Kinetic and Allosteric Cooperativity in L-Adenosine Transport in Chromaffin Cells. A Mnemonical Transporter†

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ABSTRACT: In cultured chromaffin cells and plasma membrane vesicles from chromaffin tissue, the transport of D-[^{3}H]adenosine followed Michaelis–Menten saturation kinetics, with K_m values of 1.5 ± 0.3 μM and 1.9 ± 0.2 μM, respectively. The transport of the isomer, L-[^{3}H]adenosine, showed sigmoidal kinetics in both preparations. In plasma membrane vesicles the $S_{0.5}$ was 2.5 ± 0.2 μM with a Hill coefficient of 2.8 and the V_{max} value of 0.26 ± 0.01 pmol s^{-1} (mg of protein)$^{-1}$. In cultured chromaffin cells the kinetic parameters for L-[^{3}H]adenosine were $S_{0.5}$ = 6.2 ± 0.2 μM and a V_{max} 19.7 ± 0.5 pmol/min per 10^6 cells, with a pronounced positive cooperativity. The Hill coefficient was 4.9. The transport of the L-isomer in cultured cells followed Michaelis–Menten kinetics at the lowest concentrations employed, below 2 μM. On the basis of these results, we propose a kinetic model whereby the adenosine transporter functions mnemonically.

Nucleoside transporters have been extensively characterized in various mammalian cells. They have been classified as (i) the sodium-dependent nucleoside transport mainly localized in intestinal and renal epithelial cells (Plagemann et al., 1988; Williams & Jarvis, 1991) and (ii) facilitated, sodium-independent nucleoside transport, which has been found in all types of mammalian cells investigated to date (Woffendin & Plagemann, 1987).

At least two forms of the facilitated transport by (nitrobenzyl)thioinosine[1] (NBTI) inhibition exist: one is strongly inhibited by nanomolar concentration of NBTI (NBTI-sensitive) and the second is inhibited by micromolar concentration of NBTI (NBTI-resistant). Both types of facilitated transport are expressed in different proportions in the nervous system preparations and other cell lines (Lee & Jarvis, 1988; Jones & Hammond 1992).

The adenosine transport in neurochromaffin cells is highly sensitive to NBTI inhibition and has been shown to be a widely regulated process (Torres et al., 1990; Delicado et al., 1990). In these cells, extracellular signals activating protein kinases A and C inhibit adenosine transport (Sen et al., 1990; Delicado et al., 1991; Sen et al., 1993). Moreover, thyroid hormones increase adenosine transport mediated by protein synthesis (Fideu & Miras-Portugal, 1992). The changes in adenosine transport capacity run in parallel with changes in the number of high affinity binding sites for NBTI in cultured chromaffin cells. The regulation of adenosine transport in these neural cells seems to be similar to that described for the regulatory enzymes of metabolic pathways. The activity of these enzymes are controlled by (i) allosteric changes induced by some intermediate metabolites, (ii) reversible phosphorylation by protein kinases, and (iii) modification in the protein synthesis.

No allosteric regulation for adenosine transport has as yet been reported. On the contrary, inhibitors of nucleoside transport, such as dipyridamol, and more recently, lidoflazine and mioflazine, have been found to allosterically modulate the binding of the high affinity ligand, NBTI, to nucleoside transporter in plasma membranes of calf lung and Erlich cell plasma membranes (Hammond, 1991; Ijzerman et al., 1989). A positive cooperativity in the NBTI binding to nucleoside transporters in plasma membranes of chromaffin tissue has recently been reported (Casillas et al., 1992). This finding correlated with the existence of dimeric forms that were identified by radiation inactivation analysis of erythrocyte membranes labeled with [^{3}H]NBTI (Jarvis et al., 1986).

Most studies on nucleoside transport have been carried out using D-adenosine as substrate, but this compound is rapidly metabolized by cellular enzymatic pathways (Rotllán & Miras-Portugal, 1985; Newsholme et al., 1985). This problem can be circumvented by measuring the adenosine transport during "short" time incubations, to determine initial rates of substrate translocation across the plasma membrane or by using plasma membrane vesicles to avoid cellular metabolism. The metabolic problem can also be solved by using nonmetabolizable analogs such as L-adenosine. The L-adenosine transport has been characterized in other cellular models, such as erythrocytes and synaptoneurosomal preparations (Gati et al., 1989; Gu et al., 1991, Gu & Geiger, 1992). In the present experimental work, we have characterized the transport of L-adenosine in a homogeneous neural cell population, the chromaffin cell. L-Adenosine was transported into the chromaffin cells and plasma membrane vesicles. In both preparations, the existence of a positive cooperativity for L-adenosine transport was demonstrated. A kinetic model for adenosine transport is proposed to explain the observed kinetic and allosteric cooperativity.

MATERIALS AND METHODS

Preparation of Plasma Membrane Vesicles. Subcellular membrane preparations were obtained as described previously (Torres et al., 1988; Delicado et al., 1988). Nonfrozen adrenal glands were dissected and homogenized in 0.32 M sucrose/10 mM Hepes (pH 7.4)/50 μM phenylmethanesulfonyl fluoride

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[1] Abbreviations: DMEM (Dulbecco's modified Eagle's medium), NBTI [(nitrobenzyl)thioinosine], PMSF (phenylmethanesulfonyl fluoride), ADA (adenosine deaminase).

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(1:4 w/v) with a glass-Teflon Potter homogenizer (1100 rpm). The homogenate was centrifuged at 800*g* for 10 min and the resultant supernatant was recentrifuged at 10000*g* for 30 min. The pellet obtained was resuspended in 0.32 M sucrose and layered over a discontinuous sucrose gradient (0.95–1.34–1.60 M). Centrifugation was carried out for 60 min at 100000*g*. The plasma membrane-enrichred fraction was then collected at the 0.32–0.95 M sucrose interface. To eliminate sucrose and other metabolites such as ATP, the membrane vesicle fractions were lysed twice with Hepes 10 mM pH 7.4. The membrane preparations were resuspended in the same volume of a Locke's solution doubly concentrated to reach isoosmotic concentrations. Protein determinations were carried out by the method of Bradford (Bradford, 1976).

Nucleoside Transport Experiments in Vesicles. The transport of nucleosides, D-[^{3}H]adenosine and L-[^{3}H]adenosine was measured at 37 °C by a stop-filtration technique. To determine nucleoside transport, a 100-μL portion of vesicle preparation (250 μg of protein) was preincubated for 1 min at 37 °C and transport was initiated adding 100 μL of Locke's solution containing 1 μCi of D-[^{3}H]adenosine or L-[^{3}H]-adenosine and nonlabeled compound to reach the required concentrations. At specified times, 100 μL of ice-cold Locke's solution containing 10 μM dipyridamole final concentration (stop solution) were added to terminate transport. The suspension was immediately filtered through Whatman GF/C filters that had been presoaked in 100 μM adenosine. To determine the nonspecific transport, similar experiments were carried out by incubating the vesicles in the presence of 10 μM NBTI. This diffusion component was subtracted from the assays. The filters were washed twice with 3 mL of stop solution, layered on 4.5 mL of Ready Safe and the radioactivity was measured.

Isolation and Culture of Chromaffin Cells. Bovine adrenal chromaffin cells were isolated by collagenase digestion and purified on a self-generating Percoll gradient (Miras-Portugal et al., 1986). The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% fetal calf serum containing 10 μM cytosine arabinofuraoside, 10 μM fluorodeoxyuridine, penicillin (5 units/mL), streptomycin (5 mg/mL), kanamycin (100 mg/mL), and amphotericin (2.5 mg/mL).

Cells were plated in culture wells at a density of 250 000 cells/well in 24-well Costar cluster dishes. The cells were used between 4 to 8 days of culture. Two to three hours before the experiments were begun, the medium was replaced by Locke's solution.

Adenosine Transport Experiments in Cultured Cells. Adenosine transport assays were carried out by the method of Miras-Portugal et al. (1986) and Torres et al. (1988). Essentially, 250 000 cells were incubated in 200 μL of Locke's solution containing 1 μCi/well of D-[^{3}H]- or L-[^{3}H]adenosine and nonlabeled adenosine isomer at the required final concentrations. For D-adenosine transport at very low concentrations, dilutions of D-[^{3}H]adenosine in Locke's medium were made. Transport was stopped by aspiration of the medium followed by two washes of 1 mL of cold Locke's solution containing 10 μM dipyridamole. Cells were scraped from the plastic wells, and the radioactivity was measured. The transport experiments were carried out in the presence or absence of 10 μM NBTI. The transport in the presence of NBTI corresponded to the diffusion component and was subtracted from the total component to obtain the NBTI-sensitive transport.

HPLC and Thin-Layer Chromatography Experiments. To check the purity of L-[^{3}H]adenosine and L-adenosine, the HPLC technique was used. The chromatographic system consisted of a Waters 600 E delivery system, a U6K injector, a 418-nm λ_{max} UV detector, and a 745 data module integrator. Both compounds eluted as a single peak in a spheri-10RP-18 (22-cm length, 0.46-cm i.d.) column from Brownlee (Applied Biosystem) with a mobile phase composed of 10 mM K_2HOP_4, pH 4.8, and 5% methanol, according to the procedure of Torres et al. (1990). Under these conditions, the retention time was 6.29 min, which exactly corresponded to the radioactivity counted from the column eluates.

After incubation of L-adenosine in the presence of 4 units of adenosine deaminase (ADA) for 10 min at 37 °C, the HPCL chromatograms showed that more than 95% remained as L-adenosine, indicating that the L-adenosine was enantiomerically pure.

The metabolism of L-adenosine by cultured chromaffin cells was assessed by TLC analysis. Cells were incubated with 5 and 15 μM L-[^{3}H]adenosine from 15 s to 30 min at 37 °C. Cells were scraped out of the plastic with 500 μL of $HClO_4$ 0.5 M/EDTA 25 mM, maintained for 1 min at 4 °C, and centrifuged at 15000*g* for 1 min. The supernatant was neutralized with KOH 3 M/TEA 1.5 M, maintained for 2 min at 4 °C, and pelleted (15000*g* 1 min). Aliquots of the resultant supernatant were placed on silica gel-coated plates. The solvent was butan-1-ol saturated with water. The spots were located under ultraviolet light, cut out, and placed in scintillation vials with 5 mL of scintillation fluid for counting radioactivity.

Controls of metabolism in the L- and D-adenosine transport experiments using plasma membrane vesicles were carried out in a similar way to that described for chromaffin cells.

Data Analysis. The results are expressed as the means ± SD of five experiments performed in quadruplicate. The data were calculated by a nonlinear regression program.

Chemicals. Collagenase (EC 3.4.24.3), adenosine deaminase (EC 3.5.4.4), and phenylmethanesulfonyl fluoride (PMSF) were supplied by Boehringer. Cytosine arabinofuranoside, (nitrobenzyl)thioinosine, and dipyridamole were purchased from Sigma. Culture media, fetal calf serum, and antibiotics were pruchased from Flow Laboratories. Culture plates were obtained from Costar. D-[^{3}H]Adenosine (26.7 Ci/mmol) was supplied by New England Nuclear and L-[^{3}H]-adenosine (33 Ci/mmol) and L-adenosine were purchased from Moravek Biochemicals. Ready Safe scintillation liquid for aqueous and nonaqueous samples was purchased from Beckman. All other reagents were supplied by Merck.

RESULTS

D- and L-Adenosine Transport in Plasma Membrane Vesicles. No research has as yet been done to characterize adenosine transport in membrane vesicle preparations from chromaffin tissue. These preparations do not contain the enzymes responsible for metabolizing adenosine or the second messengers that could also regulate the adenosine transport. In the present paper, the kinetic parameters of D- and L-adenosine transport were characterized in plasma membrane vesicles from chromaffin tissue.

The accumulation of D-adenosine was linear during the first 10 s (results not shown). Thus, the adenosine transport experiments in membrane preparations were measured during the linear period and the diffusion component nonsensitive to NBTI was subtracted. D-Adenosine transport studied at different nucleoside concentrations showed a classical Michaelis–Menten pattern similar to that obtained in cultured cells. The values calculated for K_m and V_{max} were 1.9 ± 0.2 μM and 0.36 ± 0.02 pmol s^{-1} (mg of protein)$^{-1}$, respectively (Figure 1).

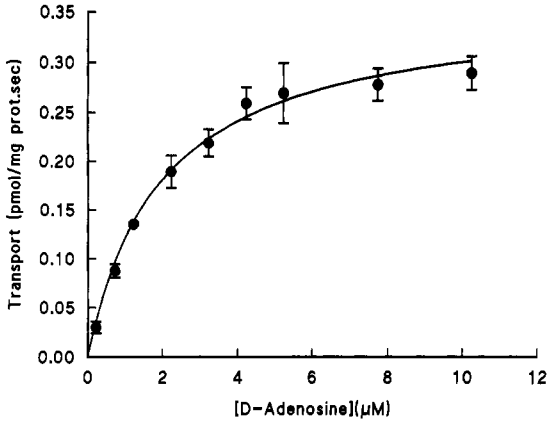

FIGURE 1: Concentration-dependence of D-[^{3}H]adenosine transport in plasma membrane vesicle preparations. The NBTI-sensitive transport was carried out with 0.25 mg protein at 37 °C as described in Methods. The transport capacity is expressed as pmol/mg of protein per second. Values are the means ± SD of four experiments performed in triplicate.

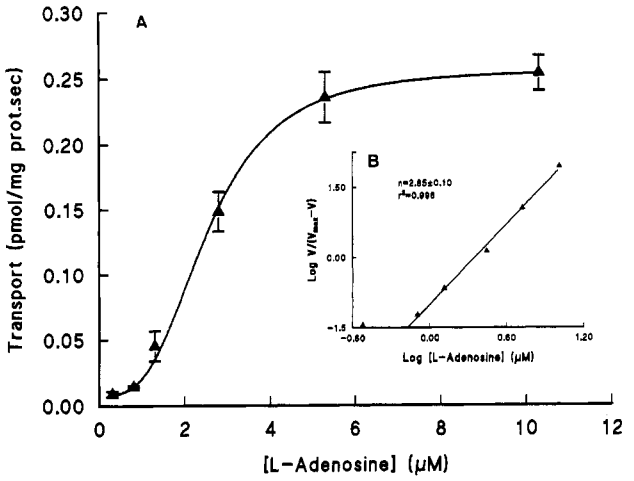

FIGURE 2: (a) Concentration-dependence of L-[^{3}H]adenosine transport in plasma membrane vesicles. An amount of 0.25 mg protein was incubated for 10 s at different concentrations of L-[^{3}H]adenosine and the NBTI-sensitive transport was determined as described in Methods. (B) The Hill representation resulting from A. These results represent a typical experiment performed in quadruplicate.

Similar experiments demonstrated that L-[^{3}H]adenosine was accumulated into vesicles. As for D-adenosine, the transport of L-adenosine was linear during the first 15-s period (not shown). The concentration dependence of L-[^{3}H]-adenosine transport showed a sigmoidal shape (Figure 2). The kinetic values obtained were $S_{0.5}$ = 2.5 ± 0.2 μM and V_{max} = 0.26 ± 0.01 pmol s^{-1} (mg of protein)$^{-1}$. The Hill coefficient was 2.8 ± 0.10 indicating that the nucleoside transporter presents a pronounced positive cooperativity for the L-isomer of adenosine. The existence of positive cooperativity with L-adenosine is a very "reproducible" result in the vesicle preparations obtained and employed with the experimental conditions described in Methods. However, as membrane preparations are submitted to a disruptive procedure that could modify the natural environment in the intact cell, it is of interest to study the L-adenosine transport in cultured chromaffin cells to exclude possible artefacts due to the process.

Nucleoside metabolism experiments by TLC demonstrated that neither D-adenosine nor the L-isomer were metabolized by plasma membrane vesicles as can be expected considering the cytosolic localization of adenosine kinase and adenosine deaminase in this tissue (Rotllán & Miras-Portugal, 1985).

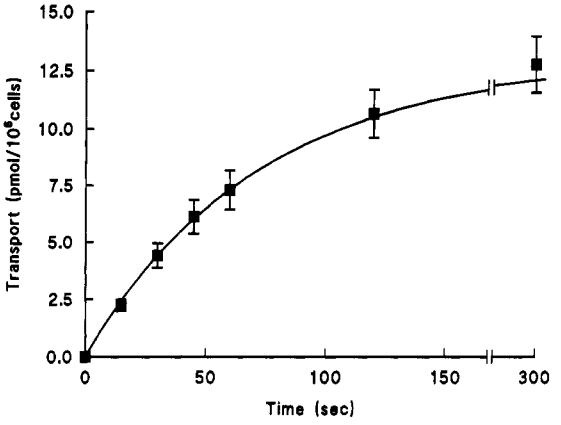

FIGURE 3: Time course of L-[^{3}H]adenosine transport by cultured chromaffin cells. An amount of 250 000 cells was incubated in the presence or absence of 10 μM NBTI and the transport at 5 μM L-[^{3}H]adenosine concentration was measured at different times as described in Methods. The transport in the presence of 10 μM NBTI was subtracted from the total component and only the NBTI-sensitive L-adenosine transport is shown. This plot is a representative experiment of four performed in quadruplicate.

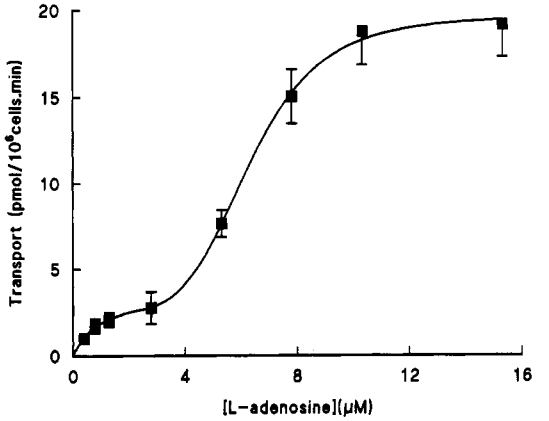

FIGURE 4: Concentration-dependence of L-[^{3}H]adenosine transport in chromaffin cells. The transport experiments were accomplished with 250 000 cells during 30 s of incubation at 37 °C at the required L-[^{3}H]adenosine concentrations. The NBTI-sensitive transport was determined as described in Figure 3. The transport capacity is expressed as pmol/10^6 cells per minute to allow comparison. Values are the means ± SD of three experiments performed in quadruplicate.

L-[^{3}H]Adenosine Transport in Cultured Chromaffin Cells. In previous studies, it has been reported that D-adenosine transport in chromaffin cells is a saturable process with high affinity (K_m = 1.5 ± 0.3 μM) that remains linear during the first minute of incubation at every studied concentration. The transport of the enantiomer L-adenosine at 5 μM extracellular concentration is shown in Figure 3, where the component nonsensitive to 10 μM NBTI was subtracted from the total component. The accumulation of L-isomer was linear for a 40-s period. When the transport as a function of L-[^{3}H]-adenosine extracellular concentration was studied, sigmoidal plot was obtained (Figure 4). There are two distinct substrate-dependence regions: one at concentrations below 2 μM and the other above this concentration. Nonlinear regression analysis of these data clearly indicated that (a) at the lowest substrate concentrations (below 2 μM) the transport of L-adenosine followed a Michaelis–Menten equation with high affinity (K_m = 1.1 ± 0.1 μM) and very low capacity (V_{max} = 3.8 ± 0.2 pmol/10^6 cells per minute); (b) at concentrations above 2 μM the transport followed a sigmoidal plot with $S_{0.5}$ = 6.2 ± 0.2 μM and V_{max} = 19.7 ± 0.5 pmol/1 0^6 cells per minute. The Hill plot, represented in Figure 5, confirms the different behavior of the transporter varying the substrate

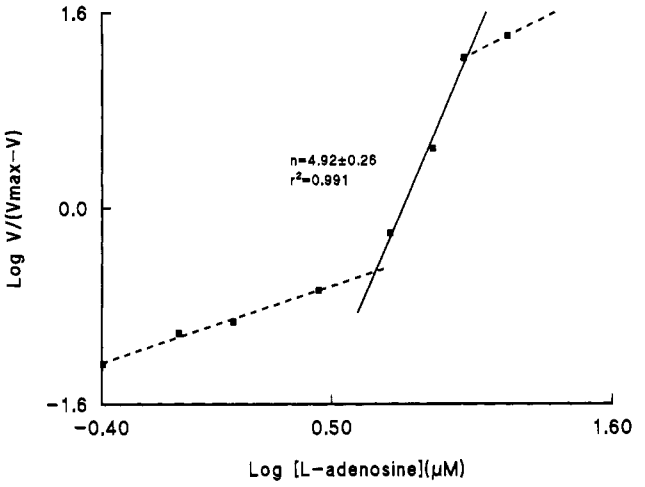

FIGURE 5: Hill representation for L-[^{3}H]adenosine transport in cultured chromaffin cells. This plot corresponds to the results from Figure 4.

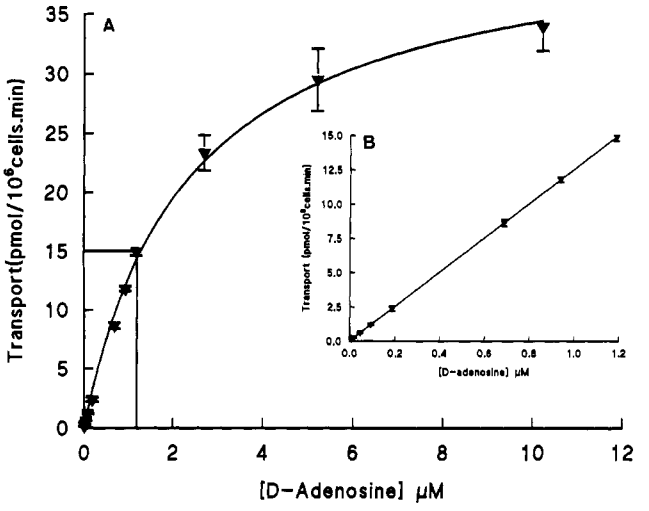

FIGURE 6: Michaelis–Menten representation for the D-[^{3}H]adenosine transport in chromaffin cells. The cells (250000) were incubated with different extracellular concentrations of D-[^{3}H]adenosine as described in Methods. Insert shows the similar representation corresponding to 0.01–1.2 μM D-adenosine concentrations. Values are the means ± SD of three experiments performed in quadruplicate.

concentrations, presenting three components. At low and high substrate concentrations the slope of Hill plot (Hill coefficient) is close to unity. In the intermediate range of substrate concentrations, the Hill coefficient is 4.9 ± 0.3, showing a pronounced positive cooperativity for the L-isomer of adenosine, was even higher than that observed in plasma membrane vesicles. These results indicate that the transporter presents a positive kinetic cooperativity and allosteric cooperativity between subunits.

To determine if the cooperativity phenomen observed in the nucleoside transporter is exclusive for the L-isomer, additional transport studies using very low concentrations of D-adenosine were carried out. The adenosine transport was measured at different extracellular concentrations of D-adenosine ranging from 0.01 to 10 μM as described in Methods. Due to the specific activity of D-[^{3}H]adenosine commercially available, 0.01 μM was the lowest concentration that could be used to measure detectable transport. As shown in Figure 6, the saturation curve followed a Michaelis kinetic, even at very low D-adenosine concentrations. Thus, the cooperativity phenomen of nucleoside transporter was not observed by using D-adenosine in our experimental conditions.

The metabolism of L-[^{3}H]adenosine by cultured chromaffin cells was also assayed as described in Methods. In contrast

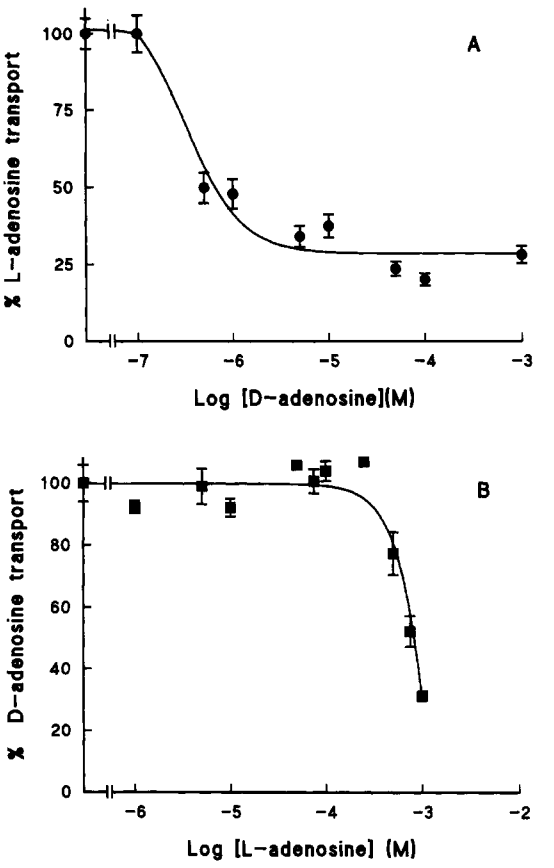

FIGURE 7: (A) Inhibition of L-[^{3}H]adenosine transport by D-adenosine in cultured chromaffin cells. An amount of 250000 cells was incubated with 5 μM L-[^{3}H]adenosine and different concentrations of the D-isomer, during 30 s at 37 °C. The NBTI-sensitive transport was determined as described in Methods. Values are represented as % transport with respect to control and are means of four experiments performed in quadruplicate. (B) Inhibition of D-[^{3}H]adenosine transport by L-adenosine. The cells were incubated with 1 μM of D-[^{3}H]adenosine in the presence of different concentrations of the L-isomer; and NBTI-sensitive transport was determined as described in Methods. Values are expressed as % transport with respect to control and are means of six experiments performed in quadruplicate.

to the D-isomer, L-adenosine was not metabolized during the transport experiments. Even when cells were incubated for 30 min at 37 °C with 15 μM of L-[^{3}H]adenosine, 100% of this isomer cochromatographed as L-adenosine.

The inhibition studies of L-[^{3}H]adenosine transport by D-adenosine and vice versa showed incomplete inhibition in both cases as is shown in Figure 7. Nevertheless, the concentrations required to obtain a 50% inhibition (IC_{50}) were very different, being 0.3 μM for D-adenosine and 2 mM for L-adenosine to inhibit the transport of L- and D-isomer, respectively (Figure 7). This inhibition pattern cannot be explained on the basis of a competitive mechanism with a simple model of the transporter. However, this behavior could fit into a more complex model of the transporter as explained in the following description.

Mnemonical Model with Multiple Conformational Steps. The kinetic model for the adenosine transporter is shown in Figure 8. This scheme is a mnemonical model based on the assumptions that:

(1) It is a zero-trans entry.

(2) The release of the substrate (A_i) from the Form AA is much faster than the internalization of the transporter AA (step k).

(3) The conformational transitions between the free transporters are very slow with respect to the binding steps of the substrate. It is also assumed that the thermodynamically stable

form, in the absence of substrate, is the form **aa**. Therefore, the velocity constants of the transitions between the form **aa** and the form **bb** are equal to 0, as occurs with the corresponding constant of the transition between **Aa** and **Ab**.

(4) Two molecules must be bound to the transporter (form **AA**) to be internalized; thus, the constant of the transition between $\mathbf{Ab}_{ext} \rightarrow \mathbf{Ab}_{int}$ is equal to 0.

If assumtion 4 is not considered then, the term α_1 [A] has to be included in the numerator of the velocity equation. Alternatively, if these assumptions were not used, the velocity equation, although similar, would have more complex α and β constants.

The velocity equation was solved with a computer program described by Varon et al. (1991).

$$\frac{V}{[T]} = \frac{\alpha_2[A]^2 + \alpha_3[A]^3 + \alpha_4[A]^4 + \alpha_5[A]^5}{\beta_0 + \beta_1[A] + \beta_2[A]^2 + \beta_3[A]^3 + \beta_4[A]^4 + \beta_5[A]^5}$$

$$\alpha_2 = 2kk'k_{11}k_{12}k_7k_5(k_2 + k_{10} + k_{13})$$

$$\alpha_3 = 2kk'[k_{11}k_7k_5(k_{12}k_3 + k_9k_{13}) + k_1k_{12}k_7k_5(k_{10} + k_{13})]$$

$$\alpha_4 = 2kk'k_7k_5(k_{11}k_9k_3 + k_1k_{12}k_3 + k_1k_9k_{13})$$

$$\alpha_5 = 2kk'k_1k_9k_7k_3k_5$$

$$\beta_0 = k_{11}k_{12}k_8k'(k_4 + k_6 + k)(k_2 + k_{10} + k_{13})$$

$$\begin{aligned}\beta_1 = {} & k'k_7k_{11}k_{12}(k_2 + k_{10} + k_{13})(k_4 + k_6 + k) + \\ & k'k_5k_{11}k_{12}[k_4(k_2 + k_{10}) + k(k_2 + k_{10} + k)] + \\ & k'k_3k_{11}k_{12}k_8(k_6 + k) + k'k_9k_{11}k_{13}k_8(k_4 + \\ & k_6 + k) + k'k_1k_{12}k_8(k_{10} + k_{13})(k_4 + k_6 + k)\end{aligned}$$

$$\begin{aligned}\beta_2 = {} & k_7k_5[k_{11}k_{12}(k_2(k + k') + k_{10}(k + k') + \\ & k_{13}(k + k') + k_4k') + k_{11}k'(k_2k + k_{10}k_4 + k_3k + \\ & k_{13}k + k_2k_4) + k_{12}k'(k_2k_4 + k_2k + \\ & k_{10}k + k_{13}k)] + k_7k_3k_{11}k_{12}k'(k_6 + k) + \\ & k_3k'[k_5k_{11}k_{12}k + k_8k_6(k_9k_{11} + k_1k_{12})] + \\ & k_1k_5k_{12}k_{10}k'(k_4 + k) + [k_9k_7k_{11}k_{13}k' + \\ & k_1k_7k_{12}k'(k_{10} + k_{13}) + k_1k_9k_{13}k_8k'](k_4 + k_6 + k)\end{aligned}$$

$$\begin{aligned}\beta_3 = {} & k_7k_3k_5[k_{11}k_{12}(k + k') + kk'(k_{11} + k_{12})] + \\ & k_9k_7k_5[k_{11}(k_{13}k + k_{13}k' + k_4k' + kk') + \\ & k_2k'(k_4 + k) + k_{13}kk'] + k'k_6k_3(k_9k_7k_{11} + \\ & k_1k_7k_{12} + k_1k_9k_8) + k_1k_7k_5[k_{12}(k_{10}k + \\ & k_{10}k' + k_{13}k' + k_{13}k + k_4k' + kk') + k_{10}k'(k + k_4)] + \\ & k_1k_9k_7k_{13}k'(k_4 + k_6 + k)\end{aligned}$$

$$\begin{aligned}\beta_4 = {} & k_9k_7k_3k_5[k_{11}(k + k') + kk'] + k_1k_7k_3k_5k_{12}(k + k') + \\ & k_1k_9k_7k_5[k_{13}(k + k') + k'(k_4 + k)] + k_1k_9k_7k_3k_6k'\end{aligned}$$

$$\beta_5 = k_1k_9k_7k_3k_5(k + k')$$

DISCUSSION

Transport studies using D-adenosine have disadvantages, because of the rapid intracellular metabolism of this nucleoside by the action of adenosine kinase and adenosine deaminase (Rotllán & Miras-Portugal, 1985; Newsholme et al., 1985). The problems derived from intracellular metabolism can be solved using L-adenosine, that is recognized by the nucleoside transporter but is a poor substrate for adenosine deaminase and adenosine kinase (Gu et al., 1991). L-Adenosine and other L-nucleosides were first synthesized to identify the metabolically stable analogs to facilitate the study of nucleic acids and increase the therapeutic usefulness of nucleosides such as mercaptopurine (Acton et al., 1964). Recently, Gu et al. (1991) and Gu and Geiger (1992) have found that L-adenosine is taken up by rat brain synaptoneurosomes and to the same extent as the D-isomer. The L-isomer remains stable during the experimental time of transport. However, L-adenosine was not accumulated into retinal cells (Blazynski, 1991).

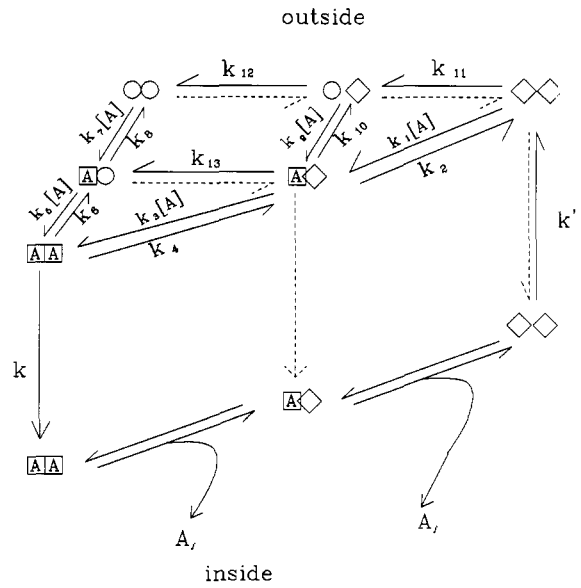

FIGURE 8: Mnemonical model for a dimeric adenosine transporter with allosteric and kinetic cooperativity. The dimer of adenosine transporter may be composed by monomers with different conformations: OO represents the **aa**-form of the adenosine transporter with high affinity and low capacity. ◇◇ represents the **bb**-form of the adenosine transporter, with lower affinity and higher capacity. Boxed A's represents the transporter with two adenosine molecules bound. A and A_i means, respectively, extracellular and intracellular adenosine.

Taking into account these findings, the characterization of D- and L-adenosine transport was done in plasma membrane vesicles from chromaffin tissue, where the metabolism of the D-isomer is also excluded. The transport of the L-isomer was also characterized in cultured chromaffin cells, where the absence of its metabolism allowed for the estimates of rates of adenosine transport.

The transport of D-[^{3}H]adenosine in plasma membrane vesicles was saturable and the K_m and V_{max} parameters were similar to those obtained in cultured chromaffin cells where the K_m values was 1–2 μM (Delicado et al., 1991; Sen et al., 1992). Moreover, these results agree well with those obtained for NBTI-sensitive adenosine transport in rat brain cortical synaptosomes, where the K_m was 1–5 μM (Shank & Baldy, 1990). The L-adenosine is also incorporated into the plasma membrane vesicles, indicating that the L-isomer is a substrate for the NBTI-sensitive nucleoside transporter expressed in chromaffin tissue. These results suggest that the nucleoside transporters in the studied neural tissues lack the marked stereoselectivity exhibited by leukemia L-1210/AM cells and mouse erythrocytes where the transport rates for D-adenosine were 17- and 22-fold higher in comparison to the L-isomer (Gati et al., 1989). It is extremely interesting to note that the L-[^{3}H]adenosine transport in chromaffin cells followed a peculiar sigmoidal kinetic in both studied preparations,

indicating that the nucleoside transporter presents a different behavior with the L-isomer, which is not observed with the natural D-isomer. The positive cooperativity observed in intact cells was even higher than that obtained in plasma membrane preparations. The shape of the Hill plot represented in Figure 5 corresponds to a hysteretic (mnemonical) enzyme. This type of kinetic cooperativity in a monomeric enzyme requires the free enzyme in at least two different conformations to be able to react with the substrate at different rates and a "slow" conformational transition between them, showing a Hill coefficient close to 2 (Ricard & Cornish-Bowden, 1987; Nari et al., 1984). However, the Hill coefficient calculated in Figure 5 for cultured cells was 4.9 which could be explained by a similar kinetic model, but with a dimeric transporter. In Figure 8, a kinetic model for the nucleoside transporter is proposed, assuming a dimeric structure. The nucleoside transporter could be present in various conformations, which have different kinetic parameters for L-adenosine (and the transition between them is "slow"). The form **aa** has a high affinity and low transport capacity for the L-isomer, that predominates at low substrate concentrations (below 2 μM) and it is responsible for the Michaelis–Menten kinetic observed in Figure 4. This substrate, L-adenosine, induces a conformational change (form **bb**). The transition between the form **bb** and form **aa** is very "slow", allowing the existence of various conformations of the dimeric transporter. The form **bb** has low affinity for the L-isomer ($S_{0.5}$ was 6 μM). Thus, as long as the substrate concentration increases, the different forms of the transporter coexist and therefore, this mechanism is responsible for the kinetic cooperativity. Moreover, in this model the allosteric cooperativity between the monomers also contributes to the great positive cooperativity observed in Figure 5. At high substrate concentrations only the form **bb** predominantes. By this means, the remarkable cooperativity observed in intact cells results from a coexistence between kinetic cooperativity as described for mnemonical enzymes and the allosteric cooperativity between the subunits of the transporter.

It is interesting to note that some hysteretic enzymes such as β-glucosidase and polyphenol oxidase modify the mnemonical behavior when they are exposed to different conditions of pH, suggesting that an ionizable group may affect the catalysis and the "slow" transition of the enzyme (Banks et al., 1979; Nari et al., 1984; Valero & García-Carmona, 1992). These data could explain the lower cooperativity observed in plasma membrane vesicles than that observed in whole cells. During the preparation of these vesicles, the tissue was submitted to a drastic treatment, such as change in osmolarity and ionic strength, which could induce the loss of the kinetic cooperativity of the transporter. Thus, in the vesicle preparations only the allosteric cooperativity between the subunits of the transporter was observed, as reported by NBTI binding to plasma membrane vesicles (Casillas et al., 1992).

Studies to characterize molecularly the nucleoside transporter have been carried out in several tissues (Jarvis & Ng, 1985; Gati et al., 1986). Photoincorporation studies with [^{3}H]-NBTI into plasma membrane polypeptides from chromaffin tissue and SDS-polyacrylamide gel electrophoresis have shown the presence of two molecular species corresponding to the monomeric and dimeric forms of the nucleoside transporter (Torres et al., 1988). Moreover, radiation-inactivation analysis "in situ" of erythrocyte membranes have shown that the transporters exist in the membrane as a functional dimer (Jarvis et al., 1986). Recently, polyclonal antibodies against the NBTI-sensitive nucleoside transporter of human erythrocytes were obtained, that also recognize the dimeric form (Kwong et al., 1992). These data are consistent with the positive cooperativity observed in the NBTI binding to plasma membranes from chromaffin tissue, suggesting that the two subunities of the dimeric form can bind the NBTI ligand (Casillas et al., 1992). However, the existence of tetrameric forms, as described for the glucose transporter, cannot be excluded (Hebert & Carruthers, 1991).

The absence of cooperativity in the D-adenosine transport could be explained if the conformational states of the nucleoside transporter had a similar affinity and transport capacity for the D-isomer. This behavior with the D-isomer can also be explained by the different affinities of the transporter (form **bb**) for the two isomers (k_1/k_2), k_1 being much higher for D-isomer than for the L-isomer. Taking into account the IC_{50} values for the two isomers, a k_i value of 3 orders of magnitude higher for D-adenosine than for L-adenosine can be estimated. Therefore, using D-adenosine as a substrate at the allowed experimental concentrations, the slow transitions between the form **bb** and the form **aa** are excluded. Nevertheless, the possible transition of the transporter at very low concentrations (below 0.01 μM) can take place. This aspect could be of particular physiological relevance suggesting that at very low D-adenosine concentrations, which are unable to induce a conformational change that would allow its transport, the nucleoside remains at the extracellular space and would interact with high affinity A_1 receptors (K_d = 0.5–4 nM) (Williams, 1987). Thus, a "damping" effect could occur on the extracellular concentrations. Although the mnemonical behavior and the cooperativity between monomers are reported here for the first time for a transporter, the adenosine transporter, these kinetic behaviors are well known for the key enzymes of metabolic pathways. Phosphofructokinase from bovine liver shows hysteretic interconversion between inactive dimer and active tetramer on the time scale of glycolytic oscillations; pyruvate kinase from red blood cell also presents hysteresis and cooperativity in tetrameric form. Other enzymes of metabolic pathways presents similar mnemonical behavior (Neet & Ainslie, 1980). All these reports increase the physiological relevance of adenosine transport as the first step in maintaining the extracellular levels of adenosine.

From these results, it can be assumed that adenosine transport is regulated by three steps: (a) by conformational changes and association between monomers, (b) by phosphorylation–dephosphorylation (Sen et al., 1990; Delicado et al., 1991; Sen et al., 1993), and (c) by the long-term effects of hormones with modifications in protein synthesis (Fideu & Miras-Portugal, 1992). It is no yet known if a relation exists between the hysteretical and cooperativity phenomenon and the phosphorylation-dephosphorylation of the adenosine transporters. Further work is necessary to fully understand all the regulatory possibilities of the adenosine transporters.

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