Presence of ε-adenosine tetraphosphate in chromaffin granules after transport of ε-ATP

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Abstract Adenosine 5′-tetraphosphate (Ap_4) is a natural constituent of chromaffin granules with concentration values of 2.2 ± 0.1 nmol/mg of protein and a ratio 245 ± 40 times lower with respect to ATP ($n = 4$). The granular transport of ε-ATP resulted in a time- and concentration-dependent production of ε-adenosine tetraphosphate (ε-Ap_4) at the intragranular level. The ε-Ap_4 formation followed a hyperbolic saturation kinetic at low ε-ATP concentrations with K_m value of 0.4 μM ε-ATP intragranular (1.15 pmol/mg of granular protein). Intragranular concentrations of ε-ATP higher than 500 pmol/mg of protein (≈ 175 μM intragranular) resulted in a non-saturable production of ε-Ap_4.

Key words: Adenosine 5′-tetraphosphate; Vesicular transport; Etheno-adenine nucleotide; Transphosphorylation; Chromaffin granule

1. Introduction

ATP is the most abundant currently reported nucleotide stored in synaptic vesicles and secretory granules; smaller, although significant, amounts of a large variety of other nucleotidic compounds such as ADP, AMP, GTP, UTP and very small amounts of adenosine 5′-tetraphosphate (Ap_4) have also been found in diverse secretory organelles [1,2]. Moreover, the presence of diadenosine polyphosphates, Ap_4A, Ap_5A and Ap_6A, has also been reported in catecholaminergic chromaffin granules and cholinergic vesicles from *Torpedo* electric organ, which are among the best characterized storage vesicles and can provide evidence about the complexity and richness of nucleotidic content in other neural or endocrine models [3–5].

The presence of such a large variety of nucleotides and dinucleotides in neural storage granules is mostly due to the low specificity of the transport process. The vesicular nucleotide transporter exhibits similar affinity values for all nucleotides triphosphate and nucleotides diphosphate (NTP and NDP) [6,7]. In contrast to its low specificity, the nucleotide vesicular transporter appears to be highly regulated by the cytosolic concentration of nucleotides [8].

Intragranular transphosphorylation between nucleotides has also been reported and it is to be considered when studying the granular content [6,9]. This phenomenon became evident when the transport studies were carried out with the fluorescent 1,N^6-ethenoderivative of ADP (ε-ADP), which once internalized to chromaffin granules was recovered mostly as ε-ATP [8].

Formation of ε-adenosine tetraphosphate (ε-Ap_4) in chromaffin granules, after ε-ATP transport, is reported in this study. The data presented here can explain the presence of Ap_4 in chromaffin granules and give physiological supplement to recent data showing that Ap_4 is a more effective agonist than ATP on the P_{2x} purinoceptors present on guinea-pig vas deferens [10].

2. Materials and methods

2.1. Preparation of chromaffin granules

Purified chromaffin granules were obtained following the method reported by Rodriguez del Castillo et al. [3], with some modifications directed to obtain granular preparations with high purity and in the best conditions to carry out the transport experiments. The buffer employed throughout the experimental procedure was 0.3 M sucrose containing 50 μM phenylmethylsulphonyl fluoride, 2 mM EDTA, 1 mM dithiotreitol, and 10 mM 1,4-piperazinediethanesulfonic acid (PIPES), pH 6.0 (buffer A) [8].

Granular preparations to measure Ap_4 levels were resuspended in 1 ml of water per each gram of original tissue, submitted to two freeze-thaw cycles and centrifuged at 100 000 × *g* for 10 min. Routinely, samples between 2 and 5 μl were taken for processing by HPLC.

2.2. Transport experiments

The transport experiments were performed with etheno-ATP (ε-ATP) as substrate and carried out with 100 μl of the granular preparation which corresponded to 0.1 g of the original adrenomedulary tissue resuspended in a buffer containing 0.3 M sucrose, 5 mM $MgCl_2$, and 10 mM Tris·HCl, pH 7.2 (buffer B). The experimental procedure for saturation- and time-dependent transport studies was carried out according to the method of Gualix et al. [8]. After the transport experiments the pellet, to measure the intragranular ε-nucleotides, was resuspended in 1 ml of a mixture of 0.1 ml of ethanol and 0.9 ml of the HPLC buffer (described below). The granular suspension was submitted to a freeze-thaw cycle and then centrifuged at 100 000 × *g* for 30 min. The samples were stored at −80°C until the process by HPLC.

2.3. HPLC procedures

The chromatografic equipment was from Waters and consisted of a 600E delivery system, an injector Autosampler 717 Plus, a Scanning fluorescent detector 474 and a Millenium 2010 Chromatography Manager System. The separation of the nucleotides and ε-nucleotides was performed using ion pair chromatography [11]. The mobile phase contained 10 mM KH_2PO_4, 2 mM tetrabutyl ammonium and 15% acetonitrile, pH 7.5. The column used was a Nova-Pak C-18 from Waters and, in some experiments, an RSiL C18 HL column from Bio-Rad. The eluents from the column were excited at 306 nm and the emission was recorded at 410 nm for all ε-nucleotides [12,13]. The peak areas were transformed to concentrations by correlation with commercial standards of nucleotides and ε-nucleotides (Sigma, St. Louis, Mo). ε-Ap_4 was synthesized by condensation of Ap_4 with chloroacethaldehyde as described for the ε-diadenosine polyphos-

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Abbreviations: Ap_4, adenosine 5′-tetraphosphate; ε-Ap_4, 1,N^6-ethenoadenosine 5′-tetraphosphate (etheno-Ap_4); ε-ATP, 1,N^6-ethenoadenosine 5′-triphosphate (etheno-ATP); HPLC, high-performance liquid chromatography

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phates [13] and purified by HPLC under the same elution conditions as described above. Non-fluorescent nucleotides were detected using a λmax 481 Spectrophotometer (Waters) at 260 nm wavelength.

3. Results

3.1. Presence of Ap_4 and ε-Ap_4 in chromaffin granules

The presence of Ap_4 as a natural constituent of chromaffin granules, already reported by other authors, was confirmed [1]. The concentration values obtained in our preparations were 2.2 ± 0.1 nmol/mg of protein and a ratio with respect to ATP of 245 ± 40 times lower (n=4). Fig. 1A shows a typical chromatogram of the chromaffin granule nucleotide content. The Ap_4 was identified by the retention time and coelution with external standard. The elution conditions described were necessary to obtain a sharper Ap_4 peak.

The presence of ε-Ap_4 was detected after transport studies

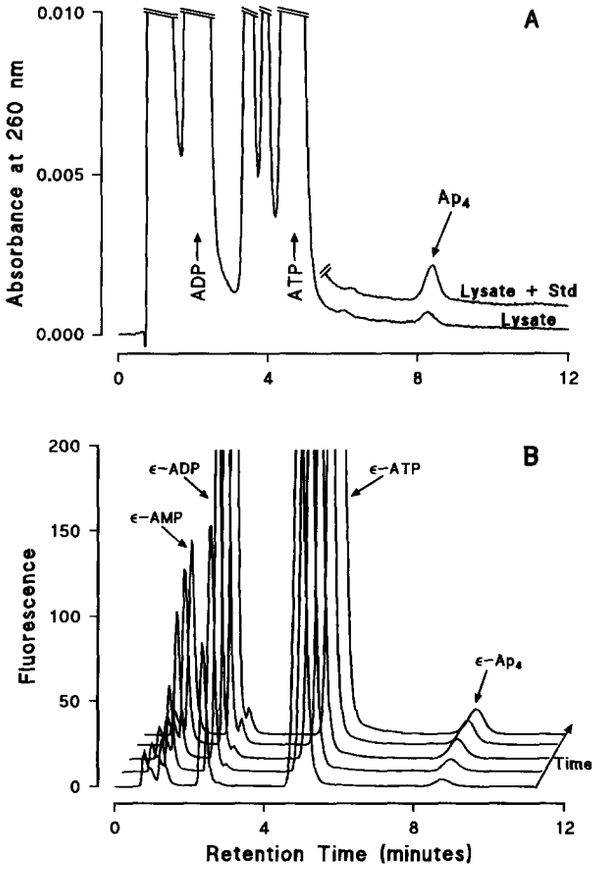

Fig. 1. ε-Ap_4 production and Ap_4 presence in chromaffin granules. (A) Detection of Ap_4 in chromaffin granules lysate: measurement of Ap_4 in chromaffin granules was carried out as described in Section 2. The HPLC profile corresponding to the chromaffin granules lysate are represented in the figure. Presence of an Ap_4 peak was confirmed by the addition to the lysate of an external standard. In this case, the first part of the chromatogram was deleted to avoid overcrowding the figure. (B) Formation of ε-Ap_4 as a function of time in chromaffin granules incubated with ε-ATP: samples of chromaffin granules containing 0.38 mg of protein were incubated in the presence of 6 mM ε-ATP as described in Section 2. The overlayed chromatograms in the figure correspond to the ε-nucleotide content, in the granular pellet, after incubation periods of 0 min, 10 min, 30 min, 1 h and 2 h. The fluorescence of ε-nucleotide peaks is expressed in arbitrary units. In A and B, the HPLC column employed was a Novapak C-18 cartridge.

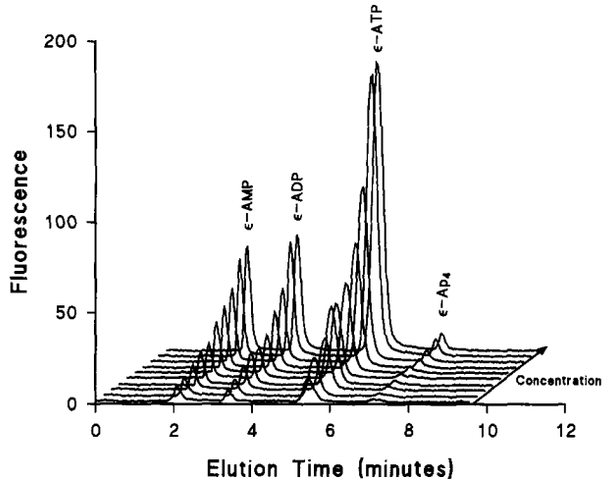

Fig. 2. HPLC chomatograms showing the appearance of ε-Ap_4 in chromaffin granules incubated with several concentrations of ε-ATP. Samples of chromaffin granules containing 0.38 mg of protein were incubated in the presence of ε-ATP concentrations ranging from 150 μM to 6 mM for 10 min, as described in Section 2. The consecutive chromatograms in the figure correspond to the chromatografic profiles obtained for the granular pellets after incubation with ε-ATP concentrations of 0.15, 0.2, 0.3, 0.4, 0.6, 0.8, 1, 1.2, 1.6 and 2 mM. Chromatograms for higher concentrations were omitted in order to avoid a crowded figure. Chromatografic profiles were obtained using an RSiL C18 HL column.

were carried out to characterize the nucleotide vesicular transport employing ε-ATP. The HPLC chromatograms showed that, although most of the nucleotide once internalized remained as ε-ATP, a significant part appeared as ε-ADP and ε-AMP. When the HPLC eluates were allowed to flow for longer periods of time, another fluorescent compound became evident, as shown in Fig. 1B. The longer time of retention suggested the existence of a more phosphorylated nucleotide. The presence of ε-Ap_4A or ε-Ap_5A was ruled out because the peak was destroyed by treatment with alkaline phosphatase (results not shown). Comparison with ε-Ap_4 standard resulted in coelution of both compounds and the peak was identified as the ε-adenosine 5′-tetraphosphate. The intragranular ε-Ap_4 levels increased up to 30 min incubation time, although the lineal period was considerably reduced. Experimental times of 10 min transport for the concentration dependence studies were routinely employed. No production of ε-Ap_4 was observed in the incubation media.

3.2. Intragranular ε-Ap_4 production in relation to ε-ATP

The HPLC distribution profiles of ε-nucleotides inside the chromaffin granules, after 10 min incubation time, with variable extragranular concentrations of ε-ATP are shown in Fig. 2. The internal concentrations of ε-Ap_4 under 150 μM extragranular ε-ATP resulted in a very small production of the nucleotide, which was difficult to quantify accurately.

The presence of ε-Ap_4 in the granule was dependent on that of ε-ATP. When production of ε-Ap_4 was plotted versus the intragranular ε-ATP concentrations, a biphasic curve was obtained (Fig. 3A). The first part of the curve, at low intragranular concentrations of ε-ATP, shows the production of ε-Ap_4 that appeared to follow a saturable kinetic. The second part corresponds to intragranular concentrations larger than 500 pmol/mg protein of ε-ATP, obtained when extragranular

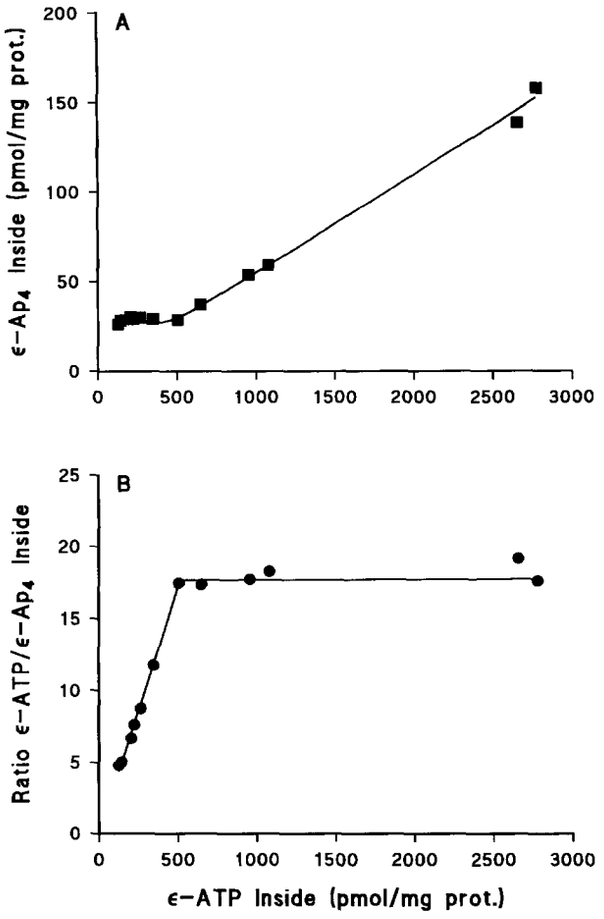

Fig. 3. Saturation studies of ε-Ap_4 formation as a function of the intragranular ε-ATP concentration. (A) Michaelis-Menten representation of intragranular ε-Ap_4 versus intragranular ε-ATP. (B) Hanes-Woolf representation of the intragranular ε-ATP/ε-Ap_4 ratio versus intragranular ε-ATP. Concentration values are means of three saturation-dependence transport experiments such is represented in Fig. 2. The standard deviation did not exceed, in any case, ± 15% of the mean values.

concentrations, in transport experiments, were higher than 1.2 mM for the same nucleotide. This part of the curve appeared to follow a non-saturable kinetic.

The Hanes-Woolf plot from Fig. 3B allows a direct representation of the intragranular ratio of ε-ATP/ε-Ap_4 (S/V) versus the intragranular ε-ATP (S) [14]. The shape of the curve indicates more clearly the existence of the biphasic process already suggested and allows the kinetic parameters to be obtained more easily. The ε-Ap_4 formation was more favourable at the lowest concentrations of ε-ATP and continuously declined until reaching the second part of the curve where a ratio of 18 times more ε-ATP than ε-Ap_4 was obtained. This proportion was maintained constant in the broad range of ε-ATP concentrations used, reaching 6 mM extragranular. The K_m value for the saturation component required the presence of 1.15 pmol ε-ATP intragranular/mg of total granular proteins. The V_{max} value obtained for the ε-Ap_4 production was 29 pmol/mg protein for the saturable component.

4. Discussion

The results presented here show the production of ε-Ap_4 after vesicular transport of ε-ATP and confirmed the already reported presence of Ap_4 as a natural constituent of the chromaffin granules [1].

The Ap_4 values reported here indicate a granular concentration close to 0.8 mM, taking into account a 245 times ATP/Ap_4 ratio and the granular concentration reported in the literature for the ATP. One explanation for the granular Ap_4 presence could be the low selectivity of the nucleotide vesicular transporter concerning the phosphate number [2,6,8]. Another could be the Ap_4 formation by phosphate interchange among granular nucleotides [6,8,9] according to the results reported here with ε-ATP.

The ε-Ap_4 formation is dependent on the intragranular presence of ε-ATP, exhibiting a well-defined kinetic. The K_m value for the saturable component is 0.4 μM when the intragranular ε-ATP concentration is considered. Moreover, it is necessary to point out that the ε-ATP values reported here correspond to the end of the transport period, and the K_m value could be significantly lower. The affinity for the natural substrate, ATP, would be similar to ε-ATP based on structural analogy.

The non-saturable component of ε-Ap_4 formation requires intragranular concentration higher than 175 μM for ε-ATP, and the quotient value for ε-ATP/ε-Ap_4 is maintained at around 18 in a very large range of concentrations. A higher ratio (ATP/Ap_4=245) was obtained for the natural nucleotides in mature chromaffin granules. The saturable component, working at very low substrate concentrations, needs to be considered in immature granules or in synaptic vesicles that have undergone replenishment after exocytosis. In this situation, the Ap_4 could not be a scarce vesicular constituent, and its actions could be physiologicaly relevant. The Ap_4 has been reported to be one order of magnitude more active than the ATP and its non-hydrolyzable analogues in the induction of contractions in guinea-pig vas deferens mediated by P_{2x} receptors [10].

An additional source of Ap_4 at the extracellular level is the action of ecto-diadenosine polyphosphate hydrolase on diadenosine pentaphosphate (Ap_5A), released from secretory granules, that produces AMP and Ap_4.

The fact that the Ap_4 is a poor substrate for the ecto-nucleotidases cascade allows its extracellular accumulation for longer periods of time and possible interaction with P_2 purinoceptors [15]. Although much work is necessary for a complete understanding of Ap_4 actions on cellular signaling, its role in the global context of purinergic transmission cannot be overlooked.

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