since
the flux through system ASC was larger than that through
system A.

Sensitivity of System ASC to Membrane Potential: Effect of
the Extracellular Substrates—The sensitivity of L-threonine
influx to the membrane potential was evaluated at different
extracellular threonine concentrations. Cell depolarization,
caused by an increase of [K+],, inhibited L-threonine uptake
to a greater degree at lower extracellular threonine concen-
trations (Table II). At 2 μM extracellular L-Thr the influx of the
amino acid was decreased by 41% when [K+] was increased
from 5 to 70 mM; the same change in external [K+] shut
down the influx of 320 μM L-Thr by only 8%. The effect of
cell depolarization on threonine influx was greater at lower
extracellular threonine concentrations also in depleted cells
(data not shown). A kinetic analysis demonstrated that cell
depolarization caused a 33% increase in the Kₘ of system
ASC for L-threonine (Fig. 3).

The influence of the membrane potential on L-threonine
influx was also examined at two concentrations of extracel-
lar Na⁺, 5 mM and 50 mM, both in replete cells and in cells
depleted for 3 h (Table III). Since at 50 μM L-Thr the half-
maximal activation of amino acid influx occurs at [Na⁺] <
20 mM (see Ref. 16, Fig. 5), the system is more than 80%
saturated at 50 mM [Na⁺] and only 20% saturated at 5 mM
[Na⁺]. The membrane potential was altered by varying extracellular K⁺ from 1.5 to 100 mM. Cell depolarization
inhibited threonine influx both in replete cells and in cells
depleted for 3 h. The percentage decrease with depolariza-
tion was greater with 5 mM than with 50 mM [Na⁺] with both
types of cells.

These data indicate that the influx of threonine was more
sensitive to the membrane potential when the loading of the
carrier at the external surface was reduced. A comparison of
fluxes in the replete and depleted cells also demonstrates that
while trans stimulation of L-threonine influx was readily
observed at 50 mM [Na⁺] in the absence of 2 μM extracellular L-Thr, it was not apparent at 5 mM [Na⁺] in the presence of MeAIB (Table III).

Sensitivity of System ASC to Membrane Potential: Effect of
the Intracellular Substrates—The influence of the membrane

| Condition | [L-Thr] | Influx of L-Thr | Total | MeAIB-sensitive | ΔF% |
|-----------|--------|----------------|-------|-----------------|-----|
| Replete   | 0.05   | 669 ± 38       | 0     | 0               | 0   |
| Replete   | 2.0    | 2664 ± 121     | 0     | 0               | 0   |
| Depleted  | 0.05   | 280 ± 18       | 0     | 0               | 0   |
| Depleted  | 2.0    | 1080 ± 87      | 535   | 17              |     |

**TABLE II**

Effect of extracellular threonine concentration on the sensitivity of the influx of L-threonine to membrane potential

Cultured human fibroblasts were washed twice with EBSS, and the influx of L-threonine was determined immediately. During the transport assay, membrane potential was changed by altering external [K⁺] as indicated. Values are shown ± S.D. (n = 3).

| [Thr] | [K⁺]₀ₓ = 5 mM | [K⁺]₀ₓ = 70 mM | Inhibition |
|-------|--------------|---------------|-----------|
| μM    | nmol/ml/min  | nmol/ml/min   | %         |
| 2     | 39.4 ± 2.9   | 23.2 ± 4.3    | 41*       |
| 320   | 1672 ± 57    | 1536 ± 79     | 8*        |

* p < 0.01; NS.

**FIG. 3.** Kinetic analysis of L-threonine influx at 5 and 70 mM [K⁺]₀ₓ. Replete cultured human fibroblasts were washed twice with EBSS and 30-s influx of L-threonine was determined immediately at two different [K⁺]₀ₓ, 5 mM (●) and 70 mM (○) with [Na⁺]₀ₓ kept constant at 50 mM. Points are means of three independent determinations. Kinetic parameters for L-threonine influx, determined with a least-squares, nonlinear regression analysis, are: for [K⁺]₀ₓ = 5 mM, Vₘₕ = 2307 ± 40 nmol/ml/min, Kₐ = 121 ± 5 μM; for [K⁺]₀ₓ = 70 mM, Vₘₕ = 2312 ± 94 nmol/ml/min, Kₐ = 162 ± 14 μM.
TABLE III
Effect of extracellular [Na+] on the sensitivity of the influx of L-threonine to membrane potential

Cultured human fibroblasts were washed twice with EBSS. The influx of L-threonine was determined either immediately (replete cells) or after a 3-h incubation in EBSS supplemented with 10% dialyzed FBS. During the transport assay, membrane potential was changed by altering external [K+] Values are shown ± S.D. (n = 3).

| Condition | [Na+] | L-Thr influx | Inhibition |
|-----------|-------|--------------|-----------|
|          | mM    | n mole/ml/min | %         |
| Replete  | 5     | 296 ± 22     | 197 ± 12  | 33*      |
| Replete  | 50    | 961 ± 15     | 741 ± 9   | 25*      |
| Depleted | 5     | 270 ± 27     | 197 ± 7   | 27*      |
| Depleted | 50    | 680 ± 37     | 605 ± 21  | 11*      |

*p < 0.01.  
*p < 0.05.

TABLE IV
Effect of intracellular Na+ and amino acids on the sensitivity of L-threonine influx to membrane potential

Cultured human fibroblasts were incubated for 24 h in EBSS supplemented with 10% dialyzed FBS. Intracellular [Na+] and amino acids were then altered with a 10-min incubation in either EBSS supplemented with 1 mM L-threonine or in a Na+-free medium (N-methylglucamine substituting for Na+) supplemented with 20 mM L-threonine. Influx was measured in EBSS either in the presence or in the absence of 1 μM gramicidin D. Gramicidin D was added 2 min before the transport assay. The values of [Na+] are those measured at the beginning of the 30-s influx of L-Thr. In these conditions, preaccumulated L-threonine had reached a concentration of 16.5 ± 1.49 mM and 14.7 ± 1.77 mM in cells incubated in the presence or in the absence of sodium, respectively. Values are shown ± S.D. (n = 6).

| [Na+] | Preincubation | Influx of L-threonine | Inhibition |
|-------|--------------|-----------------------|-----------|
| m M   | n mole/ml/min | %                     |
| 16.5  | +            | 2331 ± 569            | 1410 ± 137| 40*      |
| 5.9   | +            | 1383 ± 111            | 1206 ± 29 | 13*      |
| 16.6  | -            | 875 ± 89              | 635 ± 29  | 27*      |
| 4.3   | -            | 696 ± 53              | 657 ± 26  | 6*       |

*p < 0.01.  
NS.

The effect of membrane potential on the influx of L-threonine at different potentials (Fig. 4). For this experiment, cells were incubated with L-Thr (10 mM) for 10 min before the influx assay in order to create conditions for maximal sensitivity of the influx to membrane potential (see Table IV). The membrane potential was changed in a graded manner by varying [K+] in the presence of 50 μM valinomycin. Values of membrane potential have been calculated from the distribution ratios of L-arginine (20) obtained in parallel cultures incubated in the same conditions. At values of membrane potentials more positive than −50 mV the activity of system ASC decreased. This result indicates that the membrane potential stimulates the influx of amino acids through system ASC and that the stimulatory effect of membrane potential appears to be maximal at approximately −50 mV.

DISCUSSION

The results reported in the present study demonstrate that under the conditions employed here the influx of L-threonine through system ASC is largely electrically neutral. In contrast, comparable influxes of amino acids through system A with the same conditions are electrogenic and depolarize the cells. Assuming that the change in the membrane potential is directly proportional to the influx of sodium coupled with amino acid through system A and that a 2% relative change in fluorescence would be readily observable, then the data reported in Table I indicate that at least 97% of the influx of L-threonine at 2 mM in replete cells and 77% of the influx of L-threonine at 0.05 mM in depleted cells must be electrically neutral. Hence, although both systems A and ASC mediate coupled inward fluxes of Na+ and amino acids (22), they differ in regard to the net movement of charge during the transport cycle, i.e., there is a net movement of charge with amino acid transfer through system A but not through system ASC. Since the entrance of an amino acid through system ASC into cultured human fibroblasts is known to be accompanied by Na+ with a stoichiometry of 1:1 (22), electrical neutrality must be achieved by the inward flux of an anion or the outward movement of a cation during a single cycle of the transporter.
Two lines of evidence favor the suggestion that the cycle requires the outward movement of sodium. First, the experiments described here show that L-threonine influx is decreased by lowering intracellular [Na⁺]. Second, Wheeler and Christensen (23) have described stimulation of sodium efflux by extracellular amino acids through system ASC. These trans effects are best accomodated by the hypothesis that the operation of system ASC consists mainly of a Na⁺/amino acid exchange. If system ASC does operate in this fashion, then the electrical potential difference across the plasma membrane cannot act directly as a driving force for the transport process, and the system cannot concentrate amino acids in the cell. Our observations, however, do not preclude some movement of the empty carrier. If the free carrier can make the transit, the membrane potential would energize the system which could then be concentrative. Until measurements are made of transit through the system in the absence of trans substrates, it is not possible to ascertain the relative movements of loaded and unloaded carriers. The trans stimulation exhibited by this system, however, indicates that the loaded carrier returns to the exterior more readily than the unloaded carrier.

Although there is apparently no net movement of charge during a complete transport cycle of the carrier bearing Na⁺ and amino acid, the influx of L-threonine is influenced by the membrane potential. These two observations may be reconciled for reasons discussed by Glynn and Karlisch (24) for a pump and by Grygorczyk et al. (25) for the "electrically silent" anion exchanger of the red blood cell. According to these authors the rate of a step-carrying charge through an electric field must be sensitive to voltage. If that step is rate-limiting, the overall transport process will also be sensitive to the voltage even if the overall transport process is not electronegenic. On this premise, the dependence of the operation of the electroneutral system ASC on the membrane potential was tested following the strategy developed by Heinz et al. (17) for studying the variable dependence upon membrane potential exhibited by Na⁺/glutamate cotransport in rabbit renal brush-border membranes. These authors proposed that dependence of a flux on the membrane potential is influenced to the extent that a charge-translocating step is rate-limiting (relative rate limitation). As a consequence, the sensitivity of a transport process to the membrane potential can be modulated by procedures, such as the availability of substrates, that change the relative rate limitation of a charge-carrying step in the overall transport process.

In the case of the cotransport of sodium and amino acids via system ASC, charge translocation could conceivably occur either when a neutral carrier makes a transit bearing Na⁺ (Fig. 5, top) or a negatively charged carrier makes a transit without loading with Na⁺ (Fig. 5, bottom). If either of these two possible transits is rate-limiting in the transport cycle, the general pattern of the data discussed here would be readily explained; indeed the depolarization should retard the influx of a positively charged loaded carrier in the former case or decrease the number of carriers available at the external surface by reducing the efflux of negatively charged, unloaded carriers in the latter case. Two observations, however, argue against the latter model (Fig. 5, bottom). First, under the conditions of the experiments described here the rheogenicity of the flux is negligible, and thus the percentage of the carriers that move across the membrane in the unloaded form is small. Hence it is difficult to see how the return of negatively charged, unloaded carriers would be rate-limiting since most of the carriers would be in the loaded, neutral, more rapidly moving form. Second, if the rate-limiting transit which moves charge is the efflux of the unloaded carrier, then a reduction of the loading of the carrier at the internal surface should increase the potential sensitivity of this movement. As a consequence of this change in sensitivity, the influx which depends on the availability of carrier at the external surface should also become more potential-sensitive. The experiments here, however, demonstrate that the potential sensitivity of the influx decreases when internal Na⁺ and/or amino acids are reduced. On the other hand, the data are well accomodated by the former model (Fig. 5, top) in which the loaded carrier bears net positive charge. If the inward movement of the fully loaded carrier transfers net positive charge and is rate-limiting, the reduction of either external Na⁺ or amino acids should not only reduce influx, but it should also make the influx more sensitive to the membrane potential, because the influx would be now relatively more rate limiting. In the same model, the reduction of either internal Na⁺ or amino acids should make the influx less sensitive to the membrane potential because the influx is now relatively less rate-limiting. These predictions match the observations reported here and hence we favor this model although the data presented are not sufficient to exclude other operative models for system ASC, such as those in which the membrane potential influences binding of substrates rather than transit steps in the transport cycle.

The model proposed here for system ASC in cultured human fibroblasts is consistent with previous interpretations of the operation of the system in red blood cells (13, 23). Moreover, the same model can account for the differences reported previously about potential dependence of system ASC. Valdeolmillos et al. (15) reported that this system was insensitive to membrane potential in the Ehrlich ascites tumor cell and in the human erythrocyte. These investigators preincubated the Ehrlich cells in the absence of external amino acids for 30 min at 37 °C, a procedure which can reduce markedly the

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**Fig. 5. Models for the operation of system ASC.** It is assumed that transit of the carrier is possible only in the fully loaded and empty forms. See "text" for a discussion of the two models.
amino acid pool in these cells. According to the results presented here, this reduction in intracellular amino acids together with conditions favoring the loading of carriers at the external surface (high [Na+]_{out} and relatively high extracellular amino acid) could have rendered the system insensitive to the membrane potential. As for the human erythrocyte, the amino acid pool in this cell is not large and, again, favorable conditions for loading at the external surface could explain the lack of sensitivity to the membrane potential.

The arguments introduced here to explain the variability described for potential dependence of system ASC can also be extended to other operational features of the system, such as trans stimulation. Indeed, no trans stimulation by internal amino acids is detected when the influx is markedly reduced by low availability of substrates (i.e. at very low [Na+]_{out}). This result indicates that the effect of intracellular substrates on influx is dependent on the extent to which the influx is not limiting. In other words, trans stimulation of the influx is detected only in conditions in which the efflux of the carrier exhibits an appreciable relative rate limitation. Thus, the data presented here also point out that certain properties of transport systems, such as trans stimulation and sensitivity to the membrane potential may not be constant features of the system but may depend, in fact, on the conditions presented to the system.

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J. Biol. Chem. 1992, 267:8330-8335.

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