Nucleotide Vesicular Transporter of Bovine Chromaffin Granules

EVIDENCE FOR A MNEMONIC REGULATION

Javier Gualix‡§, Miguel Abal‡, Jesus Pintor‡, Francisco García-Carmona‡, and M. Teresa Miras-Portugal¶

From the 1Department of Biochemistry, Faculty of Veterinary, Universidad Complutense, E-28040, Madrid, Spain and the 2Department of Biochemistry, Faculty of Biology, Universidad de Murcia, E-30080, Murcia, Spain

The nucleotide vesicular transport has been studied with the fluorescent substrate analogues, the (1,N°-ethenoadenosine) nucleotides. The transport experiments were carried out with granular preparations from bovine adrenal medulla, and e-ATP, e-ADP, and e-AMP were quantified after separation by high performance liquid chromatography. The granular concentration increase of all three nucleotides was time-dependent. The concentration dependence on e-nucleotide transport to chromaffin granules did not follow the Michaelis-Menten kinetics and presented a similar three-step curve with cooperativity. This shape can be considered to be the result of the addition of three sigmoidal curves with their corresponding kinetic parameters. e-ATP exhibited K values of 0.25, 1, and 3 mM and Vmax values of 0.02, 0.04 and 0.19 nmol min⁻¹ mg of protein⁻¹, for the first, second, and third curves for each step, respectively. e-ADP exhibited K values of 0.15, 0.9, and 3.6 mM and Vmax values of 0.025, 0.035, and 0.3 nmol min⁻¹ mg of protein⁻¹, respectively for the first, second, and third curves. e-AMP exhibited K values of 0.2, 1.2, and 3.2 mM, and Vmax values of 0.01, 0.04, and 0.055 nmol min⁻¹ mg of protein⁻¹, also for the first to third steps. The Hill numbers for e-ATP, e-ADP, and e-AMP were not constant but a function of the transport saturation. The nonhydrolyzable e-nucleotide analogues AMPPPNP, ATPγS, and ADPβS were activators of e-nucleotide transport at concentrations under 1 mM and inhibitors at higher concentrations. Atractyloside and N-ethylmaleimide partially inhibited the nucleotide granular transport. High extragranular ATP concentrations specifically induced the exit of the previously transported granular e-ATP.

Studies of vesicular storage mechanisms that allow the functioning of neural and non-neural secretory tissues are essential to understand the various steps in cellular communication. It is well known that secretory vesicles containing catecholamines or acetylcholine also contain nucleotides such as ATP and ADP, their intragranular levels reaching 0.15 to 0.2 nM in some cases (Winkler and Carmichael, 1982; Zimmermann, 1994). More recently, diadenosine polyphosphates: Ap4A, Ap5A, and Ap6A have also been found (Rodríguez del Castillo, 1988; Pintor et al., 1992b, 1992c; Schlütter et al., 1994). The co-stored vesicular components are also co-released to the extracellular media (Pintor et al., 1991, 1992a; Zimmermann, 1994).

Chromaffin granules from adrenal medulla have been the most employed model by which to study the mechanisms of vesicular storage, both for aminergic and nucleotide components. The vesicular transport of catecholamines has been characterized from a physicochemical, pharmacological, and molecular biology approach in this preparation (Njus and Radda, 1978; Knott et al., 1980; Schermann and Henry, 1983; Liu et al., 1992; Henry et al., 1994). Furthermore, the nucleotide vesicular transport has been characterized using radio-labeled nucleotides and similar specificity, and affinity values have been reported in chromaffin granules and in the cholinergic vesicles from the torpedo electric organ (Aberer et al., 1978; Luqmani, 1981; Grüninger et al., 1983). Molecular biology approaches by which to characterize the nucleotide vesicular transporter have been also made (Schläfer et al., 1994).

The existence of kinetic and allosteric cooperativity was recently demonstrated, for the first time, in the equilibrative nucleoside transporter from neural tissues (Casillas et al., 1993). However, no allosteric regulation for aminergic or nucleotide vesicular transporters has yet been reported. It is possible, though, that such mechanisms exist, which could take into account the rather complex energetic and metabolic situations related to the intracellular ATP levels, as occurs with the key regulatory enzymatic steps in cellular metabolism (Ricard and Cornish-Bowden, 1987).

In this work, the nucleotide vesicular transport has been characterized by using the fluorescent (1,N°-ethenoadenosine) nucleotides (e-ATP, e-ADP, and e-AMP) as substrate analogues. The e-adenine nucleotides can be followed, once internalized, by monitoring their fluorescence (Secrist et al., 1972; Rotllán et al., 1991). Due to the existence of multiple enzymes that can modify the phosphate moiety of nucleotides and their analogues, the e-ATP, e-ADP, and e-AMP have been quantified in every assay after their separation by HPLC chromatography and fluorescent detection. This technique proved to be efficient and accurate in studying the vesicular nucleotide transport, allowing its deeper kinetic characterization. The complexity of the saturation kinetics for e-ATP, e-ADP, and e-AMP transport can be interpreted by postulating the existence of cooperative mnemonic mechanisms.

EXPERIMENTAL PROCEDURES

Preparation of Chromaffin Granules—Crude granular fractions and purified chromaffin granules were obtained as reported (Rodríguez del Castillo et al., 1988) with some modifications, as follows: fresh bovine
adrenal glands were dissected from the cortex and homogenized in
(1:50, w/v) ice-cold 0.3 M sucrose containing 50 μM phenylmethylsulfo-
nyl fluoride, 2 mM EDTA, 1 mM dithiothreitol, and 10 mM PIPES, pH
6 (buffer A). This homogenate was subjected to a centrifugation at 800
×g for 20 min. The pellet (P1) was discarded, and the supernatant (S1)
was centrifuged at 10,000 ×g for 20 min to obtain the crude granular
fraction (P2). This fraction was resuspended in the following buffer,
layered over a solution of 1.5 M sucrose, and S.D.; reducible quantity of protein, 8.6 mg from adrenomedullary tissue. These preparations contain a very repro-
ducible quantity of protein, 8.6 mg (Secrist et al., 1979) and P1, 10 min experimental time. The eluents from the column were excited at a 306 nm, and the emision at 410 nm was recorded. The peak areas were transformed to concentrations by correlation with commercial standards.

Data Analysis—All results were expressed as mean ± S.D., n being
the number of experiments in triplicate. The kinetic analysis of
the complex experimental data were processed according to the equation

\[ V = \frac{V_{max} \cdot [S]}{K_m + [S]} \]

where (Castro et al., 1979) because the V/S representation was not hyperbolic and appeared to be
the result of the addition of various Hill type curves with two or more
infection points and plateaus.

As the kinetics do not fit in a simple Hill equation, the differential
method of Kurganov (1982) was employed to calculate the \( n_v \) with respect to the saturation

\[ \frac{1}{V'} - \frac{1}{V''} = \log \frac{1}{\chi} = \log \frac{1}{\chi} \]

RESULTS

\( e \)-ATP Transport to Purified Chromaffin Granules—The purified
chromaffin granules, corresponding to the mitochondrial
fraction, have been widely employed in the characterization
of the nucleotide transport using radiolabeled nucleotides
as substrates. This fraction contains mostly mitochondria and
chromaffin granules, but lysosomes and fragments of plasma
membrane are also present. In the experimental conditions
herein described, this fraction presents important inconve-
"nences due to the quick degradation of \( e \)-ATP to lower phos-
phorylated metabolites, \( e \)-ADP and \( e \)-AMP, the \( e \)-ATP completely
disappearing between 1 and 5 min of incubation time (results not shown).

The rapid destruction of \( e \)-ATP in the extragranular incuba-
tion media of crude granular fractions (P2) indicated the neces-
sity for using purified granular preparations. Thus, the
chromaffin granules purified on density gradient were employed to
carry out the transport experiments. Fig. 1 shows the extragranular and intragranular distribution of \( e \)-adenine nucleo-
tides as a function of the experimental time. In the extragranular
media (Fig. 1A), the \( e \)-ATP concentration with purified fractions was maintained more than 80%, even after 2 h of
incubation period. The \( e \)-ADP and \( e \)-AMP are present in the
commercially available \( e \)-ATP and represent, respectively,
6.1% and 0.4% of the total. The \( e \)-ADP increased very slowly
during the incubation period and even to a lower extent was the production of \( e \)-AMP. The percentage distribution of the nucleo-
tides after a 10-min incubation was 0.5, 10, and 89.5%, respecti-
v, for \( e \)-AMP, \( e \)-ADP, and \( e \)-ATP. These data were neces-
sary to establish the optimum situation for the concentration
dependence studies. When the experiments were carried out in

\[ \frac{1}{V'} - \frac{1}{V''} = \log \frac{1}{\chi} \]

Because (Secrist et al., 1979) and P1, 10 min experimental time. The eluents from the column were excited at a 306 nm, and the emision at 410 nm was recorded. The peak areas were transformed to concentrations by correlation with commercial standards.

Data Analysis—All results were expressed as mean ± S.D., n being the number of experiments in triplicate. The kinetic analysis of the complex experimental data were processed according to the equation

\[ V = \frac{V_{max} \cdot [S]}{K_m + [S]} \]

where (Castro et al., 1979) because the V/S representation was not hyperbolic and appeared to be the result of the addition of various Hill type curves with two or more infection points and plateaus.

As the kinetics do not fit in a simple Hill equation, the differential method of Kurganov (1982) was employed to calculate the \( n_v \) with respect to the saturation

\[ \frac{1}{V'} - \frac{1}{V''} = \log \frac{1}{\chi} \]

RESULTS

\( e \)-ATP Transport to Purified Chromaffin Granules—The purified chromaffin granules, corresponding to the mitochondrial fraction, have been widely employed in the characterization of the nucleotide transport using radiolabeled nucleotides as substrates. This fraction contains mostly mitochondria and chromaffin granules, but lysosomes and fragments of plasma membrane are also present. In the experimental conditions herein described, this fraction presents important inconveniences due to the quick degradation of \( e \)-ATP to lower phosphorylated metabolites, \( e \)-ADP and \( e \)-AMP, the \( e \)-ATP completely disappearing between 1 and 5 min of incubation time (results not shown).

The rapid destruction of \( e \)-ATP in the extragranular incubation media of crude granular fractions (P2) indicated the necessity for using purified granular preparations. Thus, the chromaffin granules purified on density gradient were employed to carry out the transport experiments. Fig. 1 shows the extragranular and intragranular distribution of \( e \)-adenine nucleotides as a function of the experimental time. In the extragranular media (Fig. 1A), the \( e \)-ATP concentration with purified fractions was maintained more than 80%, even after 2 h of incubation period. The \( e \)-ADP and \( e \)-AMP are present in the commercially available \( e \)-ATP and represent, respectively, 6.1% and 0.4% of the total. The \( e \)-ADP increased very slowly during the incubation period and even to a lower extent was the production of \( e \)-AMP. The percentage distribution of the nucleotides after a 10-min incubation was 0.5, 10, and 89.5%, respectively, for \( e \)-AMP, \( e \)-ADP, and \( e \)-ATP. These data were necessary to establish the optimum situation for the concentration dependence studies. When the experiments were carried out in
the presence of Ap5A, 10 μM or 5 μM atractyloside, no changes were observed in e-ADP and e-AMP production at the extragranular level.

Fig. 1A shows the intragranular levels of etheno-nucleotides. e-ATP constituted the overwhelming majority of intragranular nucleotides. e-ADP and e-AMP were also stored and appeared at a higher ratio than outside the granule. The presence of e-ADP at such levels will be further emphasized in the results of the e-ATP transport. The intragranular transport of e-ATP was maintained linear until 30 min incubation time in the experiment reported in Fig. 1 and in general with high extragranular nucleotide concentration; with the lowest e-ATP concentration used (50 μM), the lineal period was reduced to 15 min. The total of the intragranular e-nucleotides never sur-

FIG. 1. HPLC chromatographic profiles of e-ATP transport to purified chromaffin granules as a function of time. Samples of purified chromaffin granules containing 0.38 mg of protein were incubated in the presence of 6 mM e-ATP, with 5 μM atractyloside, as described under "Experimental Procedures." The consecutive HPLC chromatographic profiles represented correspond to the experimental times of 0 min, 10 min, 30 min, 1 h, and 2 h. The transport was stopped by addition of 13 ml of cold stop solution buffer A and processed as indicated under "Experimental Procedures." The fluorescence of e-nucleotides is expressed in arbitrary units. A, HPLC chromatograms of the e-nucleotide levels in the extragranular media. B, HPLC chromatograms of the e-nucleotide content in the granular pellet. The e-nucleotides in the intragranular samples exhibited a slightly shorter time of retention when compared to the extragranular samples. This is due to the extremely high levels of endogenous nucleotides (mM) that are not detectable by fluorescence, but induce a saturation on the HPLC chromatographic column. Addition of external standards corresponds exactly to the e-adenine nucleotides in each experimental situation. These chromatograms represent a very reproducible experiment.

FIG. 2. Concentration dependence of e-ATP transport to chromaffin granules. The transport experiments were accomplished with 0.38 mg of protein of purified chromaffin granules and incubated with e-ATP concentrations ranging from 50 μM to 6 mM, for 10 min at 25°C as described under "Experimental Procedures." Both the extragranular and the intragranular e-nucleotide levels were quantified after separation. To avoid a crowded figure, only the HPLC chromatograms corresponding to the e-ATP concentrations of 0.3, 0.4, 0.6, 0.8, 1, 1.2, 1.6, 2, 4, and 6 mM, in a consecutive order have been represented. A, HPLC chromatograms of the extragranular levels of e-AMP, e-ADP, and e-ATP, as a function of e-ATP concentration. B, HPLC chromatograms of the intragranular levels of e-AMP, e-ADP, and e-ATP, as a function of e-ATP concentration.

passed 2% of that present in the incubation media. This ratio also assures the linearity of the transport process.

Saturation Studies of e-ATP Transport to Purified Chromaffin Granules—Experimental knowledge of the e-ATP stability in the incubation media of chromaffin granules, and the linear period of transport was essential to establish the best conditions to undertake the kinetic studies. Routinely, a 10-min incubation period was chosen for the transport experiments. In Fig. 2, the chromatographic profile of the concentration dependence transport studies for e-ATP are shown, both for the incubation media (Fig. 2A) and the stored e-nucleotides (Fig. 2B). The e-ATP at the extragranular media, and after a 10-min incubation time, was catalobized to e-ADP in a percentage reaching 20% at the lowest concentration employed (50 μM) and 10% at the highest concentration (6 mM). In these experimental conditions, because most of the initial e-ATP still remained at each concentration employed, saturation studies of the e-ATP transport were undertaken.

At the intragranular level, as shown in Fig. 2B, the e-ATP
The saturation curve was obtained by processing the HPLC data from Fig. 2B. The e-ATP concentration ranged from 50 μM to 6 mM. Transport velocity is expressed as nanomoles of e-ATP transported per min per mg of chromaffin granule proteins. B, three sigmoidal curves, the addition of which accounts for the experimentally observed curve (A). Their affinity values (K) or the equivalent S0.5, together with the corresponding Vmax and nH values, are summarized in Table I. C, plot of nH against the e-ATP saturation calculated for curve A, according to Kurganov (1982), Equation 2 under “Experimental Procedures.” These results represent a typical experiment performed in triplicate, which is very reproducible.

![Diagram](http://www.jbc.org/)

**Table I**

Kinetic parameters of nucleotide transport to chromaffin granules

Values of the kinetic parameters of the three curves that are the constituent parts of the experimental saturation curve for e-nucleotide transport. K is the value of the corresponding S0.5 affinity, 2Vmax corresponds to the addition of the partial Vmax, nH is the value for the cooperativity, considering each single curve. Values are means ± S.D. of three experiments in triplicate for e-AMP, four experiments in triplicate for e-ADP, and five experiments in triplicate for e-ATP.

| Curves | e-ATP | e-ADP | e-AMP |
|--------|-------|-------|-------|
| K (mM) | 1     | 0.25 ± 0.03 | 0.15 ± 0.01 | 0.20 ± 0.02 |
| Vmax (nmol · min⁻¹ · mg protein⁻¹) | 1     | 0.020 ± 0.001 | 0.025 ± 0.002 | 0.010 ± 0.001 |
| 2Vmax (nmol · min⁻¹ · mg protein⁻¹) | 1     | 0.040 ± 0.003 | 0.035 ± 0.003 | 0.040 ± 0.003 |
| nH | 1     | 3     | 2     | 2     |
| 2     | 4     | 4     | 3     |
| 3     | 5     | 5     | 5     |

The true cooperativity that is stated by the transporter depends on the saturation level, because the nH number is variable. This variation can be evaluated according to Kurganov’s differential procedure (Equation 2 under “Experimental Procedures”) and is shown in Fig. 3C.

**Fig. 3.** Saturation studies of e-ATP transport to chromaffin granules. A, the saturation curve was obtained by processing the HPLC data from Fig. 2B. The e-ATP concentration ranged from 50 μM to 6 mM. Transport velocity is expressed as nanomoles of e-ATP transported per min per mg of chromaffin granule proteins. B, three sigmoidal curves, the addition of which accounts for the experimentally observed curve (A). Their affinity values (K) or the equivalent S0.5, together with the corresponding Vmax and nH values, are summarized in Table I. C, plot of nH against the e-ATP saturation calculated for curve A, according to Kurganov (1982), Equation 2 under “Experimental Procedures.” These results represent a typical experiment performed in triplicate, which is very reproducible.

Composed the majority of the nucleotides present, but a significant fraction was as e-ADP and also e-AMP. The nucleotide distribution percentages were completely different from those at the extragranular level and corresponded to 72.5, 25.5, and 2.3 at 6 mM, respectively, for e-ATP, e-ADP, and e-AMP. As reported in the time function experiments, this distribution did not change significantly with time; even after a 2-h incubation period at 25 °C, the distribution was modified only slightly. When internal standards of e-ATP were added to the chromaffin granule pellet after transport, and afterwards processed as indicated under “Experimental Procedures,” the e-ADP production as a direct consequence of the procedure was under 0.5% and under 1% for e-AMP.

From the intragranular e-nucleotide values, the saturation curve was plotted, and a nonhyperbolic curve was observed in our experimental conditions, as shown in Fig. 3A. The complex dependence of transport velocity with respect to extragranular e-ATP concentration observed made it necessary to interpret the saturation curve as the superposition of various sigmoidal kinetics. Therefore, the processing of the experimental data was done by Equation 1 described under “Experimental Procedures” (Kurganov, 1982). This analytical procedure has been employed already with enzymes exhibiting similar kinetic behavior (Somero and Hochachka, 1969; Irving and Williams, 1973a, 1973b; Kagan and Dorožko, 1973). The addition of three sigmoidal curves was necessary, in this case, to process the experimental data, as shown in Fig. 3B. The affinity values (K), partial Vmax, and the Hill number for each curve, are summarized in Table I.

The true cooperativity that is stated by the transporter depends on the saturation level, because the nH number is variable. This variation can be evaluated according to Kurganov’s differential procedure (Equation 2 under “Experimental Procedures”) and is shown in Fig. 3C.

**e-ADP Transport to Chromaffin Granules**—The e-ADP transport to chromaffin granules was studied, during the lineal period, at 10 min experimental time, and experiments were performed as for e-ATP.

At the extragranular level, some small amounts of e-ATP appeared during the incubation time, reaching a maximum of 4% of the total e-nucleotide (Fig. 4A). The action of adenylosuccinate kinase and the improbable action of the mitochondrial adenine nucleotide translocase were excluded, because the experiments were carried out in the presence of AP5A and atractylloside.

The intragranular content of e-nucleotides, when e-ADP was the substrate to be transported, is shown in Fig. 4B. In this case, the e-ADP was present, but e-ATP was the major compound, together with very small amounts of e-AMP. The nucleotide percent distributions were around 7%, 30–40%, and 55–65%, for e-AMP, e-ADP, and e-ATP, respectively, at every concentration studied and for 10-min transport experiments. The percent distribution is shown in Fig. 4C. At longer exper-
2, 2.5, 3, 4, and 6 mM, in a consecutive order, have been represented. To control the e-ADP stability, an external standard of this nucleotide was added to the chromaffin granules pellet and processed as indicated under "Experimental Procedures." No production of e-ATP was observed to any extent, and only a minor e-AMP production, which was under 3%.

To approach the kinetic parameters for e-ADP transport, the total granular e-nucleotide content was considered to calculate the transport velocity at each e-ADP concentration used, and the data obtained are represented in Fig. 5A. A nonhyperbolic curve was obtained, very similar to that reported for e-ATP, and the data were analyzed in the same way. The kinetic parameters were obtained considering three superimposed nonhyperbolic curves (Fig. 5B) and are summarized in Table I. Fig. 5C shows the $n_H$ variability as a function of saturation ($V/V_{\text{max}}$).

**e-AMP Transport to Purified Chromaffin Granules**—The e-AMP was not modified during the incubation period either at the extrgranular incubation media or at the intragranular level, as shown in Fig. 6A. The saturation curve was very similar to that obtained for e-ATP and e-ADP, with a three-step shape as shown in Fig. 6B and processed as described for e-ATP and e-ADP. The K affinity, the $V_{\text{max}}$, and the $n_H$ values, corresponding to every curve, are summarized in Table I.

The variation of $n_H$ values with respect to saturation (V/V$_{\text{max}}$) is similar to that obtained for ATP (results not shown).

Release of Intragranular e-Adenine Nucleotides Induced by Extragranular Nucleotides—High extragranular concentrations (4 mM) of ATP or ADP induced the release of the intragranular e-nucleotides, with a corresponding decrease in their contents, as shown in Fig. 7. The total granular e-nucleotides and their distribution, after the e-ATP transport was carried out, are shown in the control of Fig. 7B. Subsequent incubation of charged granules resulted in a decrease in their content dependent on the incubation media (Fig. 7B). In all experimental situations, the e-ATP was the most abundant intragranular component. The addition of ATP or ADP reduced the initial granular content to 40% and 54%, respectively. These values are very significant when compared with no addition (buffer) or AMP (4 mM) where the granular content was 70% of the total.

The pattern of released nucleotides changes with respect to the granular one, and e-AMP and e-ADP appeared even in higher concentrations than reported in the granules (Fig. 7A). One plausible explanation is the action of membrane ATPases and other nucleotidases on the e-ATP and e-ADP when they are not protected inside the granules. In the presence of a large amount of ATP, this nucleotide, acting as a competitor, protected the released e-ATP, now appearing in large quantities (Fig. 7A).

The significant extragranular presence of e-nucleotides in buffer-resuspended granules (30% of the total content) (Fig. 7A) could be due, almost in part, to the breakdown of granules that occurred during their resuspension and later centrifugation. This hypothesis was supported by the activity values of the granular enzyme dopamine β-monooxygenase (EC 1.14.17.1). The activity of its soluble form was 19–24% ($n = 3$) of the total content in the extragranular media, independent of the nucleotide addition (results not shown).

**e-Nucleotide Transport Inhibition**—The nucleotide nonhydrolyzable analogues ATP-$\gamma$S and AMPPNP exhibited a mixed effect on the transport of e-nucleotides (Fig. 8, A and B). When the inhibition studies were carried out at an e-nucleotide concentration lower than 1 mM, the addition of a similar amount of the nucleotide analogue always resulted in a transport increase. At higher concentrations (even at 5 mM), and, in spite of
this evident similarity, the inhibition in any case reached 50% of the total transport. When the inhibition studies were carried out at ε-nucleotide concentrations of 1 or 2 mM, the addition of similar amounts of ATPγS or AMPPNP always resulted in a transport increase, and higher concentrations (up to 5 mM) were not able to produce measurable inhibitions, as shown in Fig. 8, A and B, for ε-ADP. The specific effect of ADPβS concerning the ε-ADP transport is included in Fig. 8B.

The nucleotide transport was inhibited, although not completely, by atractyloside, as shown in Fig. 8D. The IC50 values were, respectively, 0.10 ± 0.01 mM for ε-ATP, 0.033 ± 0.002 mM for ε-ADP, and 0.17 ± 0.02 mM for ε-AMP (n = 3).

N-Ethylmaleimide inhibits the transport of ε-nucleotides at maximal levels of 41, 49, and 60% for ε-ATP, ε-ADP, and ε-AMP, respectively (Fig. 8C). The IC50 values were, respectively, 0.094 ± 0.008, 0.80 ± 0.07, and 0.20 ± 0.01 mM (n = 3).

Granule treatment with ionophores such as the charge carrier valinomycin (10 μM), or the K+ /proton exchanger nigericin (20 μM), in the presence of K+, resulted in no inhibition of the ε-ATP transport. The proton translocator FCCP (0.25 mM) inhibited the transport of ε-ATP, ε-ADP, and ε-AMP to an extent of 40 ± 6%, 45 ± 7%, and 53 ± 10% (n = 3), respectively.

DISCUSSION

Transport studies using the ε-adenine nucleotides to characterize the vesicular nucleotide transport present the advantage of their direct fluorescent detection, which combined with HPLC techniques makes it possible to obtain a large set of information necessary to understand the secretory vesicle replenishment and circumvents the drawbacks of the traditional radioactive techniques.

The results presented here show that the nucleotide vesicular transport, when studied in a very broad range of substrate concentration, exhibits a saturable nonhyperbolic kinetic, for all ε-adenine nucleotides, in bovine chromaffin granules. Previous studies of nucleotide vesicular transport to chromaffin granules and torpedo synaptic vesicles reported hyperbolic saturable kinetics for both experimental models (Aberer et al., 1978; Luqmani, 1981; Weber and Winkler, 1981). The Km values described ranged between 0.9 and 1.4 mM for ATP, 1.2 and 1.4 mM for ADP, 0.7 and 2 mM for UTP, 0.3 and 1.2 mM for GTP, and, finally, 2.9 and 3.3 mM for AMP. This range of values is nevertheless comprised in the affinities reported here for the three-step saturation curves, for ε-ATP, ε-ADP, and ε-AMP (Table I).

The Vmax values reported for the ATP transport to chromaffin granules are about 0.4 nmol-min⁻¹-mg of protein⁻¹, that are in the same order as that reported here for ε-ATP. Concerning the Vmax for the ADP, the bibliographic data report lower values than for ATP (Aberer et al., 1978; Winkler and Carmichael, 1982); in our conditions, the ε-ADP transport exhibits a higher Vmax than that of ε-ATP. This result can be explained on the basis of the intragranular modifications of the ε-ADP that experimentally can be quasi-assimilated to a Zero-trans transport conditions. In fact, when ε-ADP was the extragranular nucleotide to be transported, the intragranular ε-ATP accounted for more than 55% of the total ε-nucleotides. A transphosphorylation or interchange reaction of ε-ATP with the adenine nucleotides already present in the granule can be suggested. This type of reaction has previously been described for labeled nucleotides (Aberer et al., 1978; Roisin and Henry, 1982; Winkler and Carmichael, 1982; Taugner et al., 1988). The enzyme responsible for this reaction still needs to be identified, and its physiological relevance needs to be analyzed, but from the transport studies with ε-AMP, it is certain that the enzyme cannot use ε-AMP, or to a very limited extent, as a substrate for the phosphate interchange reaction.

The ε-nucleotide transport was inhibited by the same compounds described for ATP, such as atractyloside, N-ethylmaleimide (a good inhibitor of V-ATPases), and the proton translocator FCCP, at similar concentrations (Aberer et al., 1978; Luqmani, 1981; Weber and Winkler, 1981). None of these compounds was able to inhibit the nucleotide transport completely as previously reported both in bovine and torpedo vesicles (Luqmani, 1981).

The efflux or "exchange" of granular stored compounds is still an unresolved question. In the case of monoamines, both processes are independent of the transport inhibitor reserpine and "exchange" has been observed only when an excess of substrate was added (Schuldiner et al., 1995). In the case of granular nucleotides, their fluorescent labeling allowed a first experimental approach to the problem and, as in the previous case, a high extragranular ATP or ADP concentration is necessary, with the peculiarity that AMP was ineffective. The physiological relevance of these data still needs to be established.
Concerning the transport saturation studies, the three-step curve that was obtained for each of the e-nucleotides studied needs to be analyzed and interpreted in several ways. First, the chromaffin granule model itself needs some discussion. It is well known that adrenergic and noradrenergic cells exist in adrenal medulla, storing the specific catecholamines in their granules (Moro et al., 1991). The noradrenergic granules appear to be more dense with a higher proportion of nucleotides than the adrenergic granules (Terland et al., 1979). Nothing is yet known about the different transport properties of both granules. Nevertheless, the superposition of two different transporters with their respective affinities is not compatible with the positive kinetic cooperativity exhibited by the saturation curve. Recently, the presence of different vesicular monoamine transporters has been reported in the large secretory chromaffin granules (VMAT1) and in the small synaptic vesicles (VMAT2), both present in bovine adrenal glands (Henry et al., 1994). Thus, the existence of different vesicular nucleotide transporters, related to the secretory organelle size in the same cell, cannot be ruled out. As in the previous hypothetical situation, the existence of two different transporters cannot explain the positive cooperativity in the transport saturation studies.

The second aspect under analysis and discussion is the cooperative phenomenon. e-ATP, e-ADP, and e-AMP exhibit similar shapes in their saturation curves. In allosteric enzymes, the occurrence of various intermediate plateaus on $V$ versus $[S]$ plots has been explained by the presence in the system of forms of the enzyme which exhibit differing degrees of kinetic cooperativity toward the substrate, due to the comparatively slow isomerization induced by the substrate. The enzymes with such a kinetic behavior are known as hysteretic ormnemonic Vesicular Nucleotide Transporter.


Plateaus have already been reported, as in the case of l-threonyl-plexitrols and saturation curves exhibiting various intermediate complexities and saturation curves exhibiting various intermediate forms, and they adapt well to a transporter model as has been cloned successfully, it is difficult to make an exact and accurate transport model. The analysis of the saturation curve required a new approach to the n_H concept and notion (Kurganov, 1982). The idea of three superimposed curves with cooperation was necessary to analyze and obtain the kinetic parameters (Silonova et al., 1991; Kurganov, 1982).

The transport activation by ATP-nonhydrolyzable analogues at low concentrations, together with their poor inhibitory effect at the highest concentrations (Aberer et al., 1978), also confirm the hypothesis of conformational cooperative transitions. Similar behavior has already been reported for some inhibitors of l-threonine dehydratase at low substrate concentrations (Kagan and Dorozhko, 1973). Based on the previous discussion, the complexity of the saturation kinetics for -nucleotides can be interpreted by postulating the existence of a mnemonic mechanism. Nevertheless, the heterogeneity of the chromaffin granule model, on account of their amnigeric content, size, and the breakdown and subsequent vesicle ghost formation, could be at the root of complex additive kinetic behaviors, except for the cooperativity phenomena that cannot be explained in this way.

The third aspect to be considered concerns the physiological relevance and the advantages of such kinetic behavior. In our opinion and in agreement with the results reported here, the mnemonic behavior is essential to establish the priority order on the remaining cellular ATP after physiological situations demanding large amounts of energy, as neurosecretion or in anomalous situations as anoxic episodes. The rational is as follows.

(a) It is well known that glucose is the main energetic substrate in neurosecretory cells, and the remaining ATP in the situations previously described is necessary for the phosphorylation of glucose; the K_m value for the neural type I hexokinase for ATP is close to 0.1 mM. This isoenzyme accounts for 90% of the total hexokinase activity in the adrenomedullary tissue; the remaining 10% corresponds to hexokinase II (Millaruelo et al., 1986). Thus, the priming of glycolysis as the main neural catabolic energetic pathway is assured because the K (S_0.5) for the first intermediate plateau of transport is about 0.25 mM for ATP (it is assumed that e-ATP and the natural substrate ATP have a similar kinetic behavior). Only when the glycolysis goes on and the ATP level recovers, does the nucleotide vesicular transporter increase its capacity in a highly cooperative way. As the affinities for all nucleotides are very similar, this kinetic behavior presents the advantage of preventing the massive entrance to the granule of lower phosphorylated metabolites.

(b) The presence for the last plateau with K (S_0.5) values for the ATP transport (about 3 mM) is in our opinion related to the phosphofructokinase reaction. This enzyme needs ATP as a substrate with a K_m value in the range of 10 to 20 μM, but ATP also regulates the glycolytic flux by its inhibitory action on this enzyme. The K_m values for ATP reported for the brain isoenzymes are in the 2–3 mM range (Vora et al., 1985). Thus, when the high ATP levels are on the edge of inhibition of the glycolytic flux, they are in the best situation to be transported.

In the absence of a full theoretical development of the kinetic mechanism operating in membrane transport, the possibility of a mnemonic behavior opens new perspectives in this field. Moreover, the mnemonic behavior in the specific case of the nucleotide transporter allows a security threshold in the cellular energetic metabolism and sheds some light on the harmony existing in cellular functioning.

Acknowledgments—We thank Erik Lundin and Duncan Gilson for help in the preparation of this manuscript.

REFERENCES

Aberer, W., Kostron, H., Huber, E., and Winkler, H. (1978) Biochem. J. 172, 353–360
Apps, D. K., and Percy, J. M. (1987) Ann. N. Y. Acad. Sci. 493, 179–187
Banks, R. D., Blake, C. C. F., Evans, P. R., Hase, R., Rice, D. W., Hardy, G. W., Merrett, M., and Phillips, D. C. (1979) Nature 279, 773–777
Castro, E., Torres, M., Miras-Portugal, M. T., and Gonzalez, M. P. (1990) Br. J. Pharmacol. 100, 360–364
Cidon, S., Ben-David, H., and Nelson, N. (1983) J. Biol. Chem. 258, 11684–11688
Grüssinger, H. A., Apps, D. K., and Phillips, J. H. (1983) Neuroscience 9, 917–924
Hebert, D. N., and Carruthers, A. (1991) Biochemistry 30, 4654–4658
Henry, J. P., Botton, D., Sagne, C., Isambert, M. F., Desnos, C., Blanchard, V.,...
Raisman-Vozari, R., Krejci, E., Massoulie, J., and Gasnier, B. (1994) J. Exp. Biol. 196, 251–262
Irving, M. G., and Williams, J. F. (1973a) Biochem. J. 131, 287–301
Irving, M. G., and Williams, J. F. (1973b) Biochem. J. 131, 303–313
Kagan, Z. S., and Dorozhko, A. I. (1973) Biochim. Biophys. Acta 302, 110–128
Klingenberg, M., Grebe, K., and Scherer, B. (1975) Eur. J. Biochem. 52, 351–363
Knoth, J., Handloser, K., and Njus, D. (1980) Biochemistry 19, 2938–2942
Kurganov, B. I. (1982) Kinetic Behaviour (Yakovlev, V. A., ed) John Wiley and Sons, New York
Kwong, F. Y. P., Fincham, H. E., Davies, A., Beaumont, N., Henderson, P. J. F., Young, J. D., and Baldwin, S. A. (1992) J. Biol. Chem. 267, 21954–21960
Lienhard, G. E., and Secemski, I. I. (1973) J. Biol. Chem. 248, 1121–1123
Liu, Y. J., Peter, D., Roghani, A., Schuldiner, S., Prive ´, G. G., Eisenberg, D., Brecha, N., and Edwards, R. H. (1992) Cell 70, 539–551
Luqmani, Y. A. (1981) Neuroscience 6, 1011–1021
Millaruelo, A. I., Sagarra, M. R., Delicado, E., Torres, M., and Miras-Portugal, M. T. (1986) Mol. Cell. Biochem. 70, 67–76
Miras-Portugal, M. T., Millaruelo, A. I., and Vara, F. (1980) Mol. Cell. Biochem. 33, 25–33
Moro, M. A., Garcia, A. G., and Langley, O. K. (1991) J. Neurochem. 57, 363–369
Nari, J., Noat, G., and Ricard, J. (1984) Eur. J. Biochem. 145, 319–322
Neet, K. E., and Ainslie, G. R. (1976) Trends Biochem. Sci. 7, 145–147
Nelson, N. (1992) J. Exp. Biol. 172, 149–153
Njus, D., and Radda, G. K. (1970) Biochim. Biophys. Acta 243, 219–244
Pintor, J., Torres, M., and Miras-Portugal, M. T. (1992a) Neurosci. Lett. 136, 141–144
Pintor, J., Kowalewski, H. J., Torres, M., Miras-Portugal, M. T., and Zimmermann, H. (1992b) Neurosci. Res. Commun. 10, 9–15
Pollard, H. B., Zinder, O., Hoffman, P. G., and Nikodejevic, O. (1976) J. Biol. Chem. 251, 4544–4550
Ricard, J., and Cornish-Bowden, A. (1987) Eur. J. Biochem. 166, 255–272
Rodriguez del Castillo, A., Torres, M., Delicado, E. G., and Miras-Portugal, M. T. (1989) J. Neurochem. 51, 1696–1703
Roshin, M. P., and Henry, J. P. (1982) Biochim. Biophys. Acta 681, 292–299
Rotllán, P., Ramos, A., Pintor, J., Torres, M., and Miras-Portugal, M. T. (1991) FEBS Lett. 280, 371–374
Scherman, D., and Henry, J. P. (1983) Biochimistry 22, 2805–2810
Schröder, M., Volknandt, W., and Zimmermann, H. (1994) J. Neurochem. 63, 1924–1931
Schröder, H.Offers, E.,Brüggemann,G.,Van der Giet,M.,Tepel,M.,Nordhoff,E.,Karas,M.,Spleker,C.,Witzel,H.,andZidek,W.(1994)Nature 367, 186–188
Schuldiner, S., Shirvan, A., and Linial, M. (1995) Physiol. Rev. 75, 369–392
Secrist, J. A., III, Barrio, J. R., Leonard, N. J., and Weber, G. (1972) Biochimistry 11, 3499–3506
Silonova, G. V., Livanova, N. B., and Kurganov, B. I. (1969) Mol. Biol. (Mosc.) 3, 768–784
Somero, G. N., and Hochachka, P. W. (1969) Nature 223, 194–195
Taugner, G., Heym, CH., Kummer, W., and Wunderlich, Y. (1988) Biol. Amines 5, 409–426
Terlind, O., Flatmark, T., and Kryvi, H. (1979) Biochim. Biophys. Acta 553, 460–468
Torres, M., Delicado, E. G., Fideu, M. D., and Miras-Portugal, M. T. (1992) Biochim. Biophys. Acta 1105, 291–299
Valero, E., and García-Camona, F. (1992) Biochem. J. 286, 623–626
Vora, S., Oskam, R., and Staal, G. E. J. (1985) Biochem. J. 229, 333–341
Weber, A., and Winkler, H. (1981) Neurosci. Res. Commun. 6, 2269–2276
Winkler, H., and Carmichael, S. W. (1982) in The Secretory Granule (Poisner, A. M., and Trifaro ´, J. M., eds) pp. 3–79, Elsevier-North Holland Biomedical Press, New York
Zimmermann, H. (1994) Trends Neurosci. 17, 420–426
Nucleotide Vesicular Transporter of Bovine Chromaffin Granules: EVIDENCE FOR A MNEMONIC REGULATION
Javier Gualix, Miguel Abal, Jesus Pintor, Francisco Garcia-Carmona and M. Teresa Miras-Portugal

J. Biol. Chem. 1996, 271:1957-1965.
doi: 10.1074/jbc.271.4.1957

Access the most updated version of this article at http://www.jbc.org/content/271/4/1957

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 11 of which can be accessed free at http://www.jbc.org/content/271/4/1957.full.html#ref-list-1