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Tyrosine transport into isolated rat brain synaptosomes. Ionic dependence and kinetic studies

F. Javier Diez-Guerra, Federico Mayor, Jr. and Celilio Giménez

Departamento de Bioquímica y Biología Molecular, Centro de Biología Molecular, Facultad de Ciencias, Universidad Autónoma, 28049 Madrid (Spain)

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The ionic dependence and kinetics of the uptake of L-tyrosine into isolated rat brain synaptosomes has been investigated. L-Tyrosine has been found to enter the synaptosomes through three different transport systems showing distinct ionic requirements and kinetic characteristics. The one with the lowest affinity for tyrosine (K_m 0.6 mM) showed a strong Na^+ dependence. This system seems to provide the nerve cell with a safety mechanism that ensures the supply of tyrosine even in the presence of high levels of competing amino acids. The second one (K_m 50 μM) does not appear to exhibit any strong ionic requirements and features most of the characteristics of the L-system for large neutral amino acids. Finally, the third shows the most interesting ionic dependence. Its activity increases at very low Na^+ external concentrations, but this increase is prevented by the removal of divalent cations, Ca^{2+} and Mg^{2+}. This ionic behaviour, along with the affinity constant of this system (K_m 6 μM) (within the range of tyrosine extraneural concentrations), suggests that it is an initial regulatory step in the synthesis of catecholamines.

Introduction

Tyrosine is taken up by the nerve cell through transport systems that have been extensively studied during the past years using different preparations [1–4]. From the kinetic analysis of tyrosine uptake data, the existence of several different transport systems has been postulated [3–8]. In addition, tyrosine uptake has been found to be inhibited by the presence of large neutral amino acids (phenylalanine, tryptophan, leucine, etc.) and the possibility that these amino acids share the same transport systems has been suggested on the basis that they inhibit each other's influx competitively [9–12]. However, in spite of the number of studies reported on tyrosine transport into nerve cells, the ionic dependence of the process has not been completely elucidated. Earlier studies proposed a Na^+ dependence from experiments carried out in brain slices [13], but more recent investigations reported that tyrosine uptake into rat brain synaptosomes was Na^+ independent [14] or that it even decreased by its presence [15]. Finally, a Na^+-electrochemical gradient (out > in) has been shown to be able to drive tyrosine accumulation in plasma membrane vesicles derived from rat brain synaptosomes [4].

All these discrepancies have prompted us to reexamine the role of Na^+ on tyrosine uptake into the nerve cell. We have chosen a highly purified fraction of rat brain synaptosomes [16] as the ideal preparation for this study, since the synaptosomes retain most of the biochemical and physiological characteristics of the nerve terminals [17,18]. The

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

results obtained in the present study strongly suggest the existence of three kinetically different transport systems for L-tyrosine in rat brain synaptosomes, each one showing distinct characteristics with respect to its ionic dependence.

Materials and Methods

Materials

L-[U-^{14}C]Tyrosine (specific activity, 510 mCi/mmol) was purchased from Amersham International, Amersham, Bucks, U.K. Ficoll 400 was obtained from Pharmacia and was exhaustively dialysed against water before use. All others reagents used here of the highest purity available.

Methods

Preparation of synaptosomes. Synaptosomes from adult male Wistar rats (150–200 g) were prepared essentially as previously described [16]. Rats were killed by decapitation and the forebrain rapidly removed and chopped finely with scissors. 3 g of tissue were gently homogenized with 30 ml of an ice-cold medium containing 0.32 M sucrose/1 mM K^+-EGTA/10 mM Tris-HCl (pH 7.4) (isolation medium) in a Dounce-type glass homogenizer. The homogenate was centrifuged at 1300 $\times g$ for 3 min, the pellet was discarded and the supernatant centrifuged at 17000 $\times g$ for 10 min. The pellet (P_2 fraction) was resuspended with 5 ml of isolation medium and layered on the top of a discontinuous Ficoll gradient consisting of 3 ml of 7.5% (w/v) Ficoll in isolation medium on 3.5 ml of 13% (w/v) Ficoll in isolation medium. Tubes were placed in a Beckman Swing-out rotor (SW 40 Ti) and centrifuged at 98000 $\times g$ for 30 min. The synaptosomal fraction was then sucked off after carefully removing the myelin layer, diluted 10-fold with the isolation medium and centrifuged at 15000 $\times g$ for 10 min. Synaptosomes were kept in an ice bath either as pellets or they were resuspended with 0.32 M sucrose at 40–60 mg protein/ml until utilization. Synaptosomes were used with a maximum of 3 h after preparations.

Transport assays. Unless otherwise stated, 20 μl portions of the synaptosomal suspension (0.1 mg of protein) were preincubated for 5 min at 37°C in the final incubation medium. The uptake was started by the addition of 80 μl of a solution kept at 37°C and containing the desired ionic composition together with labelled L-tyrosine. After incubation with gentle agitation, uptake was terminated by diluting the incubation mixture with 3 ml of a modified Krebs-Henseleit medium (125 mM NaCl/5 mM KCl/1.3 mM $MgSO_4$/2.7 mM $CaCl_2$/11.1 mM D-glucose/25 mM Hepes-Tris (pH 7.4)) kept at room temperature and then immediately filtering through a moistened Millipore filter (HAWP 02500, 0.45 μm pore size) attached to a vacuum assembly. Filters were rinsed once with another 3 ml of the Krebs-Henseleit medium. The dilution, filtration and washing procedures were performed within 15 s. Filters were dried at 60°C, placed in microvials and their radioactivity was measured by scintillation spectrometry. Results were corrected for a control obtained by diluting the synaptosomal suspension before adding the radioactive substrate solution. All solutions used in the preparation procedures and uptake experiments were made up with distilled de-ionized water previously filtered through Millipore filters (0.45 μm) to avoid possible bacterial contamination. The osmolarity of all solutions was kept constant during the uptake experiments. The modified Krebs-Henseleit medium described above was termed Na^+-containing medium, while the resultant of isoosmotically replacing NaCl, by sucrose was termed Na^+-free medium.

Incubations were carried out in triplicate. Each experiment was repeated at least three times with different membrane preparations. Agreement among experiments was good; thus, typical experiments are reported.

The metabolism of L-tyrosine within the synaptosomes was studied by extracting the radioactivity previously accumulated by means of a hypotonic stock and analysing it by thin-layer chromatography. More than 95% of the radioactivity was found associated with L-tyrosine under the different conditions used for transport assays.

Protein determination. Synaptosomal and membrane proteins were determined according to the method of Resch et al. [19].

Analysis of the data. Statistical analysis was performed by Student's 't'-test for non-paired samples and comparison was made with the control group. P values of 0.05 or less were taken as

significant, and the results are expressed as the mean ± S.E. Fitting of the experimental kinetic results to predicted theoretical formulae was performed with the aid of the FORTRAN program NLIN.

Results

Ionic dependence

As shown in Fig. 1, L-tyrosine was taken up actively by rat brain synaptosomes. At 5 μm L-tyrosine (Fig. 1A), a value assumed to be similar to the concentrations of extraneuronal space [8,20]; the uptake reached an equilibrium after 5 min of incubation, when the apparent internal concentration was 18 times greater than the external one (measured internal synaptosome volume: 3.3 μl/mg protein). The uptake reached an equilibrium after 7 min of incubation when the L-tyrosine concentration was 0.5 mM (Fig. 1B). At both concentrations, the assumulation observed at 2°C showed a linear relationship with time, reflecting the presence of binding and diffusion phenomena. The incubation with 1 mM L-phenylalanine or L-tryptophan during the time course resulted in both L-tyrosine concentrations with a similar pattern of uptake to that obtained at 2°C. Thus, for all subsequent measurements we adopted a control at 2°C that was subtracted from determinations at 37°C.

The addition of 1 mM ouabain, a $(Na^+ + K^+)$-ATPase inhibitor, to the incubation medium resulted in a decreased value at equilibrium. Interestingly, this inhibition was greater at 0.5 mM L-tyrosine (4.74 ± 0.23 and 2.97 ± 0.133 nmol L-tyrosine taken up per mg of protein in 7 min, mean ± S.E. for control and ouabain, respectively) than at 5 μM (328 ± 25 and 249 ± 16 pmol L-tyrosine per mg of protein in 7 min, mean ± S.E. for control and ouabain, respectively).

Fig. 2 shows the time course of the uptake of L-tyrosine in situations where external sodium was isoosmotically substituted by sucrose, choline or lithium. The curves obtained fitted an exponential

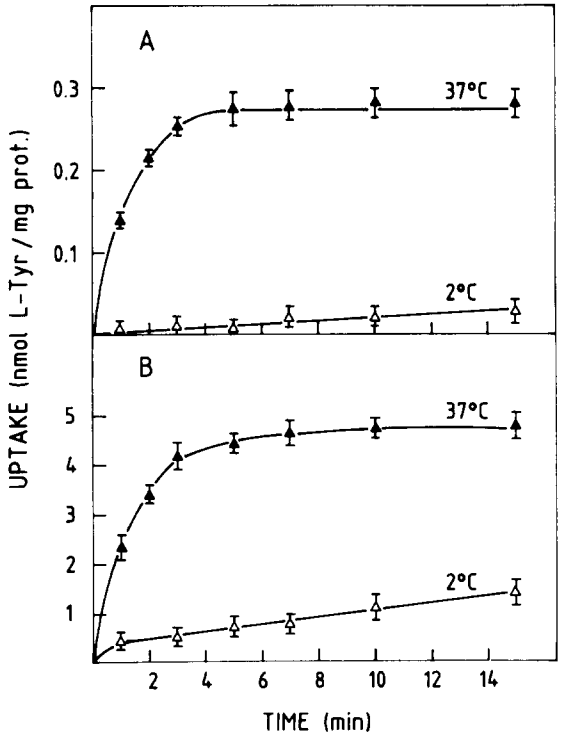

Fig. 1. Time course of L-tyrosine uptake. Synaptosomes (1 mg protein/ml, final concentration) were preincubated for 5 min at 37°C and subsequently incubated with (A) 5 μM and (B) 0.5 mM L-[U-^{14}C]tyrosine either at 2°C (ice bath) (△) or 37°C (▲). Results are expressed as the mean ± S.E. of three experiments.

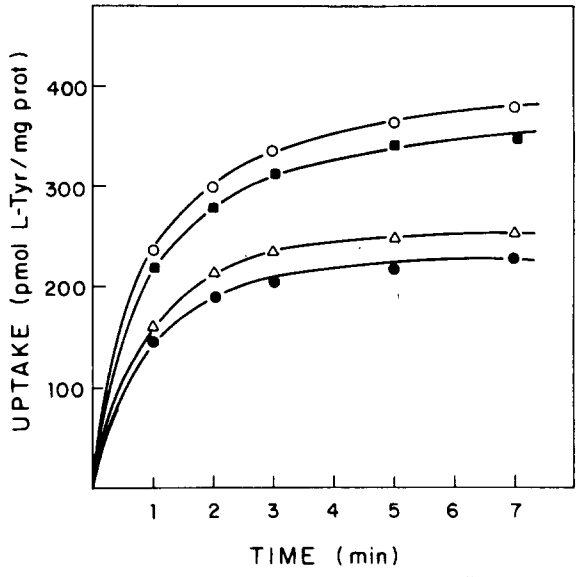

Fig. 2. Effect of isoosmotic replacement of sodium on synaptosomal uptake. Synaptosomes (1 mg protein/ml, final concentration) were preincubated for 5 min at 37°C and subsequently incubated with 5 μM L-[U-^{14}C]tyrosine in (△) modified Krebs-Henseleit medium and media in which NaCl was replaced by (●) 125 mM lithium chloride, (□) 125 mM choline chloride or (○) 250 mM sucrose.

model well, giving a time constant around 1.00 min^{-1}, with no significant difference for the various conditions (mean value $\pm$S.D. = 0.999 $\pm$ 0.015). Choline and sucrose substitutions gave similar maximum values (339 and 365 pmol L-tyrosine, respectively) and these were significantly higher than those with lithium and sodium substitutions (222 and 249 pmol L-tyrosine, respectively). All four exponentials showed a nearly linear relationship with time during the initial 45 s ($P < 0.05$), a result that was experimentally confirmed by uptake measurements at short time intervals (data not shown).

The study of the uptake of L-tyrosine as a function of the external potassium concentration revealed the existence of an optimal value around 1 mM and an increasing inhibition of the uptake as the potassium concentration was increased beyond 5 mM (Fig. 3). The results obtained in the study of the sodium dependence of the uptake are shown in Fig. 4. An initial decay in the uptake was observed when the sodium concentration was lowered to 25 mM. Beyond this concentration, further substitution of sodium by sucrose resulted in an increasingly enhanced uptake until values 45% greater than those obtained at 125 mM sodium were obtained at 0.1 mM sodium.

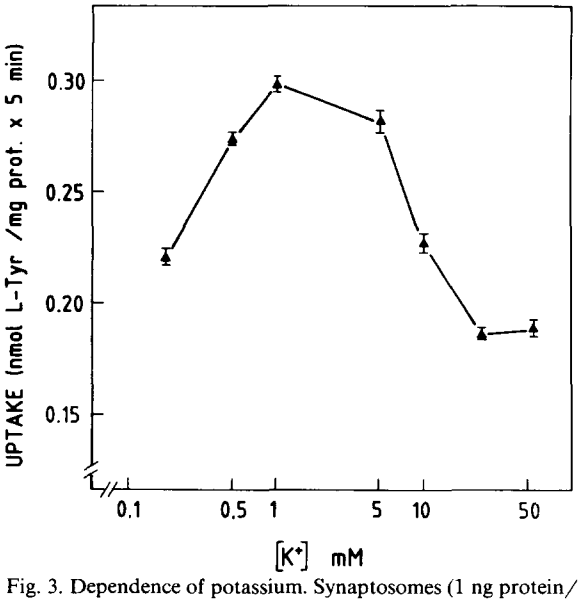

Fig. 3. Dependence of potassium. Synaptosomes (1 ng protein/ml, final concentration) were preincubated for 5 min at 37°C in the final uptake media and then incubated at the different K^+ concentrations shown in the presence of 5 μM L-[U-^{14}C]tyrosine during 5 min. Results are expressed as the mean $\pm$ S.E. of three determinations. Osmolarity was maintained constant by equimolar replacement of sodium and potassium.

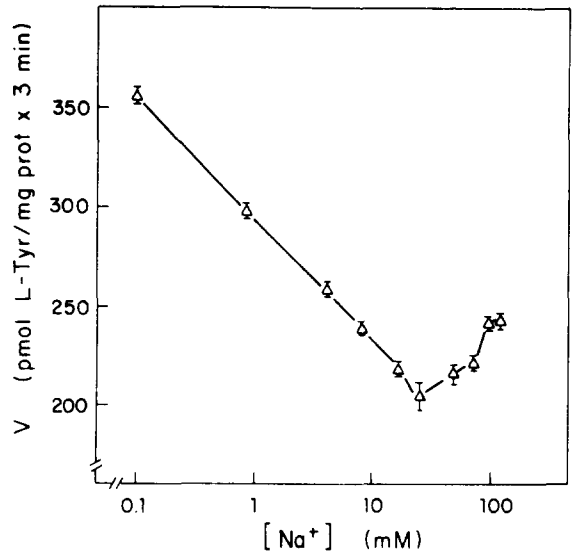

Fig. 4. Effect of partial isoosmotic replacement of sodium on synaptosomal uptake. Synaptosomes (1 mg protein/ml, final concentration) were preincubated for 5 min at 37°C and subsequently incubated with 5 μM L-[U-^{14}C]tyrosine for 5 min. Osmolarity was kept constant by replacing NaCl by sucrose. Results are expressed as the mean $\pm$ S.E. of three determinations.

The effect of removing the divalent cations Ca^{2+} and Mg^{2+} was investigated in Na^+-free and Na^+-containing media. Table I shows that changes

TABLE I

EFFECT OF Ca^{2+} AND/OR Mg^{2+} REMOVAL ON SYNAPTOSOMAL UPTAKE

Synaptosomes (1 mg protein/ml, final concentration) were preincubated in the media with final ionic composition for 5 min at 37°C and were subsequently incubated with 5 μM L-[U-^{14}C]tyrosine for 5 min. NaCl, $MgSO_4$ and $CaCl_2$ in modified Krebs-Henseleit media were present or isoosmotically replaced by sucrose, as indicated. Results are expressed as the mean $\pm$ S.E. of three determinations. Uptake units are pmol L-tyrosine taken up per mg of protein in 5 min.

Isoosmotical removal	Na^+-containing medium	Na^+-free medium
None	312 $\pm$ 12	420 $\pm$ 8
Ca^{2+}	290 $\pm$ 6	408 $\pm$ 12
Mg^{2+}	296 $\pm$ 8	412 $\pm$ 9
Ca^{2+} and Mg^{2+}	252 $\pm$ 4	196 $\pm$ 5 *

* $P < 0.0005$.

in the divalent cations composition did not affect the uptake when assayed in Na^+-containing medium. Nevertheless, the removal of both Ca^{2+} and Mg^{2+} significantly decreased the uptake in Na^+-free medium, whereas individual removal of Ca^{2+} or Mg^{2+} was devoid of effect.

Kinetic analysis

In order to investigate how the different transport systems for L-tyrosine contribute to the total uptake under different ionic conditions, we carried out kinetic studies. Fig. 5 is an Eadie-Hofstee plot of initial velocities of uptake (determinations at 30 s) measured in Na^+-free and Na^+-containing media. At lower substrate concentrations (40 μM and lower) uptake was greater in a Na^+-free medium, whereas at higher substrate concentrations (0.1 mM and higher) uptake was greater in Na^+-containing medium. Computer analysis of the data indicated a best fit to a three-parameter Michaelis-Menten transport system in Na^+-containing medium and to a two-parameter model in Na^+-free medium. In order to achieve a more accurate estimate of the kinetic parameters defining the different transport systems, separate kinetic experiments at two L-tyrosine concentration ranges were undertaken (Fig. 6). Table II summarizes the kinetic parameters obtained. It is worthy of note that the low-affinity transport system ($K_m = 0.6$ mM) does not appear to be active when Na^+ is absent from the incubation medium. On the other hand, an increase in the V_{max} of the high-affinity transport system was observed in Na^+-free medium. The transport system with intermediate affinity showed similar kinetic constants under both ionic conditions.

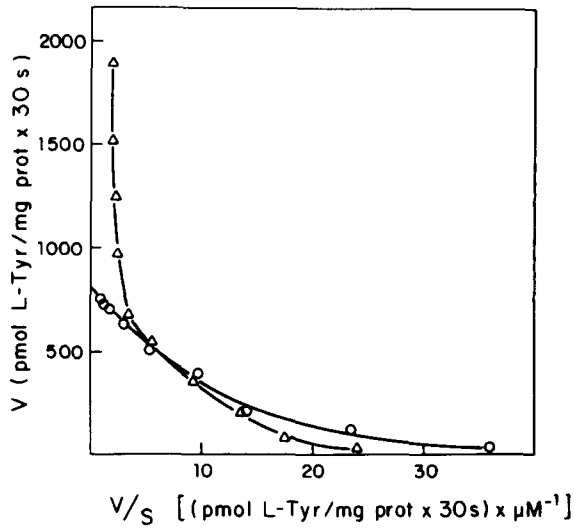

Fig. 5. Kinetic study of synaptosomal tyrosine uptake. Effect of sodium ions. Synaptosomes (1 mg protein/ml, final concentration) were preincubated for 5 min at 37°C and subsequently incubated for 30 s with concentrations of L-[U-^{14}C]tyrosine ranging from 1 μM to 1 mM in (○) Na^+-free or (△) Na^+-containing modified Krebs-Henseleit media. Results are epressed as the mean of three determinations.

Fig. 7 represents an Eadie-Hofstee plot of the initial rates of uptake obtained when the divalent cations, Ca^{2+} and Mg^{2+}, were removed from the incubation medium. The analysis of the data indicates the presence of a single transport system either in Na^+-free or Na^+-containing media with similar K_m values. K_m and V_{max} values were, respectively, 33 μM and 0.9 nmol/mg of protein per 30 s in Na^+-containing medium, and 30 μM and 0.7 nmol/mg of protein per 30 s, in Na^+-free medium.

TABLE II

KINETIC CHARACTERISTICS OF THE TYROSINE UPTAKE SYSTEMS IN ISOLATED SYNAPTOSOMES

K_m values are given in μM and V_{max} values in pmol tyrosine taken up per mg of protein in 30 s. Constants were estimated from the graphical plots in Figs. 5 and 6.

Systems	Na^+-containing medium		Na^+-free medium	
	K_m	V_{max}	K_m	V_{max}
High-affinity	5, 7	147	5, 8	247
Intermediate-affinity	45–54	775–1206	51–54	1124–1206
Low-affinity	600	2650	–	–

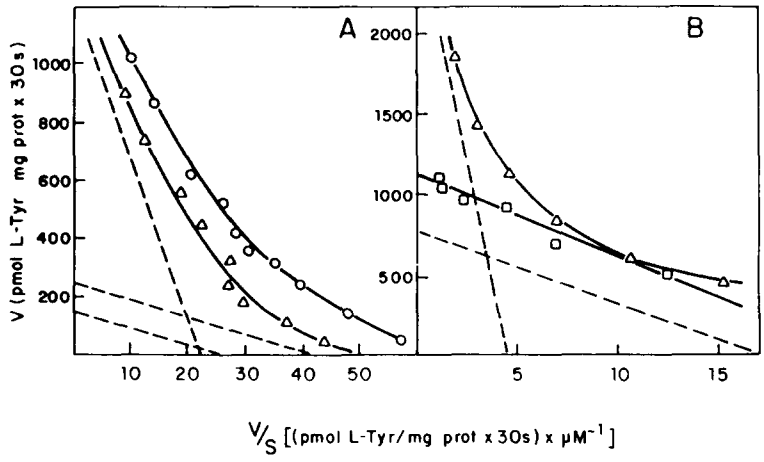

Fig. 6. Detailed kinetic study of synaptosomal tyrosine uptake. Effect of sodium ions. Synaptosomes (1 mg protein/ml, final concentration) were preincubated for 5 min at 37°C and subsequently incubated for 30 s with concentrations of L-[U-^{14}C]tyrosine ranging from (A) 1 μM to 0.1 mM and (B) 40 μM to 1 mM in (○) Na^+-free or (△) Na^+-containing Krebs-Henseleit media. Dotted lines indicate computer-fitted single transport systems. Results are expressed as the mean of three determinations.

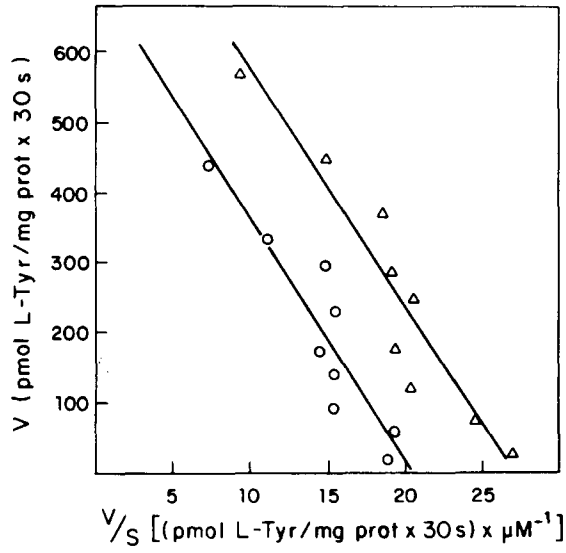

Fig. 7. Kinetic study of synaptosomal tyrosine uptake. Effect of calcium and magnesium ions. Synaptosomes (1 mg protein/ml, final concentration) were preincubated for 5 min at 37°C and subsequently incubated for 30 s with concentrations of L-[^{14}C]tyrosine ranging from 1 μM to 60 μM in (○) Na^+-free or (△) Na^+-containing Krebs-Henseleit media, where $CaCl_2$ and $MgSO_4$ were isoosmotically replaced by sucrose. Results are expressed as the mean of three determinations.

Discussion

We have studied the ionic dependence and kinetic characteristics of tyrosine transport in rat brain synaptosomes and found that L-tyrosine is taken up into the synaptosomes by three different transport systems. The one showing the lowest affinity has a K_m value of 0.6 mM, in good agreement with previous observations of Belin and Pujol [5]. Several pieces of evidence support the hypothesis that this system is Na^+ dependent. First of all, it has been demonstrated by kinetic analysis that this system is not active in Na^+-free medium. Secondly, it has been previously shown that synaptosomal L-tyrosine uptake showed a partial sensitivity to ouabain [25]. Our results demonstrate that this sensitivity is greater as the substrate concentration is increased, thus suggesting an involvement of the low-affinity system. The existence of this system would explain the initial decay of the uptake observed when the external Na^+ concentration is lowered from 125 to 25 mM. The physiological relevance of a transport system with such a K_m value could be to ensure the supply of L-tyrosine when abnormally high levels of competing amino acids are present. The second transport system presented a K_m value of 50 μM. The relative affinity of this system for L-tyrosine and the fact that it showed no relevant ionic dependence support the idea that this is the L-system proposed by Christensen et al. [26] for large neutral amino acids, where the driving forces are exchange phenomena with structure-related amino acids.

Finally, the transport system showing the highest affinity for L-tyrosine presented a K_m value of 6 μM, thus appearing to be the most relevant at physiological concentrations. The K_m value obtained is in good agreement with previous studies in synaptosomes [3] and plasma membrane

vesicles derived from synaptosomes [4]. This system seems to be responsible for the enhanced uptake observed in synaptosomes incubated in Na^+-free medium, since its V_{max} showed a 68% increase in that medium. Furthermore, the kinetic data show that the system needs the presence of divalent cations to be active, thus suggesting the involvement of this system in the drastic inhibition of the utpake in a Na^+-free medium after removal of the divalent cations, Ca^{2+} and Mg^{2+}.

While the driving forces for the transport of L-tyrosine through the low and and intermediate affinity systems appear to be the electrochemical Na^+ gradient and amino acid heteroexchange phenomena [26], respectively, the energy sources linked to the high-affinity system remain to be established. It seems that synaptosomal ATP levels are not likely to be directly coupled to high-affinity uptake, since addition of metabolic inhibitors results in a rapid decay of synaptosomal ATP content that is not parallelled by the uptake (Ref. 21, data not shown). Neither does $\Delta\Psi$ appear to be involved in high-affinity uptake, since the incubation with the protonophore CCCP, that specifically collapses $\Delta\Psi$ by creating a H^+ gradient, does not modify significantly the uptake of L-tyrosine at low concentrations (data not shown). Na^+ dependence has been a matter of controversy during the last years [13,14]. Aragón et al. [4] have proposed a Na^+ gradient dependence from their observations in plasma membrane vesicles. However, studies carried out in synaptosomes strongly suggest that Na^+ inhibits high-affinity uptake [3,8,15]. Our results indicate an activation of the high-affinity system in Na^+-free medium without change in its K_m value; this is in good agreement with the last proposal. This activation is only possible when the divalent cations Ca^{2+} or Mg^{2+} are present in the medium. The possibility of the formation of a complex (divalent cation-L-tyrosine) that might improve efficiency of the carrier, has been suggested previously [8]. However, our results do not support this hypothesis, since the removal of divalent cations did not modify the levels of uptake in Na^+-containing medium. On the other hand, changes in synaptosomal morphology and alterations of the internal volume should be disregarded.

In conclusion, the versatility displayed by the high-affinity transport system for tyrosine, now beginning to be understood, is suggested to be most likely involved in regulation at the physiological level. This could ensure that intracellular levels of L-tyrosine are maintained under the particular ionic and electrical conditions prevailing during nerve activity.

Acknowledgments

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