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The effect of the intracellular sodium level on the activity of amino acid transport systems L and A in SV40 3T3 cells

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The rate of transport of phenylalanine and leucine, pertinent amino acids of System L, has been measured in SV40 3T3 cells as a function of the presence of Na^+ ions during the reloading phase that precedes the influx determination. The presence of Na^+ ions during the reloading phase resulted in an increase of the subsequent substrate influx through System L. This effect was related to the intracellular Na^+ level and was found to be independent by the presence of a chemical sodium gradient outside-inside during influx determination; furthermore, this effect could not be ascribed to a difference between control and Na^+-treated cells in the internal levels of those amino acids that participate in the exchange phenomena of transport System L. The transport of phenylalanine appeared to have the ability to accept Li^+ for Na^+ substitution in the *'trans'* position. The presence of Na^+ ions in the *'trans'* position was not required to optimize the transport of System A-reactive substrates, whose influxes are dependent on the presence of the cation in *'cis'* position. Analysis of the relationship between influx and substrate concentration indicated that the Na^+-dependent increase of substrate influx was associated with an enlarged capacity of the high-affinity component of transport System L.

Introduction

At least three independent systems participate in the transport of neutral amino acids across the cell membrane in animal cells [1]. Two of them. Systems A and ASC, are known to be Na^+-dependent, whereas for System L there is a broad consensus on its Na^+-independent property [1,2]. Na^+-independence, substrate specificity toward amino acids with apolar branches or rings on the side chain and strong exchange properties are the main characteristics consistently associated with the activity of System L. However, the Na^+-independence is widely regarded as the most striking property differentiating System L from other transport systems for neutral amino acids.

The Na^+-independence of System L was first described by Oxender and Christensen [3] and in a more detailed study by Inui and Christensen [4] in Ehrlich ascites cells. Since then, a large number of reports have described the activity of System L in different cell types, using different experimental conditions such as Na^+-absence or Na^+-presence during uptake determinations and employing undepleted or depleted cells as well as cells reloaded with specific substrates of System L. An analysis of these studies reveals some results that question the Na^+-independence of System L. For instance, Jacquez et co-workers [5] found that in Ehrlich ascites cells 60–65% of the influx of phenylalanine required Na^+; and McClellan and Schafer [6] re-

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ported that uptake of BCH, an amino acid analogue regarded as a pertinent substrate of System L, was inhibited by minimum of 60% when Na^+ in the medium was replaced by other cations. However, a plausible interpretation of these results suggests that the upake of phenylalanine and BCH is heterogeneous, and then it might occur to some degree by Na^+-dependent pathways.

The presence of two distinct Na^+-independent transport systems for neutral amino acids has recently been reported in primary culture of rat hepatocytes [7]. By kinetic analysis we have shown that two saturable components of transport System L contribute to the entry of neutral amino acids in SV40 3T3 cells. Furthermore, the relation between the activity of Na^+-independent components for the transport of neutral amino acids and the internal concentration of substrates available for exchange has been described in detail [8]. Initial velocity transport determinations have been performed in the absence of Na^+ ions in cells depleted and/or reloaded with pertinent substrates [8]. During that investigation we noted in certain circumstances variable results for leucine or phenylalanine uptake. This variability was found to be dependent on the presence or absence of Na^+ ions in the reloading phase, but independent of the presence of Na^+ during influx determination.

Here we present evidence that the presence of Na^+ ions during the reloading phase is essential for the optimal activity of the high-affinity component of amino acid transport System L. This effect is related to the intracellular Na^+ level and is independent of the presence of the chemical Na^+ gradient outside-inside. Furthermore, the requirement for the presence of Na^+ in the intracellular compartment ('*trans*' position) to optimize substrate influx does not appear to be a property shared by transport System A.

Experimental

Materials

L-[4,5-^{3}H]Leucine, L-phenyl[2,3-^{3}H]alanine, L-[5-^{3}H]proline and 3-*O*-methyl-D[1-^{3}H]glucose were obtained from Amersham International, U.K. 3-*O*-Methyl-D-glucose and unlabelled amino acids were purchased from Sigma, St. Louis, MO, U.S.A., except for 2-methyl-aminoisobutyric acid obtained from Aldrich-Europe, Beerse, Belgium. Media, salt mixture and fetal calf serum for cell culture were purchased from GIBCO, Grand Island, N.Y., U.S.A.

Cell culture

Monolayers of simian virus-40 (SV40) transformed Balb/c 3T3 cells (SV40 3T3) were maintained in culture and propagated as previously described [7,8]. Cells were usually passaged twice a week.

Incubation and influx assay

The measurement of amino acid influx in cell monolayers was essentially as previously described [8].

In all the experiments described in this work a preincubation step lasting 60 min in Earle's solution with 1% fetal calf serum to reduce the intracellular levels of amino acids, was employed (depletion phase). Following the depletion phase, in most of these experiments a reloading phase preceded the uptake determination. During the reloading phase cells were incubated in Earle's solution (Na^+-containing medium) or in Na^+-free Earle's solution (where choline replaced Na^+ in the Earle's mixture) and in the presence of the amino acid under study. Amino acid uptake was assayed under conditions approaching initial velocity (1 min) in a Na^+-free Earle's solution as previously described [8].

Protein content, cell volume measurements and calculation

Cell monolayers were dissolved in 0.5 M NaOH and an aliquot was taken for protein determination following the method of Lowry et al. [10] using bovine serum albumin as standard.

The volume of the cells still attached to the substratum was measured by the method described by Erlinger and Saier [11] with slight modifications and using 3-*O*-methyl-D-glucose as an intracellular water space marker. By this method a value of 5.85 ± 0.6 μl of cell water per mg of cell protein was obtained, an estimate that is close to the values obtained previously with the same cell line by a completely different procedure [12].

Rates of substrate uptake, expressed as μmol

per ml of intracellular water ± S.D. of the mean, and kinetic parameters of amino acid transport were calculated as previously described [8].

Determination of intracellular Na^+ and Li^+ level

The procedure described by Owen and Villereal [13] was used with slight modifications. At the end of depletion step and/or after the reloading phase, cell monolayers (9 cm^2) were quickly washed four times with 5 ml ice-cold 0.1 M $MgCl_2$. The cells were then extracted with 2 ml 5% trichloroacetic acid. This procedure was completed in less than 12 s. The cell extract was then clarified by centrifuging at $3000 \times g$ for 10 min and the supernatant, after suitable dilution, was analyzed for its sodium of lithium content in a Varian atomic absorption spectrometer model A275.

Results

Effect of sodium presence during the reloading phase on phenylalanine transport

The activity of transport System L is closely related to the intracellular level of pertinent amino acids as phenylalanine, leucine or BCH that participate in exchange phenomena. When the intracellular amino acid level has been artificially altered by experimental procedures causing depletion (incubation in amino acid-free medium) or accumulation of amino acids (incubation in amino acid-enriched medium) the activity of transport System L decreased in depleted and increased in reloaded cells, respectively [8].

The presence of Na^+ during the depletion step does not appreciably affect the subsequent phenylalanine uptake. On the contrary, the presence of Na^+ during the reloading phase that follows depletion, significantly increases the phenylalanine transport. Similar results have been obtained with leucine, another preferred substrate of System L in these cells [12].

Effect of intracellular phenylalanine level on the sodium mediated increase in transport

We have previously reported that the influx of phenylalanine in SV40 3T3 cells appeared to be related to the internal level of amino acids available for exchange up to a definite concentration, beyond which transport activity did not parallel a further increase in internal substrate level [8]. The possibility that the increased phenylalanine uptake seen after reloading cells in the presence of Na^+ could be ascribed to different internal levels of exchangeable substrates was analyzed in detail. Indeed, cells reloaded in the presence of Na^+ accumulated phenylalanine to higher levels than cells reloaded in the absence of Na^+ [8]. Fig. 1 shows the initial entry of phenylalanine as a function of the apparent molarity of the reloaded phenylalanine. The data presented in this figure, however, show that whatever the molarity of internal substrate, the presence of Na^+ during the reloading phase significantly increases the initial rate of phenylalanine uptake. Therefore, this effect appears to be related to the cation itself and cannot be ascribed to a difference between control and Na^+-treated cells in the internal levels of those

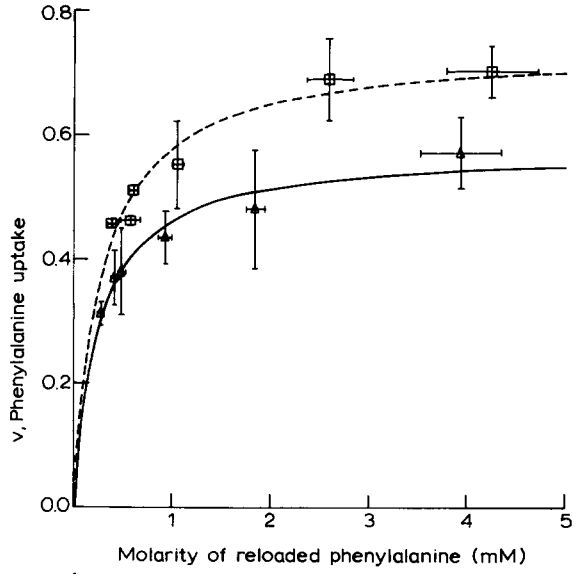

Fig. 1. Transport of phenylalanine as a function of the molarity of the reloaded substrate. SV40 3T3 cells were depleted of intracellular amino acids by a 60 min incubation at 37°C in Earle's basal salt solution containing 1% fetal calf serum. Influxes of 0.05 mM phenylalanine (1 μCi/ml) were performed at 37°C in a Na^+-free medium as described in the Experimental section, at the end of a 15 min reloading phase which took place in the presence of different concentrations of unlabelled phenylalanine and in Na^+-free (△) or in Na^+-containing medium (□). The levels of the reloaded phenylalanine were determined at the end of the reloading phase in sister cultures incubated in the presence of labelled phenylalanine. The values are the means of three independent determinations ± S.D.

amino acids that participate in the exchange phenomena.

Effect of extracellular Na^+ on the initial rate of entry of phenylalanine

The presence of extracellular Na^+ during the initial entry of phenylalanine does not affect the transport activity, as shown in Table I. Moreover, one can note that the presence of MeAIB (an amino acid analogue known as a characterizing substrate of the Na^+-dependent System A) does not inhibit the entry of phenylalanine in these cells. These results suggest that phenylalanine is poorly or not at all transported by System A in SV40 3T3 cells and confirm our previous results that is a pertinent substrate of a Na^+-independent transport system [8].

It should be stressed that the presence of the Na^+ chemical potential during uptake did not modify significantly the increase in phenylalanine transport seen after a reloading phase in the presence of Na^+. Therefore, in these cells the role of Na^+ in increasing the influx of phenylalanine appears to be restricted to the reloading phase, its presence being negligible during the depletion step and/or the initial entry of substrate.

TABLE I

EFFECT OF THE PRESENCE OF Na^+ DURING THE INFLUXES OF PHENYLALANINE IN CELLS EXPOSED TO Na^+-FREE OR Na^+-CONTAINING MEDIUM DURING THE RELOADING PHASE

Influxes of 0.1 mM phenylalanine were determined in Na^+-free or Na^+-containing medium at the end of a reloading phase which follows a 60 min depletion step as described in the Experimental section. When present during influx determination, MeAIB was at 20 mM final concentration. The reloading phase lasted 15 min and the concentration of phenylalanine during this period was 2 mM. The values are the means of three independent determinations ± S.D. MeAIB, methylaminoisobutyric acid.

Condition during influx determinations	v, phenylalanine	
	Na^+-free reloading phase	Na^+-containing reloading phase
Sodium presence	0.69 ± 0.07	1.09 ± 0.15
Sodium and MeAIB presence	0.67 ± 0.10	1.06 ± 0.08
Sodium absence	0.65 ± 0.05	1.04 ± 0.15

Effect of intracellular Na^+ level on the initial rate of phenylalanine uptake

Since the increased uptake of phenylalanine has been shown to be independent of the presence of Na^+ in the extracellular medium during measurements of the initial entry rate, the possibility that the effect of Na^+ during the reloading phase could be ascribed to its intracellular presence during phenylalanine uptake has been analyzed. Fig. 2 shows the initial entry of phenylalanine in reloaded cells as a function of the internal Na^+ level. The internal Na^+ concentration has been varied by adding the physiological concentration of Na^+ at different times before uptake measurements. As shown in Fig. 2, it appears that a significant correlation ($r = 0.94$) exists between the rate of phenylalanine transport and the molarity of the intracellular Na^+. Moreover, increasing the internal Na^+ level beyond physiological values by blocking the cation pump with oubain did not

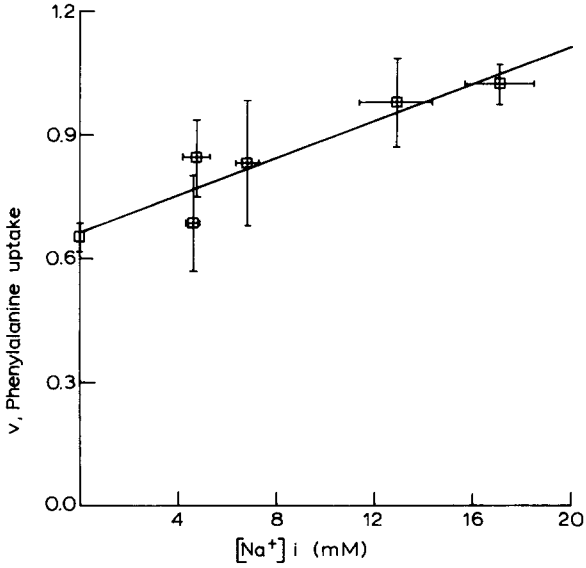

Fig. 2. Transport of phenylalanine as a function of the molarity of the intracellular sodium concentration. Influxes of 0.1 mM phenylalanine were determined at the end the reloading phase as described in the Experimental section. The levels of intracellular sodium were determined as described in the Experimental section in sister cultures at the end of a 15 min reloading phase which took place in the presence of 2 mM phenylalanine. Different levels of intracellular sodium were achieved by exposing the cells during the reloading phase for different periods of time to Na^+-free medium. The values are the means of three independent determinations ± S.D.

TABLE II

TRANSPORT OF PROLINE AS A FUNCTION OF INTRACELLULAR CONCENTRATION OF SODIUM AND SUBSTRATE

Condition during the reloading phase	v, Proline influx in the presence of Na^+	v, Proline influx in the absence of Na^+	Intracellular sNa^+ concentration (mM)	Reloaded proline (μmol/ml)
Sodium presence				
− Proline	0.24 ± 0.03	0.04 ± 0.01	19.2 ± 2.9	-
+ Proline	0.23 ± 0.04	0.04 ± 0.01	25.7 ± 3.5	5.63 ± 0.29
Sodium absence				
− Proline	0.21 ± 0.02	0.03 ± 0.01	4.4 ± 1.2	-
+ Proline	0.27 ± 0.03	0.03 ± 0.01	2.7 ± 0.5	3.28 ± 0.13

Influxes of 0.1 mM proline were determined in Na^+containing or Na^+-free medium as indicated, at the end of a reloading phase which followed a 60 min depletion step (cf., the Experimental section). The concentrations of reloaded proline were determined in sister cultures at the end of a 15 min reloading step in the presence of 0.5 mM proline (Na^+-containing medium) or 4 mM proline (Na^+-free medium, where choline replaced Na^+ in the sodium salt of the mixture). The concentrations of intracellular Na^+ were determined in sister cultures and according to the procedure described in the experimental section. The values are the means of 3–5 independent determinations ± S.D.

appear to increase the activity of transport Systems L further (result not presented here).

Effect of intracellular Na^+ level on the initial rate of proline uptake

As expected for a Na^+-dependent transport system, the absence of external Na^+ during the initial entry of proline affects markedly the activity of System A (cf. Table II). Moreover, the influx of proline, a preferential substrate of System A in these cells [14], appears to be independent of the level of the intracellular Na^+. However, it should be noted that increasing the internal Na^+ level beyond physiological values by treatment with 1 mM ouabain for 15 min or more, decreased the influx of proline significantly (results not presented here). Furthermore, as shown in this table, the presence of a high internal concentration of proline does not appear to inhibit the substrate influx, thus suggesting that feed-back regulation of System A by trans-inhibition is not operative in these cells.

Li^+ as a substitute for Na^+ during phenylalanine influx

The requirement for Na^+ ions in the intracellular compartment to optimize the influx of phenylalanine does not appear to be absolute. As shown in Table III, the use of Li^+ as an intracellular substitute for Na^+ does not abolish the observed

TABLE III

Li^+ AS A SUBSTITUTE FOR Na^+ DURING PHENYLALANINE INFLUX

Influxes of 0.05 mM phenylalanine were determined in Na^+-free medium at the end of a reloading phase which followed a 60 min depletion step as described in the experimental section. The concentrations of the reloaded phenylalanine were determined in sister cultures at the end of a 15 min reloading step in presence of 1 mM (Na^+-containing medium) or 2 mM phenylalanine (Na^+-free medium, where Li^+ or choline replaced Na^+ in the sodium salt of the mixture). The levels of intracellular Na^+ or Li^+ were determined in sister cultures and following the procedure described in the experimental section. The values are the means of 3–5 independent determinations ± S.D.

Conditions during the reloading phase	v, Phenylalanine influx	Reloaded phenylalanine (μmol/ml)	Intracellular Na^+ or Li^+ concentration (mM)
Sodium presence	0.32 ± 0.09	1.09 ± 0.12	19 ± 2
Lithium presence	0.32 ± 0.06	1.60 ± 0.07	27 ± 2
Choline presence	0.22 ± 0.02	1.65 ± 0.12	0

increase of phenylalanine influx. Since Na^+ has been replaced by a similar charged cation, the preservation of the stimulatory effect on phenylalanine transport indicates that the specificity of the intracellular recognition site of the carrier is not absolute.

Initial rate kinetics

Initial velocities of phenylalanine transport as a function of substrate concentrations were measured in cells reloaded with saturating amounts of substrate in the absence and in the presence of Na^+. As previously noted [8] the Eadie-Hofstee plot of the initial velocity of transport, v, against $v/[S]$ was curvilinear in these cells (see Fig. 3). Assuming that two independent families of carriers with different capacities and/or affinities participate in the transport of phenylalanine, by computer analysis it is possible to resolve the experimental curve into two linear components: a high-affinity, low capacity and a low-affinity, high-capacity component. As shown in this figure, the intercept on the y axis (V) of the low-capacity, high-affinity component appears modified by the presence of intracellular Na^+ during uptake, whereas the slope (K_m) of this component remains substantially unchanged. The kinetic parameters of the high-capacity, low-affinity component appear only marginally modified. Thus, these results suggest that in reloaded cells internal sodium affects the capacity of the high-affinity component of System L, while the affinity of substrate for the inner receptor site of the transport system appears to remain almost unchanged.

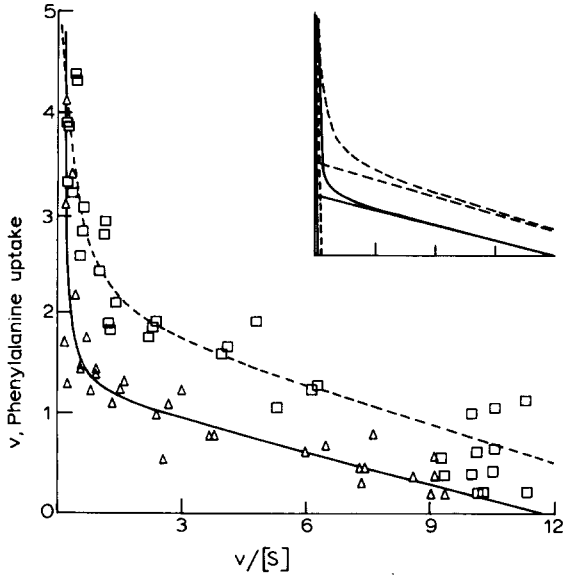

Fig. 3. Kinetic analysis of phenylalanine influxes in SV40 3T3 cells. Influxes of phenylalanine corrected for the non-saturable component as previously described [8] were determined in cells exposed to Na^+-free (△) or Na^+-containing medium (□) during the reloading phase that follows a 60 min depletion. The reloading phase lasted 15 min and the concentration of phenylalanine during that period was 2 mM. Data were analyzed by the Eadie-Hofstee method. The range of phenylalanine concentrations tested for influx was 0.01 to 40 mM. Lines relating the variation of influx of phenylalanine to the ratio of velocity to substrate concentration ($v/[S]$) were drawn according to the fitting of the data obtained by computer analysis assuming that two independent Michaelis-Menten components contribute to transport [8]. Inset: two Michaelis-Menten components obtained after resolution of the curvilinear plot by computer analysis were presented. For both curvilinear and linear plots, solid or broken lines represent cells exposed to Na^+-free or Na^+-containing medium, respectively, during the reloading phase.

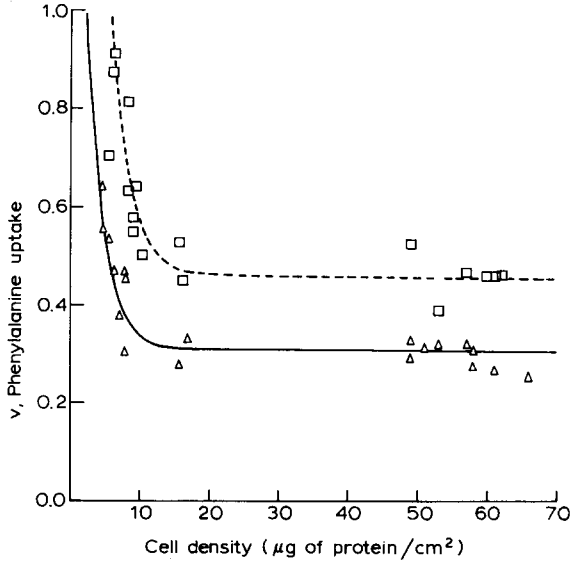

Fig. 4. Phenylalanine transport as a function of cell density. SV40 3T3 cells were seeded over a range of densities and were assayed 24 hours later. Influxes of phenylalanine at 0.05 mM were measured, as described in the experimental section, following the reloading phase. Computer-drawn curves represent the best fitting of the experimental points as obtained according to a double-exponential equation of the type $y = Ae^{-k_1 t} + Be^{-k_2 t}$, with a 90% confidence limit for the final estimate of parameters, for cells exposed to Na^+-free (△) or Na^+-containing medium (□) during the reloading phase.

Effect of cell density on system L transport activity

We have recently shown that the activity of System L in reloaded SV40 3T3 cells is regulated by cell density. Furthermore, the higher phenylalanine influx observed in sparse cells has been ascribed to the intrinsic properties of the transport system rather than to a more active *trans*-stimulatory effect responding to a higher internal level of substrate available for exchange [8].

Here we present evidence (see Fig. 4) that the effect of the Na^+ present during the reloading phase on the subsequent phenylalanine entry is observed at all the cell densities tested. In other words, the influence exhibited by the intracellular Na^+ molarity on the influx of phenylalanine appears to be independent of the cell density regulation of transport activity known to be present in these transformed cells.

Discussion

The results recounted in this paper showing that the influx of phenylalanine is modulated by the presence of Na^+ ions in the intracellular compartment suggest that the Na^+-independence of transport System L could be reconsidered. Taking into account that the influx of System L pertinent amino acids is strictly related to the state of the intracellular pool of the exchangeable substrates, and considering that the steady-state level of neutral amino acids is higher in cells reloaded in the presence of Na^+ ions than in those reloaded in its absence [8], we initially studied the effect of cell reloading in different cationic conditions on the subsequent influx of L-site reactive amino acids. We found that phenylalanine or leucine influx, as determined in the absence of Na^+ '*cis*' (i.e., absence of Na^+ on the external face of the membrane), was significantly higher in cells in which the reloading phase took place in the presence rather than in the absence of Na^+. This effect did not appear to be related to the internal level of the exchangeable amino acids, but to the intracellular level of sodium ions up to a definite concentration beyond which phenylalanine influx did not parallel a further increase of intracellular Na^+ level.

If phenylalanine is differently compartmentalized during loading in the presence of Na^+, a higher level of substrate adjacent to the inside of the plasma membrane could result. This higher level of exchangeable substrate could, in turn, affect by *trans*stimulation influx of pertinent substrates of System L [8]. In Ehrlich ascite tumour cells under normal conditions, the neutral amino acid analog aminoisobutyric acid appeared to be distributed rather equally all over the cell [16]. In addition, our results described in Fig. 1, have shown the persistence of Na^+-driven effect on phenylalanine influx even at internal substrate concentrations higher that those required for maximal transtimulatory activity [8]. Thus, these outcomes are not well compatible with an intracellular compartmentation of phenylalanine affecting substrate influx and suggest as unlikely the hypothesis opening this paragraph.

The requirement for intracellular Na^+ was not found to be highly specific, since it can be replaced by Li^+, a smaller cation than Na^+ in the non-hydrated form, but larger when hydrated. Furthermore, it should be noted that only the presence of Na^+ '*trans*' (i.e., presence of Na^+ on the internal face of the membrane) during influx optimizes substrate transport activity, whether Na^+ is present in the '*cis*' position or not.

Recently, it has been shown the presence of two components of the Na^+-independent transport System L for neutral amino acids in several cell types including human red blood cells [15], rat hepatocytes [7] and transformed fibroblasts [8]. The inhibition of phenylalanine influx by reducing or deleting Na^+ in the '*trans*' position appeared to be based upon a decrease in the capacity of the high-affinity component of transport System L, whereas the affinity of the substrate for the internal receptor site of the carrier appeared to remain almost unchanged. This result is consistent either with a reduced number of functional carriers or a lower mobility of the substrate-carrier complex in the cell membrane.

That this effect of the intracellular Na^+ level is of general interest and not related to peculiar characteristics of transformed cells was shown by the presence of similar Na^+-driven regulatory properties of phenylalanine and leucine influx in the 'normal' heteroploid 3T3 cell line and in diploid chick embryo fibroblasts (Petronini, P.G., Gandolfi, S.A. and Borghetti, A.F., unpublished observations).

The stimulation by intracellular Na^+ on the phenylalanine influx through the high-affinity component of transport System L in substrate loaded cells could be attributed to changes in membrane potential capable to modify transport activity. However, it has been recently shown that amino acid uptake by System L is not dependent on the electrical membrane potential in human fibroblasts [18]. Of course, further studies are needed to clarify the role, if any, of membrane potential on the influx of neutral amino acids such as phenylalanine or leucine in cells loaded in the presence of Na^+.

The possibility that the presence of Na^+ ions in the intracellular compartment will introduce a functional asymmetry in the operation of the transport carrier by modifying the kinetic properties for the receptor site at the inner face of the membrane or the complex interactions of the substrate with the carrier should be considered. Our preliminary results suggest that the presence of Na^+ on the intracellular side of the plasma membrane affects in a significant way the efflux of L-phenylalanine.

Finally, the influx of amino acids known as good substrates of System A appeared to be dependent, as expected, on the presence of Na^+ ions on the '*cis*' face of the plasma membrane but was found to be completely independent of Na^+ presence (up to physiological concentration) on the '*trans*' face of the membrane. These results, introducing for the amino acid influx through Systems A and L important complementary roles of sodium in the '*cis*' or '*trans*' position in respect to the plasma membrane, add a further criterion for discriminating the fluxes of neutral amino acids.

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