Nucleoside transporter and nucleotide vesicular transporter: Two examples of mnemonic regulation

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Research Overview

Nucleoside Transporter and Nucleotide Vesicular Transporter: Two Examples of Mnemonic Regulation

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ABSTRACT According to their relevant roles in the regulation and availability of extracellular levels of purinergic signals, the nucleoside transporter and the nucleotide vesicular transporter are subject to acute regulation. The plasma membrane nucleoside transporter has been shown to exhibit several regulatory mechanisms, such as regulation by long-term signals, phosphorylation/dephosphorylation processes, and allosteric modulation. The present work reviews studies concerning allosteric modulation of nucleoside and nucleotide vesicular transporters, as the first reported examples of mnemonic behavior in transporter proteins, presenting kinetic and allosteric cooperativity. This fact implies that the protein can exhibit different conformations, each one with specific kinetic parameters. Transport substrates are able to induce slow conformational changes between the different forms of the transporter. This kinetic mechanism can provide several physiological advantages, since it allows strict control of transport capacity by changes in substrate concentrations. This allosteric modulation has been confirmed in several experimental models, the nucleoside transporter in chromaffin and endothelial cells from adrenal medulla and the nucleotide vesicular transporter in the chromaffin cell granules and rat brain synaptic vesicles. Taking into account these considerations, the mnemonic regulation described here could be a widespread mechanism among transporter proteins. Drug Dev. Res. 52:11–21, 2001.

Key words: ATP; diadenosine; polyphosphates; adenosine; purinergic transmission; nucleotide vesicular transport; nucleoside transport; mnemonic transporter

INTRODUCTION

Purinergic neurotransmission accounts for the release of ATP and dinucleotides by exocytosis from nerve terminals and their action through specific plasma membrane receptors [Pintor et al., 1992; Zimmermann, 1994; North and Barnard, 1997; Pintor et al., 1999]. The widely distributed family of ectonucleotidases finishes the extracellular actions of nucleotides and dinucleotides and yields adenosine as the final product [Zimmermann, 1999]. In addition, adenosine performs its neuromodulatory actions interacting through specific receptors [Palmer and Stiles, 1995]. Finally, the regulation of extracellular levels and the availability of these purinergic signals are achieved mainly by two transport processes.

First there is the plasma membrane nucleoside transporter, which finishes adenosine extracellular actions by its internalization into the cell, which can be considered the last step in purinergic neurotransmission. This transport process is also necessary for the recovery of the nucleotide pool and the cellular energetic state, since the incorporated adenosine is efficiently phosphorylated into mononucleotides by the action of adenosine kinase. Second there is the vesicular nucleotide and dinucleotide transporter, which is involved in the replenishment of secretory vesicles in ATP and dinucleotides. This step is essential for the subsequent recovery of cellular secre-

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tory activity and prepares the cell to undergo exocytosis. Taking into account these considerations, the study and characterization of these two intracellular steps in the Ap_nA/ATP/adenosine cycle and their regulation are also necessary for a complete understanding of the relevant role of purinergic signaling.

Much work on these two transporter systems has been done in neurochromaffin cells from bovine adrenal medulla, which are counterparts of sympathetic neurons. The main function of these cells is exocytotic release of catecholamines in response to nicotinic receptor stimulation. ATP and dinucleotides also are released, together with catecholamines, indicating the importance of purinergic signals in these cells [Burgoyne, 1991; Pintor et al., 1991]. The aim of this article is to review the most relevant findings on the functioning of the plasma membrane nucleoside transporter and the nucleotide vesicular transporter in chromaffin cells, especially with respect to their regulatory mechanisms. These two transporters were the first examples in which the coexistence of allosteric and kinetic cooperativity, characteristic of hysteretic or mnemonic enzymes, has been established. This implies that the transporters can exist in several conformations, differing in their kinetic properties, and slowly isomerize under the action of substrates. The same allosteric modulation has been reproduced in other experimental models, such as nucleoside transport in microvascular endothelial cells of the adrenal medulla and nucleotide transport of synaptic vesicles, as will be discussed later herein. This suggests that mnemonic regulation could be a general property of transporter proteins and may have important physiological implications.

REGULATORY STUDIES OF NUCLEOSIDE TRANSPORTERS: ALLOSTERIC MODULATION

In neurochromaffin cells the only process accounting for nucleoside internalization is a facilitated-diffusion transport system that is sensitive to the transport inhibitor nitrobenzylthioinosine (NBTI), since it is an NBTI-sensitive equilibrative nucleoside transporter (named the *es*-type) [Torres et al., 1990]. The *es*-transporter in chromaffin cells has been the aim of many regulatory studies, which have shown that it is a process with several levels of regulation, as shown in Fig. 1.

First there is long-term regulation of the *es*-transporter in chromaffin cells by hormones acting through

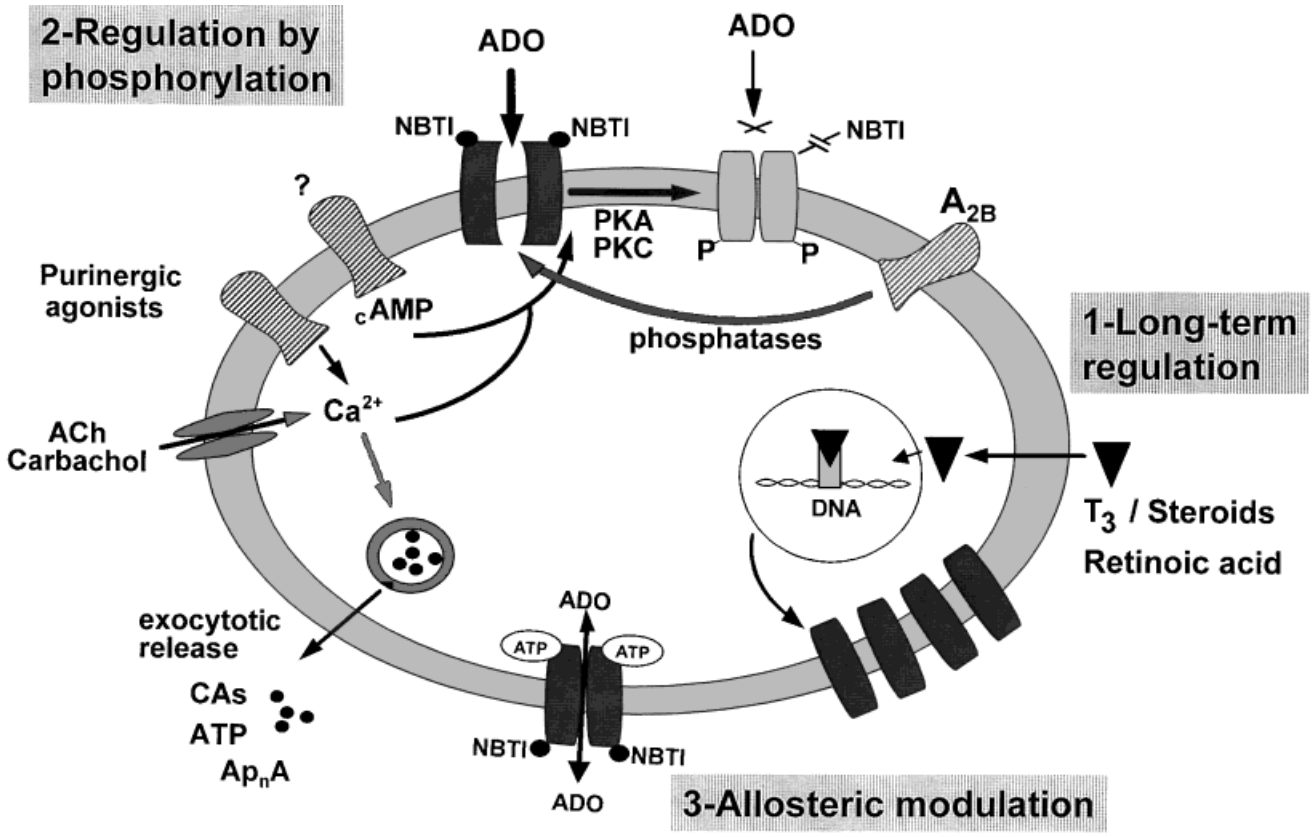

Fig. 1. Different levels of regulation of the *es*-transporter in neurochromaffin cells. NBTI, transport inhibitor nitrobenzylthioinosine. ADO, adenosine; ACh, acetylcholine; cAMP, cyclic AMP; CAS, catecholamines; Ap_nA, diadenosine polyphosphates; T_3, thyroid hormone T_3; A_{2B}, A_{2B} adenosine receptors.

nuclear receptors involved in the synthesis of new transporter molecules. Long-term treatment of cells with thyroid hormones, which are crucial for the development of aminergic neurons, increased adenosine transport in chromaffin cells. In contrast to the stimulatory effect of T3, other long-term treatments, such as those with glucocorticoids, steroids, and retinoic acid, significantly inhibited adenosine transport and also were able to antagonize stimulation by T3 [Fideu and Miras-Portugal, 1992, 1993].

Second, it has been described that the *es*-transporter in chromaffin cells is the target of several signals of physiological relevance in these cells. Extracellular signals, such as secretagogues and purinergic receptor agonists, which are coupled to an increase in the intracellular calcium concentration and the activation of protein kinase C, significantly modified adenosine transport by decreasing transport capacity and, at the same time, decreasing the number of high-affinity binding sites for NBTI [Delicado et al., 1991; Sen et al., 1993]. However, transport affinity was not modified. The same inhibitory effect was obtained with intracellular signals, such as forskolin, which directly activated the cyclic AMP–protein kinase A pathway [Sen et al., 1990]. This regulation has been confirmed at the single-cell level by flow cytometry [Sen et al., 1998]. These findings indicated that a regulatory mechanism by phosphorylation/dephosphorylation processes was taking place, because the opposite effect, namely, an increase in adenosine transport capacity, was obtained with the activation of protein phosphatases through the stimulation of A_{2B} adenosine receptors [Delicado et al., 1990].

Similarly, in neuro-2A neuroblastoma cells, which have different transport systems, only the *es*-component, and not the Na^+-dependent one, was susceptible to inhibition by protein kinases A and C [Sen et al., 1999]. However, in some non-neural *es*-systems and Na^+-dependent processes the regulation by phosphorylation/dephosphorylation seems to present some variability [Lee, 1994; Sayós et al., 1994; Coe et al., 1996; Soler et al., 1998]. Furthermore, the non-neural *es*-transporter of microvascular endothelial cells from the adrenal medulla was not regulated to any extent by protein kinases A and C [Sen et al., 1996], indicating that not all the *es*-sytems are susceptible to regulation by the same kind of mechanisms.

Finally, short-term regulation implies allosteric modulation by direct effectors. It was described that [^{3}H]NBTI binding to purified chromaffin cell plasma membranes showed a curvilinear plot, with a Hill coefficient higher than unity [Casillas et al., 1992]. This finding was indicative of positive cooperativity between transporter subunits, first suggested in previous studies [Jarvis et al., 1980; Ijzermann et al., 1989]. In addition, transport studies of plasma membrane vesicle preparations of chromaffin cells that were resealed in the presence of ATP showed a significant increase in uridine transport and a parallel increase in [^{3}H]NBTI binding [Casillas et al., 1993a]. These findings suggested for the first time the existence of an intracellular ATP binding site on the nucleoside transporter [Delicado et al., 1994]. More recent studies have shown that besides allosteric modulation by intracellular effectors, such as ATP, the adenosine transporter in chromaffin cells is regulated by extracellular levels of its own substrate, adenosine. This has led to the proposition of a mnemonic model for the *es*-nucleoside transporter in which kinetic and allosteric cooperation coexist [Casillas et al., 1993b]. The question arises whether allosteric modulation can be a general property of nucleoside transporters. The present article describes a comparative study of two *es*-systems, those present in neurochromaffin and microvascular endothelial cells from adrenal medulla, in terms of allosteric modulation by extracellular adenosine levels.

L-Adenosine Transport Studies of Allosteric Modulation in Endothelial Cells: Comparison with Chromaffin Cells

The allosteric modulation of *es*-transporter was done by means of transport studies using the L-isomer of adenosine as substrate. Although the nucleoside transporter has been shown to be highly stereoselective, with a strong preference for the D-enantiomer of adenosine [Plagemann et al., 1988], L-adenosine was incorporated efficiently into chromaffin and endothelial cells from adrenal medulla via the NBTI-sensitive equilibrative nucleoside transporter; in contrast to the situation with the D-isoform, however, L-adenosine was not metabolized [Casillas et al., 1993b].

Similarly to what was found in chromaffin cells, a sigmoidal plot was obtained when the concentration dependence of L-adenosine transport was studied in endothelial cells (Fig. 2). The shape of the curve was characteristic of the typical kinetics of a mnemonic enzyme, and this implies that kinetic behavior varies with substrate concentration. In fact, at very low substrate concentrations (below 2 μM), L-adenosine transport followed Michaelis-Menten kinetics, with high affinity and very low capacity (K_m and V_{max} values of 0.7 μM and 3.48 pmol/min $\times$ 10^6 cells, respectively). At concentrations above 2 μM, the transport exhibited sigmoidal kinetics, with $S_{0.5} = 5.77 \pm 0.61$ μM and $V_{max} = 22.64 \pm 2.64$ pmol/min $\times 10^6$ cells. The Hill plot analysis (Fig. 2, insert) confirmed the different behavior of the transporter with the extracellular adenosine concentrations. At very low and high substrate concentrations, the Hill coefficient was close to unity. But in the intermediate range of substrate concentrations, a remarkable positive cooperativity with a Hill coefficient of 4.5 ± 0.75 was noted. The mnemonic

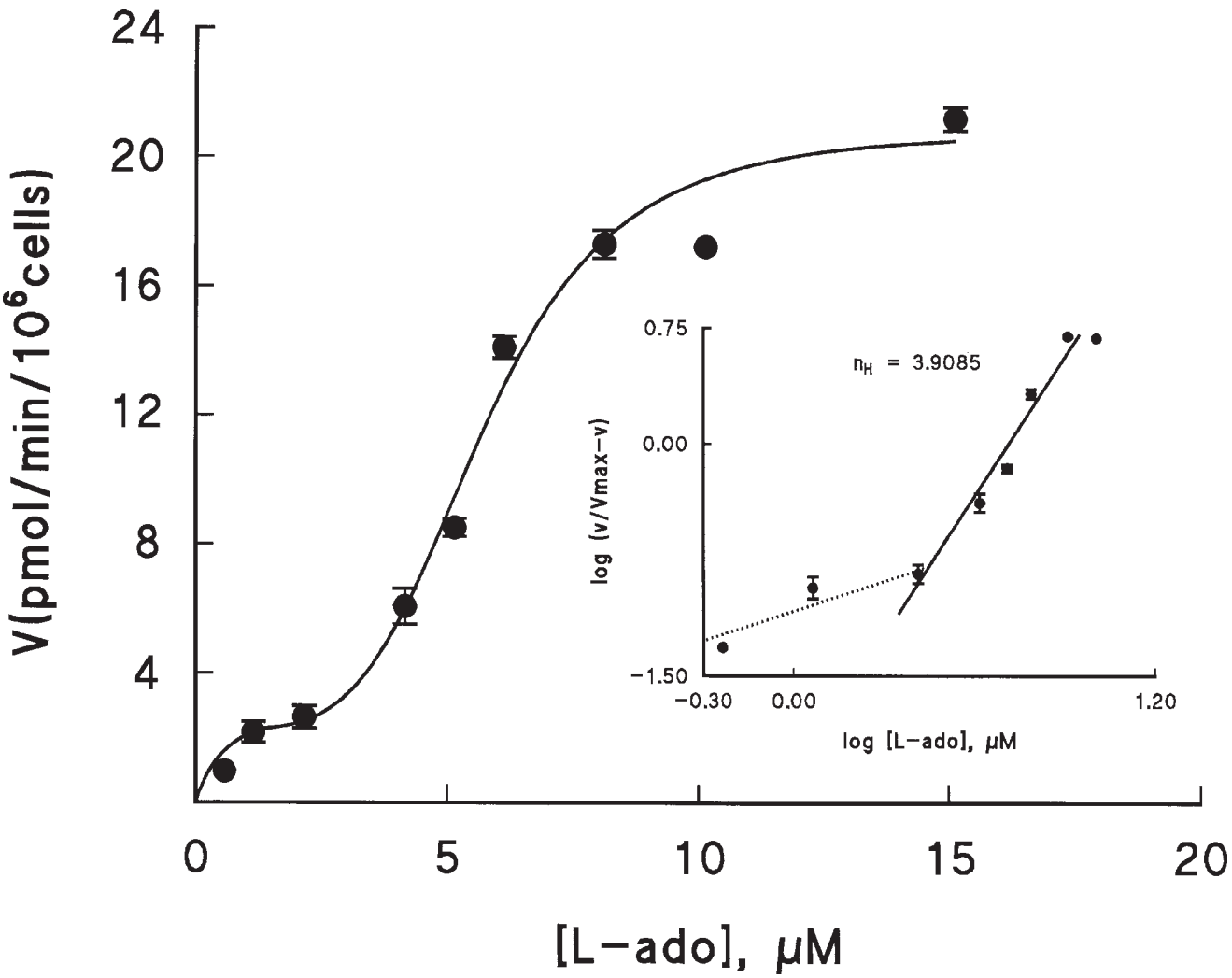

Fig. 2. Concentration dependence of L-adenosine transport in microvascular endothelial cells of the adrenal medulla. Cells were incubated with different concentrations of L-[^{3}H]adenosine, and nitrobenzylthioinosine–sensitive transport was determined for 30 sec of incubation at 37°C. The data were analyzed by nonlinear regression of a sigmoid equation. The insert shows the transformation of the data for the L-adenosine transport according to the Hill equation. This represents a typical experiment performed in quadruplicate.

nucleoside transporter previously described in chromaffin cells also showed high positive cooperativity (Hill coefficient = 4.9) and kinetic parameters similar to those found in endothelial cells for L-adenosine transport [Casillas et al., 1993b].

As with chromaffin cells, cross-inhibition studies between L- and D-adenosine transport in endothelial cells showed incomplete inhibition in both cases; IC_{50} values of 30–40 μM for D-adenosine and 3 mM for L-adenosine were needed to inhibit the transport of the L- and D-isomer, respectively. The pseudo-Hill coefficients calculated from the inhibition curves were also different, being close to unity (n_H = 0.92) for the D-isomer (Fig. 3A) and significantly greater than unity (n_H = 2.08) for the L-isomer (Fig. 3B).

On the other hand, the physiological D-isomer of adenosine presented saturable Michaelis-Menten kinetics [Sen et al., 1996]. The absence of cooperativity does not exclude its possible existence at very low D-adenosine concentrations, in the low nanomolar range. Taking into account that cross-inhibition studies of D- and L-adenosine transport presented a difference of four orders of magnitude in the respective IC_{50} values, the conformational changes of the transporter that take place in the micromolar range for L-adenosine are expected to occur at the low nanomolar range for D-adenosine. Taking into account the high specific activity of commercial D-[^{3}H]adenosine, accurate measurements of adenosine transport at concentrations below the nanomolar range cannot be obtained in radiometric studies.

The mnemonic behavior of the nucleoside transporter in endothelial and chromaffin cells from adrenal

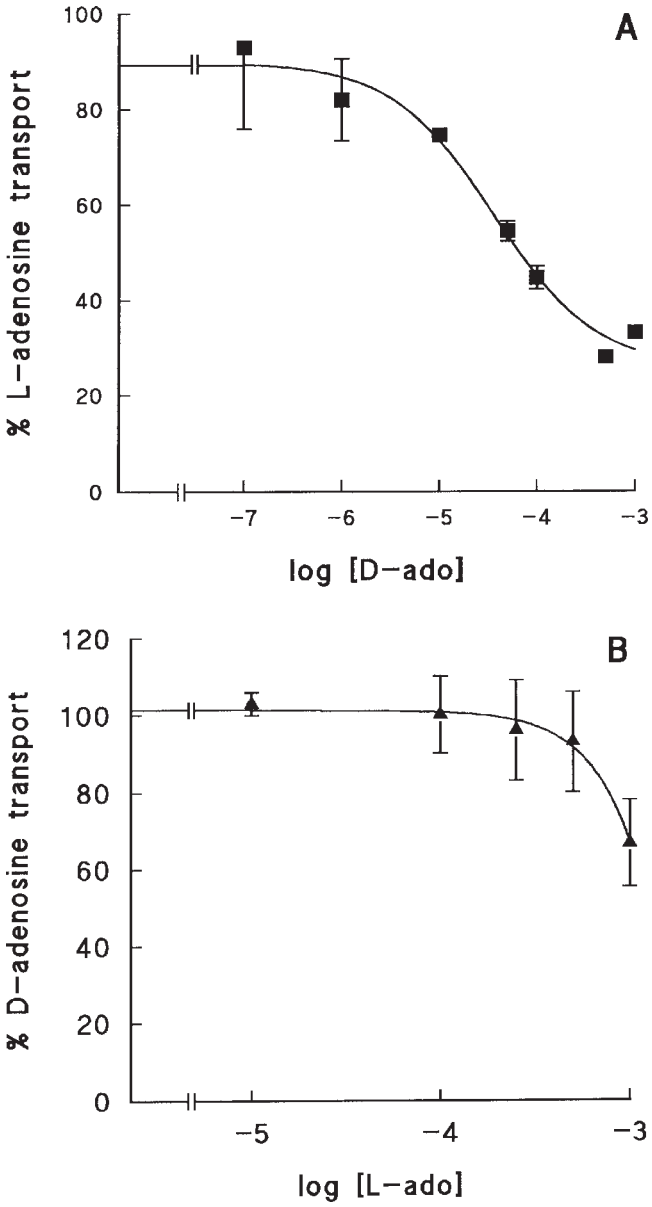

Fig. 3. Inhibition studies of D- and L-adenosine transport in bovine adrenomedullary endothelial cells. **A:** The transport of 5 μM of L-[^{3}H]adenosine was determined for 30 sec in the presence of graded concentrations of D-adenosine. Values are the means ± s.d. of three experiments performed in quadruplicate. **B:** The transport of 5 μM D-[^{3}H]adenosine was measured for 1 min in the presence of graded concentrations of the L-isomer. This represents a typical experiment of three performed in quadruplicate. In both panels only the nitrobenzylthioinosine–sensitive component is shown. The values are represented as percentage transport with respect to control.

medulla indicates that it is not tissue-specific but substrate-specific and can be reproduced in other tissues, as occurs with mnemonic enzymes, which display these kinetics with certain substrates or when they are exposed to specific conditions [Neet and Ainslie, 1980; Valero and García-Carmona, 1992]. In contrast, *es*-transporters of chromaffin and endothelial cells markedly differed from regulation by protein kinases and phosphatases. It is clear from these results that the two *es*-transporter proteins could have similar structural elements responsible for the kinetic characteristics, but they can differ in the presence of certain specific residues susceptible to phosphorylation by protein kinases.

Kinetic Model for Nucleoside Transporter

The mnemonic model for the *es*-transporter is based on the following assumptions:

- It is a zero-*trans* entry.
- Two substrate molecules must be bound to the transporter to be internalized, and their release is much faster than the internalization of the transporter.
- The conformational changes between the free transporters are very slow with respect to the binding steps of the substrate, allowing the existence of different conformations of the transporter.

The velocity equation was solved with the computer program described by Varon et al. [1991].

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

The nucleoside transporter could be present in several conformations, which have different kinetic parameters for L-adenosine, and they are controlled by the extracellular substrate concentrations (Fig. 4). At very low substrate concentrations (below 2 μM), the *form aa*, which has high affinity (close to 1 μM), predominates and is responsible for the Michaelis-Menten kinetics. The substrate L-adenosine induces a conformational change (*form bb*), as described for mnemonic enzymes [Ricard and Cornish-Bowden, 1987]. The *form bb* has low affinity ($S_{0.5}$ close to 6 μM) and predominates at very high substrate concentrations. As mentioned previously, the transition between *form bb* and *form aa* is very slow, allowing for the existence of various conformations of the transporter. As long as the substrate concentration increases, different forms of the transporter coexist; this mechanism of action is responsible of kinetic cooperativity. However, the high positive cooperativity observed ($n_H = 4.5 \pm 0.75$) cannot be explained on the basis of a monomeric transporter exhibiting kinetic cooperativity, but it indicates the presence of at least a dimeric form of the transporter and therefore accounts for allosteric cooperativity between the subunits of the transporter.

NUCLEOTIDE VESICULAR TRANSPORTER

Nucleotide Transport Into Chromaffin Granules

During the late 1970s and early 1980s, Winkler and colleagues studied the incorporation of radioactive nucle-

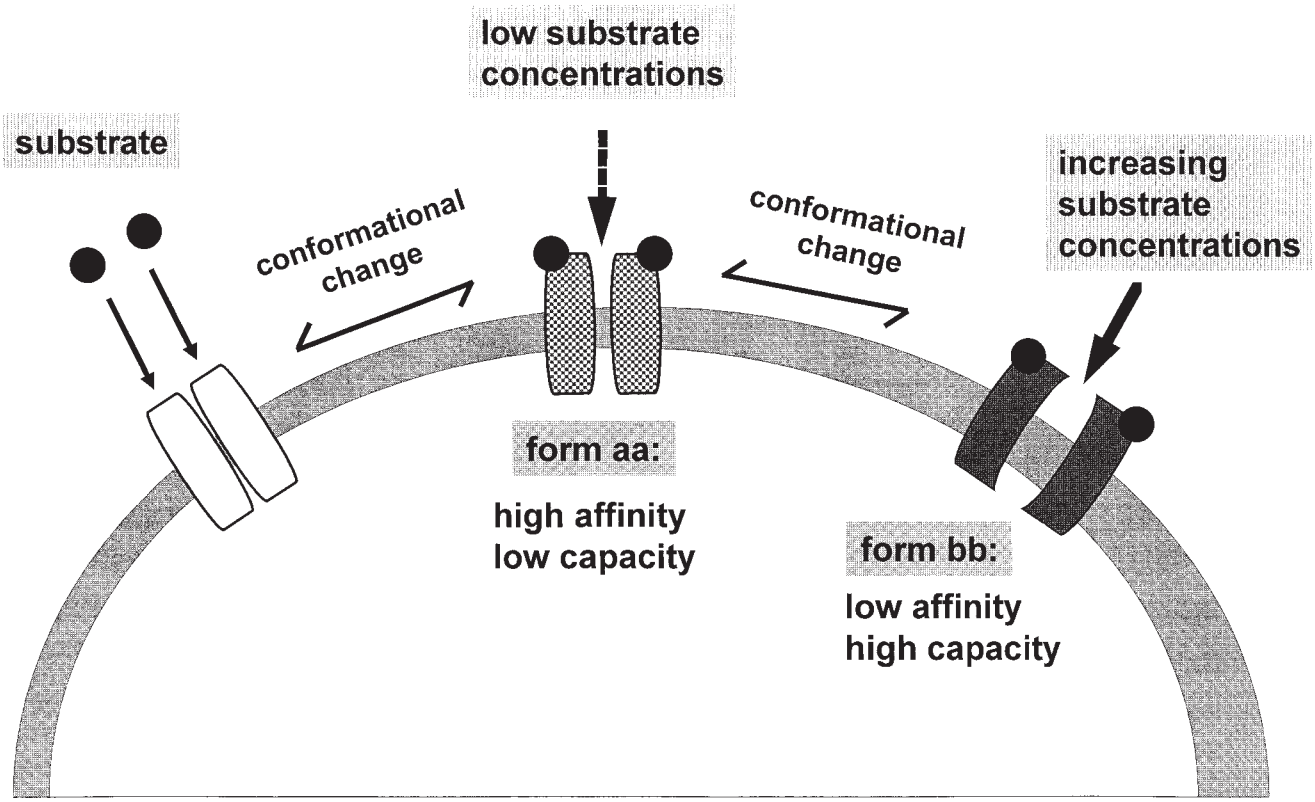

Fig. 4. Mnemonic model for the nucleoside transporter.

otides into mature intact chromaffin granules. The temperature dependence of nucleotide uptake and the inhibition of nucleotide incorporation by atractyloside, a blocker of mitochondrial nucleotide exchanger, indicated the existence of a carrier-mediated process. Further studies showed that nucleotide transport into chromaffin granules was driven by the electrical part of the proton gradient, generated by vacuolar type H^+-ATPase. Chromaffin granule nucleotide transporter showed a broad range of specificity, being able to internalize a large variety of nucleotides (ATP, ADP, AMP, GTP, UTP) with K_m values in the mM range [Aberer et al., 1978; Weber and Winkler, 1981].

Studies of nucleotide vesicular transport also were carried out in chromaffin granule "ghosts." These ghosts constitute a model to study the mechanisms of vesicular storage without the interference of endogenous components, and they have been used widely in the characterization of catecholamine uptake into the granules [Henry et al., 1998]. However, early studies done with chromaffin granule "ghosts" failed to show a transport-mediated uptake mechanism, concluding that nucleotides crossed the granule "ghost" membrane mainly by passive diffusion [Grüninger et al., 1983]. It was not until 1996 that Bankston and Guidotti clearly established the presence of a membrane potential–dependent nucleotide transporter in the chromaffin granule "ghosts" [Bankston and Guidotti, 1996].

This was the state of the field when a new approach to investigating nucleotide transport in chromaffin secretory granules was developed by the research group of Gualix and co-workers. This experimental approach was based on the use of fluorescent derivatives of adenine nucleotides as transport substrates and the analysis and measurement of the internalized nucleotides by high-performance liquid chromatography. The fluorescent nucleotide analogs contained an additional etheno bridge in the purine ring and were known as etheno-nucleotides (ε-nucleotides). Studies of etheno-nucleotide transport were done in intact chromaffin granules, since they may reflect the situation of the granules *in vivo*, and these studies established the incorporation of the fluorescent derivatives of ATP, ADP, and AMP (ε-ATP, ε-ADP and ε-AMP) into chromaffin granules [Gualix et al., 1996a]. When studied in a broad range of substrate concentrations, transport of ε-adenine nucleotides did not follow Michaelis-Menten kinetics; instead, a complex non-hyperbolic saturation curve was obtained (Fig. 5). The dependence of transport velocity with respect to extra-granular nucleotide concentration made it necessary to interpret the saturation curve as the superposition of several sigmoidal kinetics. There-

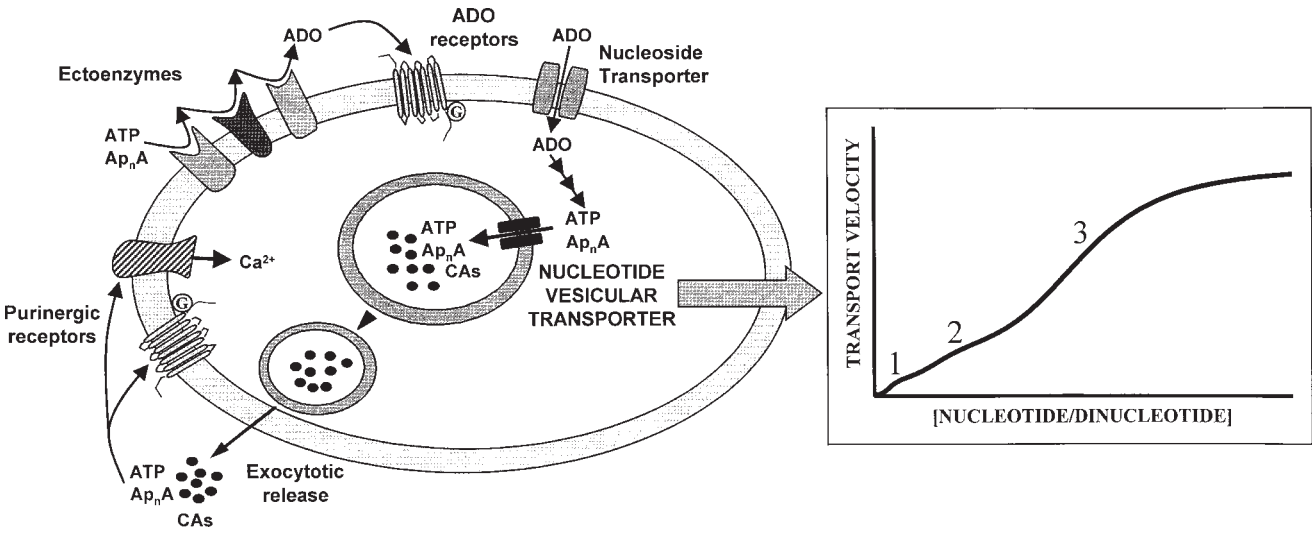

Fig. 5. The nucleotide vesicular transporter in the Ap_nA/ATP/adenosine cycle in chromaffin cells. ADO, adenosine; Ap_nA, diadenosine polyphosphates; CAs, catecholamines.

fore, the experimental data was processed according to the following equation:

$$V = \sum \frac{V_{max} \cdot S^n}{K^n + S^n}$$

The kinetic parameters for each of the sigmoidal components the addition of which accounted for the experimental saturation curves of ε-ATP, ε-ADP, and ε-AMP transport are summarized in Table 1.

Diadenosine Polyphosphate Transport Into Chromaffin Granules

Despite the increasing physiological relevance of diadenosine polyphosphates as extracellular signals, no bibliographic data were available concerning their storage mechanism into the secretory vesicles, a step necessary for these compounds to reach the extracellular space. In 1997, by means of a combination of radiometric and etheno-derivative-based fluorimetric techniques, we described for the first time the transport of diadenosine polyphosphates into secretory granules, the chromaffin granules [Gualix et al., 1997].

When studied in a broad range of substrate concentrations, diadenosine polyphosphate transport into chromaffin granules showed complex non-hyperbolic saturation curves (Fig. 5). The shape of these curves was similar to that noted for ε-mononucleotide transport, and the analysis of the experimental data was done according to the same considerations as described for ε-ATP, ε-ADP and ε-AMP transport (see the previous section). The kinetic parameters of the sigmoidal curves that are the constituents of the saturation curves for diadenosine polyphosphate transport also are summarized in Table 1. It is interesting to note that the affinity values for dinucleotide transport appeared to be adapted to the intracellular concentrations of Ap_nA. The cytosolic concentrations of these substances have been described to be in the low micromolar range in resting cells. However, dinucleotide levels can increase several times in cells with high proliferative activity or under certain conditions, such are

TABLE 1. Kinetic Parameters of Nucleotide and Dinucleotide Transport Into Chromaffin Granules

	Curves	ε-ATP	ε-ADP	ε-AMP	ε-Ap_4A	[^{3}H]Ap_5A
K (μM)	1	250	150	200	16	16
	2	1,000	900	1,200	75	125
	3	3,000	3,600	3,200	—	545
V_{max} (pmol · min^{-1} · mg prot.$^{-1}$)	1	20	25	10	18	15
	2	40	35	40	13	8
	3	190	300	55	—	102
n_H	1	3	2	2	2	2
	2	4	4	3	4	4
	3	5	5	5	—	6

Values of the kinetic parameters of the curves that are the constituents of the experimental saturation curve for nucleotide and dinucleotide transport. K, V_{max} and n_H are, respectively, the values of the corresponding $S_{0.5}$ affinity, partial V_{max}, and Hill number, considering each single curve.

environmental stress, and the role of cytosolic Ap_nA levels in the cellular decision to trigger proliferation, quiescence, differentiation, and apoptosis is still under discussion [McLennan, 2000]. The transport of diadenosine polyphosphates into secretory vesicles regulates the accessibility of these compounds to the extracellular space but also could be a mechanism for finishing their cytosolic actions, which would give this transport process a relevant physiological meaning.

On the other hand, diadenosine pentaphosphate (Ap_5A) transport into chromaffin granules could be inhibited by other diadenosine polyphosphates (Ap_3A and Ap_4A) and, to the same extent, by the non-hydrolizable analogs of ATP and ADP, ATPγS and ADPβS (Gualix et al., 1997). The inhibitory pattern of nucleotide analogs on Ap_nA transport suggested that both types of substances, adenine mono- and dinucleotide, share a common vesicular transporter.

Nucleotide/Dinucleotide Transport in Chromaffin Granules

From the results described in the preceding sections, the nucleotide transporter in chromaffin granules appears to show a broad range of specificity, since it is able to internalize a large variety of mononucleotides as well as the diadenosine polyphosphates. In contrast to this lack of specificity, the nucleotide/dinucleotide vesicular transporter showed complex behavior when the dependence of the transport velocity with respect to the substrate concentration was analyzed, with the appearance of saturable non-hyperbolic kinetic curves exhibiting various intermediate plateaus (Fig. 5). This high degree of complexity was shared by all the substrates assayed, as has been described in the previous sections. The existence of different transporters, with their respective affinities, acting on the same substrates is not compatible with the positive cooperativity shown by the saturation curves. In allosteric enzymes, the occurrence of various intermediate plateaus on V versus $[S]_0$ plots has been explained by the presence of at least two different forms of the enzyme, differing in their kinetic properties, with slow conformational transitions between them depending on the substrate concentration. The enzymes with such kinetic behavior are known as hysteretic or mnemonic enzymes [Neet and Ainslie, 1980].

Regarding membrane transporters, the existence of mnemonic kinetic behavior has been reported for the facilitated-diffusion nucleoside transporter (see the previous sections and Casillas et al. [1993b]). A model similar to that described for the nucleoside transporter (Fig. 4) could be used to explain the results reported here for the nucleotide vesicular transporter. Nevertheless, a higher degree of complexity is required, owing to the high level of cooperativity, which can reach values of $n_H = 6$ (Table 1). This fact also implies the existence of a dimeric structure for the transporter as the minimum requirement [Gualix et al., 1997]. This proposed mnemonic model for the nucleotide vesicular transporter has been confirmed recently by the application of flow cytometry. The technique has been adapted to the study of vesicular transport in chromaffin granules by measuring the intensity of granule-associated fluorescence due to the internalization of the fluorescent etheno-nucleotides into the granules. Flow cytometry analysis of ε-ATP transport into chromaffin granules showed a complex non-hyperbolic saturation curve, similar to that obtained by high-performance liquid chromatography analysis, indicating that the peculiar kinetic behavior previously described is not due to experimental artifacts but is an intrinsic property of the granular transporter [Gualix et al., 1999a].

Intragranular Metabolism of Nucleotides and Dinucleotides

The methodology used for the study of ε-nucleotide transport into chromaffin granules involved the lysis of the organelles after the transport experiments and the separation and quantification of the granular ε-nucleotides by high-performance liquid chromatography. Thus, this methodology also provides a useful approach to the study of nucleotide metabolism after internalization into the granules. These studies showed the existence of intragranular enzymatic activities that exchange phosphate groups among the stored nucleotides, indicating that the intragranular nucleotide content may not reflect the distribution of nucleotides in the cytosol. In this sense, high levels of ε-ATP could be measured in chromaffin granules after ε-ADP transport [Gualix et al., 1996a]. The presence of ATP inside the granules after ADP transport has been reported by other authors using radiolabeled nucleotides [Aberer et al., 1978]. Moreover, the presence of etheno-adenosine tetraphosphate (ε-Ap_4) in the granules after ε-ATP transport has also been deseribed [Gualix et al., 1996b].

Concerning diadenosine polyphosphates, our studies showed that once internalized in the granules, no further metabolism of these compounds took place [Gualix et al., 1997]. On the other hand, there is no evidence of the presence of any of the enzymes known to synthesize cytosolic Ap_nA in secretory vesicles. However, it is not clear how significant quantities of higher polyphosphates (Ap_6A or Ap_7A) might accumulate in the secretory vesicles as the result merely of its transport from the cytosol, given the low intracellular concentrations of these compounds. Thus, the existence of yet unidentified Ap_nA-synthesizing activities in the secretory vesicles has been suggested. Alternatively, granular Ap_nA could be the result of non-enzymatic-mediated chemical reactions among the mono-

nucleotides stored at extremely high concentrations inside the granules, under conditions of low humidity and pH [McLennan et al., 2000]. Despite all these considerations, intragranular formation of etheno-Ap_nA after ε-mononucleotide transport could not be found under our experimental conditions [Gualix et al., 1996a,b].

Nucleotide Transport Into Synaptic Vesicles

Nucleotide and dinucleotide vesicular transport has special relevance in the central nervous system, to explain the exocytotic release of nucleotide compounds into the synaptic cleft and their role as neurotransmitters and neuromodulators. The first approaches to the study of nucleotide transport into synaptic vesicles were undertaken in the cholinergic model of the *Torpedo marmorata* electric organ [Luqmani, 1981]. In more recent studies, the same methodologic approach used to characterize nucleotide transport into chromaffin granules was applied to the study of the transport system in mammalian brain synaptic vesicles [Gualix et al., 1999b]. The nucleotide transporter present in these vesicles shares many similarities with that previously described in the chromaffin granules, particularly in terms of its kinetic mechanism. Once assayed in a broad range of substrate concentrations, ε-ATP transport into rat brain synaptic vesicles showed a non-hyperbolic two-step saturation isotherm. The addition of two sigmoidal curves was necessary to process the experimental data; the $K_{0.5}$ value of each curve were 0.39 and 3.8 mM, respectively, the corresponding Hill numbers being 2.3 and 12.7 [Gualix et al., 1999b]. The high values obtained for the Hill numbers and the complex shape of the kinetic curve for ε-ATP transport suggested the existence of a multimeric form of the transporter with mnemonic kinetic behavior, as described in the chromaffin granule model. Finally, the presence of ε-ATP in the synaptic vesicles after ε-ADP transport [Gualix et al., 1999b] indicated the existence of intravesicular enzymatic activities that equilibrate the levels of the different nucleotides present in these organelles, as has also been reported for the chromaffin granules.

MNEMONIC TRANSPORTERS. PHYSIOLOGICAL MEANING

The physiological relevance of the allosteric modulation of nucleoside transporters can be explained on the basis of the proposed model shown in the Fig. 4. For the nucleoside transporter it can be concluded that certain levels of the physiological substrate, adenosine at the nanomolar range, are required to induce a conformational change that allows its transport. Below 10 nM concentrations, which are unable to induce the conformational change of the transporter, adenosine is not internalized in the cell and remains in the extracellular space. These low adenosine concentrations are coincident with the concentrations needed to assure the continuous occupancy of A1 adenosine receptors, therefore giving an explanation of the "purinergic" tone seen in the nervous and the vascular systems.

For the vesicular nucleotide transporter it is necessary to take into account that ATP is important for cellular metabolism and the recovery of the cellular energetic state after some situations demanding large amounts of energy, such as neurosecretion or anomalous situations. When ATP concentrations are low, below 0.1 mM in the chromaffin model, the transporter does not work (it is assumed that ε-ATP and the natural substrate ATP have similar kinetic behavior). These ATP levels are coincident with the K_m value of hexokinase in chromaffin cells (K_m = 0.1 mM), indicating that ATP is necessary for the phosphorylation of glucose in the glucolytic pathway. Therefore, the energetic state of the cell is too low to undergo an exocytotic process, and ATP is not transported into the secretory granules. Only when the cellular energetic state recovers as glycolysis proceeds can ATP be transported into the vesicles, assuming that transport capacity increases with ATP availability in a highly cooperative way. The last plateau for ATP transport (K = 3 mM) can be related to the levels of ATP required for inhibition of the phosphofructokinase reaction, as glycolysis is submitted to feedback regulation by ATP-mediated phospho-fructo-kinase inhibition. Only when the cell has recovered its energetic state and functionality and is fully prepared to continue with its secretory activity can nucleotides and dinucleotides be released again and exert their extracellular actions.

Taking into account that adenosine, ATP, and dinucleotides, as well as relevant signals in the nervous system, are important intracellular metabolites, allosteric modulation of nucleoside and nucleotide transporters provides a regulatory mechanism that controls the availability of these compounds for intracellular metabolic requirements. It also assures the required levels of these purinergic signals at the extracellular space.

Although few studies have been done with respect to allosteric modulation, the fact that such modulation is reproduced in chromaffin and endothelial cells of the adrenal medulla and presents similar characteristics in both cell types seems to indicate that it could be a more general property of *es*-transporters—despite the strong differences found in the regulation by phosphorylation/dephosphorylation, In addition, the fact that at the level of the vesicular nucleotide transporter this modulation also occurs in two neural models, the chromaffin granule and the synaptic vesicle, suggests that this property could be a phenomenon that extends to transporter proteins more than expected. Finally, besides allosteric modulation, other levels of regulation have been described for the nucleoside transporter, such as long-term regulation

and regulation by phosphorylation/dephosphorylation, as reviewed herein. Although these possibilities have not yet studied, they cannot be excluded for the vesicular nucleotide transporter. This acute regulation shows the relevance of these transporter processes as key steps in the ATP/Ap_nA/adenosine cycle.

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