Diadenosine polyphosphate hydrolase from presynaptic plasma membranes of *Torpedo* electric organ

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The diadenosine polyphosphate hydrolase present in presynaptic plasma membranes from the *Torpedo* electric organ has been characterized using fluorogenic substrates of the form di-(1,*N*⁶-ethenoadenosine) 5',5'''-*P*^{*n*}-polyphosphate. The enzyme hydrolyses diadenosine polyphosphates (Ap_{*n*}A, where *n* = 3–5), producing AMP and the corresponding adenosine (*n*–1) 5'-phosphate, Ap_(*n*-1). The *K*_m values of the enzyme were 0.543 ± 0.015, 0.478 ± 0.043 and 0.520 ± 0.026 μM, and the *V*_{max} values were 633 ± 4, 592 ± 18 and 576 ± 45 pmol/min per mg of protein, for the etheno derivatives of Ap₃A (adenosine 5',5'''-*P*³-triphosphate), Ap₄A (adenosine 5',5'''-*P*⁴-tetraphosphate) and Ap₅A (adenosine 5',5'''-*P*⁵-pentaphosphate) respectively. Ca²⁺, Mg²⁺ and Mn²⁺ are enzyme activators, with EC₅₀ values of 0.86 ± 0.11, 1.35 ± 0.24 and 0.58 ± 0.10 mM respectively. The fluoride ion is an inhibitor with an IC₅₀ value of 1.38 ± 0.19 mM.

The ATP analogues adenosine 5'-tetraphosphate and adenosine 5'-[γ-thio]triphosphate are potent competitive inhibitors and adenosine 5'-[α,β-methylene]diphosphate is a less potent competitive inhibitor, the *K*_i values being 0.29 ± 0.03, 0.43 ± 0.05 and 7.18 ± 0.8 μM respectively. The P2-receptor antagonist pyridoxal phosphate 6-azophenyl-2',4'-disulphonic acid behaves as a non-competitive inhibitor with a *K*_i value of 29.7 ± 3.1 μM, and also exhibits a significant inhibitory effect on *Torpedo* apyrase activity. The effect of pH on the *K*_m and *V*_{max} values, together with inhibition by diethyl pyrocarbonate, strongly suggests the presence of functional histidine residues in *Torpedo* diadenosine polyphosphate hydrolase. The enzyme from *Torpedo* shows similarities with that of neural origin from neurochromaffin cells, and significant differences compared with that from endothelial vascular cells.

INTRODUCTION

Although ATP is the most abundant nucleotide in synaptic vesicles, the presence of other compounds, such as ADP, GTP and the adenosine 5',5'''-*P*^{*n*}-polyphosphates (diadenosine polyphosphates; Ap_{*n*}A) Ap₄A (adenosine 5',5'''-*P*⁴-tetraphosphate), Ap₅A (adenosine 5',5'''-*P*⁵-pentaphosphate) and Ap₆A (adenosine 5',5'''-*P*⁶-hexaphosphate), has been described [1–4]. The physiological meaning of such diversity is not yet completely understood. Perhaps the non-selective behaviour of the nucleotide vesicular transporter is the most likely explanation for such diversity [1,5]. Nevertheless, the various nucleotide (P2) receptors with diverse localizations and affinities for the stored nucleotides and dinucleotides suggest a more complex situation [2,6,7]. Moreover, the existence of ecto-nucleotidases, not only for mononucleotides but also for diadenosine polyphosphates, strengthens this argument.

The enzymes responsible for the extracellular hydrolysis of nucleotides, i.e. ecto-ATPase, ecto-ADPase and the ecto-5'-nucleotidase, are widely distributed and have been characterized in a large variety of tissues and species [8,9]. When the activities

of ATPase and ADPase are located in the same protein, sharing a single catalytic centre, the enzyme is then called ATP diphosphohydrolase or apyrase; this enzyme exhibits a broad biological distribution [10]. The ecto-diadenosine polyphosphate hydrolase responsible for the destruction of Ap_{*n*}A compounds has not been fully characterized, and has been described only in endothelial cells from blood vessels and in cultured neurochromaffin cells from the adrenal medulla [11–14]. Nevertheless, in spite of the small number of studies in this field, it is important to note that the enzymes of endothelial and neural origin exhibit significant differences [12,14,15]. Intracellular enzymes that specifically hydrolyse Ap_{*n*}A at the phosphate chain have been characterized and suggested to play an important role in preventing the accumulation of dinucleoside polyphosphates [16,17]. These dinucleotides are powerful effectors of many cellular processes, such as DNA replication and the inactivation of kinase enzymes responsible for phosphate interchange among nucleotides [18–20].

Although the existence of a pure nucleotide synaptic terminal is still controversial, the *Torpedo* electric organ could be a suitable model with which to study purinergic co-transmission.

Abbreviations used: Ado, adenosine; Ap₄, adenosine 5'-tetraphosphate; ATP[S], adenosine 5'-[γ-thio]triphosphate; p[CH₂]pA, adenosine 5'-[α,β-methylene]diphosphate; PPADS, pyridoxal phosphate 6-azophenyl-2',4'-disulphonic acid; DEPC, diethyl pyrocarbonate; Ap_{*n*}A, adenosine 5',5'''-*P*^{*n*}-polyphosphate (diadenosine polyphosphate); Ap₃A, adenosine 5',5'''-*P*³-triphosphate (diadenosine triphosphate); Ap₄A, adenosine 5',5'''-*P*⁴-tetraphosphate (diadenosine tetraphosphate); Ap₅A, adenosine 5',5'''-*P*⁵-pentaphosphate (diadenosine pentaphosphate); Ap₆A, adenosine 5',5'''-*P*⁶-hexaphosphate (diadenosine hexaphosphate); ε-, 1,*N*⁶-etheno derivative; ε-Ap₄, 1,*N*⁶-ethenoadenosine 5'-tetraphosphate; ε-(Ap_{*n*}A), di-(1,*N*⁶-ethenoadenosine) 5',5'''-*P*^{*n*}-polyphosphate (diethenoadenosine polyphosphate); ε-(Ap₃A), di-(1,*N*⁶-ethenoadenosine) 5',5'''-*P*³-triphosphate (diethenoadenosine triphosphate); ε-(Ap₄A), di-(1,*N*⁶-ethenoadenosine) 5',5'''-*P*⁴-tetraphosphate (diethenoadenosine tetraphosphate); ε-(Ap₅A), di-(1,*N*⁶-ethenoadenosine) 5',5'''-*P*⁵-pentaphosphate (diethenoadenosine pentaphosphate).

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The cholinergic synaptic vesicles from the *Torpedo* electric organ also contain nucleotides and Ap_nA as co-transmitters, and the presence of high-affinity receptors for Ap_nA has been described in this system [2,21,22]. Moreover, ATP degradation by ectonucleotidases has been studied extensively in these synaptic terminals, and the existence of an ecto-ATP diphosphohydrolase, or apyrase, has been described and characterized [23,24]. In addition, the extracellular hydrolysis of Ap_nA in *Torpedo* synaptic terminals is necessary to terminate their action on receptors, thus adding support to an extracellular signalling role. The aim of the present work is to study and characterize the diadenosine polyphosphate hydrolase from the plasma membranes of *Torpedo* synaptic terminals, considering this enzyme as the first constituent of the ecto-nucleotidase cascade.

MATERIALS AND METHODS

Materials

Adenosine-derived nucleotides, including adenosine 5'-[γ -thio]-triphosphate (ATP[S]), adenosine 5'-[α,β -methylene]diphosphate ($\text{p}[\text{CH}_2]_2\text{pA}$), adenosine 5'-tetraphosphate (Ap_4), ϵ -ATP, ϵ -ADP, ϵ -AMP and ϵ -Ado (where ϵ - indicates the 1, N^6 -etheno derivative and Ado is adenosine), EDTA, EGTA, diethyl pyrocarbonate (DEPC) and N -ethylmaleimide were all from Sigma (St. Louis, MO, U.S.A.). The fluorogenic ϵ -(Ap_nA) [di-(1, N^6 -ethenoadenosine) 5',5''- P^1,P^n -polyphosphate] dinucleotides were obtained according to recently published procedures [14,17]. Pyridoxal phosphate 6-azophenyl-2',4'-disulphonic acid (PPADS) was from RBI (Natick, MA, U.S.A.). Alkaline phosphatase from calf intestine, *Crotalus durissus* phosphodiesterase and Pipes were from Boehringer (Mannheim, Germany). DEAE-Sephacel was from Pharmacia (Uppsala, Sweden). All other products were reagent grade from Merck (Darmstadt, Germany).

Animals and isolation of presynaptic plasma membranes

Torpedo marmorata specimens were caught in the Mediterranean Sea and maintained alive in artificial sea water. Electric organs were dissected out under anaesthesia (tricaine; 0.33 g/l of sea water) (MS22; Sandoz) and immersed in 10 mM Tris/HCl buffer, pH 7.5, containing 1 mM EDTA. Presynaptic plasma membranes were isolated from the electric organ as previously described [25]. The enriched presynaptic plasma membrane fractions, at a protein concentration of 10 mg/ml, were stored in the same buffer at -80°C until use. Before measuring enzymic activity, a corresponding aliquot was washed twice with 20 mM Tris/HCl, pH 7.5, and centrifuged at 100 000 g for 20 min at 4°C in a Beckman L8-M Ultracentrifuge (rotor type 50.4 Ti) to eliminate EDTA that could interfere with the enzymic assay.

Ap_nA hydrolase fluorimetric assays and calculation of kinetic parameters

Fluorimetric assays were performed essentially as published [14,26], with minor modifications. Presynaptic plasma membranes were resuspended in a standard medium composed of 20 mM Tris/HCl, pH 7.5, 2 mM CaCl_2 and 4 mM MgCl_2 , containing an appropriate amount of enzyme, and assayed continuously for the hydrolysis of ϵ -(Ap_nA) using initial substrate concentrations ranging between 0.5 and 2.5 μM , in a final cuvette volume of 1.5 ml fortified with 5 units of alkaline phosphatase. Alkaline phosphatase was required to obtain reliable kinetic parameters, as it prevented enzyme inhibition caused by accumulation of the released ϵ -Ado 5'-phosphate products. Omission

of the phosphatase resulted in anomalous non-linear plots of the integrated Michaelis-Menten equation (see below); the enzyme was not required when studying the release of hydrolysis products by HPLC. The incubation was maintained at 37°C under continuous stirring. Usually a concentration of 100 μg of protein/ml per assay was used.

The progress of the reaction was followed by recording the increase in fluorescence emission at 410 nm using excitation at 305 nm and band-pass widths of 2.5–10 nm in an LS 50 fluorimeter (Perkin-Elmer, Beaconsfield, Bucks., U.K.). An increase in fluorescence emission is only associated with the cleavage of ϵ -(Ap_nA) compounds producing ϵ -Ado 5'-phosphate moieties; the subsequent hydrolysis of the released moieties up to ϵ -Ado did not result in fluorescence changes. Fluorescence intensity measurements were converted into substrate concentration according to the formula:

$$[\text{S}]_t = [\text{S}]_0(I_t - I_0)/(I_t - I_0) \quad (1)$$

where $[\text{S}]_0$ and $[\text{S}]_t$ are the substrate concentrations at time zero and time t respectively, and I_0 , I_t and I_t are the fluorescence values at time zero, time t and once all substrate had been consumed (final). I_t was measured after the addition of 1 μl containing 1 m-unit of *Crotalus durissus* phosphodiesterase to ensure complete degradation of the substrate.

To calculate K_m and $V_{\max}$ values, substrate concentration as a function of time was analysed and plotted according to the integrated form of the Michaelis-Menten equation:

$$\ln \frac{[\text{S}]_0}{[\text{S}]_t} \cdot \frac{1}{t} = \frac{V_{\max}}{K_m} - \frac{1}{K_m} \cdot \frac{([\text{S}]_0 - [\text{S}]_t)}{t} \quad (2)$$

To analyse the effect of pH on the hydrolysis of diadenosine polyphosphates, ϵ -(Ap_3A) [di-(1, N^6 -ethenoadenosine) 5',5''- P^1,P^3 -triphosphate] was used as the substrate. Pipes (20 mM; between pH 6.0 and 6.5) and Tris/HCl (20 mM; between pH 7.0 and 9.0), each containing 2 mM CaCl_2 and 4 mM MgCl_2 , were employed as buffers. The effect of pH on $V_{\max}$ and K_m was analysed using a Dixon-Webb representation [27] of the data, obtained as described above.

The effect of a histidine-modifying reagent, DEPC, on the enzyme was studied by measuring residual activity after a 5 min preincubation of membrane preparations with the reagent at concentrations of 1–20 mM. Residual activity was calculated as the slope of the linear decay of the substrate concentration with time obtained from the fluorimetric recording using eqn. (1), and was expressed as a percentage of the control.

HPLC instrumentation and chromatographic Ap_nA hydrolase assays

The following HPLC equipment was from Waters (Milford, MA, U.S.A.): a 600 E solvent delivery system, a 474 fluorescence detector, an automatic injector 717 plus Autosampler and a Millennium 2010 Chromatography Manager System. Bio-Sil C-18 HL 90-10 (250 mm $\times$ 4.6 mm; 10 μm particle size) columns from Bio-Rad (Richmond, CA, U.S.A.) were used throughout.

The separation of ϵ -(Ap_nA) and their degradation products was performed using a basic reverse-phase HPLC protocol previously described [28], using as eluent 0.1 M KH_2PO_4 and 7% (v/v) methanol, pH 6.0 adjusted with KOH, at a flow rate of 1.5 ml/min. Fluorescence detection was performed at excitation and emission wavelengths of 305 and 410 nm respectively. To study the time course of the release of degradation products from ϵ -(Ap_nA), 100 μg /ml presynaptic plasma membranes from *Torpedo* were washed and incubated with the standard medium (20 mM

Tris/HCl, pH 7.5, 2 mM CaCl_2 and 4 mM MgCl_2) containing a 1 μM concentration of the required ϵ -(Ap_nA) (where $n = 3-5$), during fixed time periods of 0, 5 and 15 min at 37 °C under continuous stirring. The final incubation volume was 1.5 ml. Alkaline phosphatase was omitted from these assays. At the end of the incubation, the reaction medium was filtered through a 0.22 μm -pore-size Millex-GS filter (Millipore, Molsheim, France) and 10 μl aliquots were injected into the chromatographic system in order to analyse substrate and degradation products.

In those experiments in which the inhibitory effects of ATP analogues and PPADS on ϵ -(Ap_nA) hydrolysis were studied, the HPLC technique was doubly valuable: (1) it allows very sensitive measurement of the hydrolysis products owing to the large fluorescence increase produced [6–9-fold, depending on the ϵ -(Ap_nA) used] when the ϵ -dinucleotide is broken into ϵ -mononucleotides [14,26]; and (2) it allows detection of the accumulation of any intermediate hydrolysis products due to the action of the inhibitors on any enzyme(s) of the ecto-nucleotidase cascade.

Protein determination

Protein content was measured by the method of Bradford [29], using BSA as standard.

RESULTS

Ap_nA hydrolysis by *Torpedo* synaptic membranes

Presynaptic plasma membranes from *Torpedo* electric organ were able to degrade the ϵ -(Ap_nA) (where $n = 3-5$) compounds. The hydrolytic reaction was analysed by both HPLC and continuous fluorimetric methods.

Figure 1 shows the HPLC chromatograms obtained following hydrolysis of ϵ -(Ap_nA). In all cases, ϵ -Ado accumulated as the final product as a function of the incubation time, due to the presence of the ecto-nucleotidase cascade in these membrane preparations [23,24]. The ϵ -(Ap_nA) compounds, when

hydrolysed, produced ϵ -AMP and the corresponding ϵ - $\text{Ap}_{(n-1)}$ nucleotide. The amounts of ϵ -AMP were very small, perhaps due to the high rate of ϵ -AMP degradation by the ecto-5'-nucleotidase. In the case of ϵ -(Ap_3A), a peak of ϵ -ADP was present, but only small amounts of ϵ -AMP were measured (Figure 1a). The simplicity of the hydrolysis of ϵ -(Ap_3A) made it the most suitable compound for studying the effects of inhibitors on Ap_nA hydrolase and other ecto-nucleotidases of the cascade. The hydrolysis of ϵ -(Ap_4A) [di-(1, N^6 -ethenoadenosine) 5',5'''- P^1 , P^4 -tetraphosphate] (Figure 1b) resulted in small peaks of ϵ -ATP and ϵ -ADP and, as in the previous case, insignificant amounts of ϵ -AMP. As always, the ϵ -Ado peak increased with incubation time. The hydrolysis of ϵ -(Ap_5A) [di-(1, N^6 -ethenoadenosine) 5',5'''- P^1 , P^5 -pentaphosphate] (Figure 1c) gave a prominent peak of ϵ - Ap_4 (1, N^6 -ethenoadenosine 5'-tetraphosphate), as well as smaller amounts of the intermediate reaction compounds produced by the other ecto-enzymes in forming ϵ -Ado.

The continuous fluorimetric assay, based on the increase in fluorescence caused by degradation of the phosphate bridge between ϵ -Ado moieties, gave significantly improved kinetic data for ϵ -(Ap_nA) hydrolysis. Since the fluorescence of ϵ -nucleotides does not depend on their phosphorylation state and is the same as that of ϵ -Ado, this technique measured Ap_nA hydrolysis directly. The enzyme present in *Torpedo* synaptic membranes showed a similar capacity for hydrolysis for all of the ϵ -(Ap_nA) substrates, as can be seen in the continuous recordings of the fluorescence increases (Figure 2a) and in the transformations to substrate disappearance (Figure 2b) for ϵ -(Ap_3A), ϵ -(Ap_4A) and ϵ -(Ap_5A).

Saturation studies of ϵ -(Ap_nA) hydrolysis

The kinetic parameters for ϵ -(Ap_nA) hydrolysis were obtained from the fluorescence recordings shown in Figure 2(a), transformed into the corresponding concentration values as a function of time (Figure 2b). The data were then treated according to the integrated form of the Michaelis–Menten equation (see the

Figure 1 Fluorescence reverse-phase HPLC profiles illustrating the time-dependent hydrolysis of ϵ -(Ap_nA) by presynaptic plasma membranes from *Torpedo* electric organ

Plasma membranes (100 μg of protein/ml) were incubated in standard medium (see the Materials and methods section) containing the required ϵ -(Ap_nA) (where $n = 3-5$) at 1 μM . Aliquots of 10 μl were taken after 0, 5 and 15 min of incubation and injected into the chromatographic system to analyse substrate and degradation products. (a) ϵ -(Ap_3A) hydrolysis; (b) ϵ -(Ap_4A) hydrolysis; (c) ϵ -(Ap_5A) hydrolysis. These traces are from one of three experiments giving identical results. A.U., arbitrary units.

Figure 2 Determination of kinetic parameters for the hydrolysis of ϵ -(Ap_nA) by presynaptic plasma membranes from *Torpedo* electric organ

Suspensions of plasma membranes containing 100 μg of protein/ml of standard medium were incubated in the fluorimeter cuvette at 37 °C under continuous stirring in the presence of 1 μM ϵ -(Ap_nA) (where $n = 3-5$), and the time-dependent increase in fluorescence due to substrate hydrolysis was recorded. Results from a single representative experiment are shown. (a) Traces of fluorescence increases associated with ϵ -(Ap_3A), ϵ -(Ap_4A) and ϵ -(Ap_5A) cleavage by these membrane preparations. (b) Decline of ϵ -(Ap_nA) concentration as a function of time, calculated from the fluorescence increase traces depicted in (a). (c) Plot of reaction time and ϵ -(Ap_3A) concentration data obtained from curve in (b) treated according to the integrated form of the Michaelis–Menten equation to determine K_m and $V_{\max}$ (see the Materials and methods section for details). S_0 and S_t are the substrate concentrations at time zero and time t respectively. For clarity, transformations of the ϵ -(Ap_4A) and ϵ -(Ap_5A) data are not shown. A.U., arbitrary units.

Materials and methods section), and linear plots for the three Ap_nA substrates were obtained, indicating hyperbolic kinetics. Figure 2(c) shows the plot for ϵ -(Ap_3A); those for ϵ -(Ap_4A) and ϵ -(Ap_5A) were similar (not shown).

The K_m and $V_{\max}$ values for the hydrolysis of ϵ -(Ap_nA) are summarized in Table 1. The affinity and $V_{\max}$ values were very similar for the three dinucleotides. The $V_{\max}/K_m$ ratios obtained from the experimental data were 1.16, 1.24 and 1.11 for ϵ -(Ap_3A), ϵ -(Ap_4A) and ϵ -(Ap_5A) respectively, indicating almost equal substrate suitability among them.

Table 1 Kinetic parameters for the hydrolysis of ϵ -(Ap_nA) compounds by plasma membranes from *Torpedo* synaptic terminals

Values are means $\pm$ S.D. for three determinations.

ϵ -(Ap_nA)	K_m (μM)	$V_{\max}$ (pmol/min per mg of protein)
ϵ -(Ap_3A)	0.543 ± 0.015	633 ± 4
ϵ -(Ap_4A)	0.478 ± 0.043	592 ± 18
ϵ -(Ap_5A)	0.520 ± 0.026	576 ± 45

Table 2 Effects of some bivalent cations and fluoride on the hydrolysis of ϵ -(Ap_4A) by plasma membranes from *Torpedo* synaptic terminals

Suspensions of plasma membranes (50 μg of protein/ml) were incubated in 20 mM Tris/HCl, pH 7.5, containing 10 μM ϵ -(Ap_4A) and continuously assayed in the fluorimeter cuvette at 37 °C under continuous stirring. Activities were calculated from the fluorescence increase as a function of time and expressed as a percentage of control activity without added ions for Ca^{2+} (CaCl_2), Mg^{2+} (MgCl_2) and Mn^{2+} (MnCl_2), or as the percentage decrease with respect to control activity in the presence of 2 mM Ca^{2+} and 4 mM Mg^{2+} (standard medium) for F^- (NaF). EC_{50} and IC_{50} values are means $\pm$ S.D. of three experiments performed in duplicate.

Ion	Effect	$\text{EC}_{50}/\text{IC}_{50}$ (mM)	Maximal effect (%)
Ca^{2+}	Activator	0.86 ± 0.11	500
Mg^{2+}	Activator	1.35 ± 0.24	400
Mn^{2+}	Activator	0.58 ± 0.10	400
F^-	Inhibitor	1.38 ± 0.19	70

Effects of bivalent cations and fluoride on Ap_nA hydrolase activity

The enzyme from presynaptic plasma membranes of the *Torpedo* electric organ was stimulated to a similar extent by Ca^{2+} , Mg^{2+} and Mn^{2+} . The $\text{EC}_{50}/\text{IC}_{50}$ values and maximal effects are summarized in Table 2. When the enzymic activity was determined in the absence of these bivalent cations (in 20 mM Tris/HCl, pH 7.5), the activity was $21 \pm 3\%$ of that in the standard medium (plus 2 mM CaCl_2 and 4 mM MgCl_2). The activity in the presence of only one bivalent cation in the medium was $70 \pm 3\%$ for 4 mM Mg^{2+} and $80 \pm 4\%$ for 2 mM Ca^{2+} compared with that in the presence of both (standard medium); this indicated only a slight additive effect of the two cations. In the presence of EGTA or EDTA the hydrolytic activity was almost completely abolished.

Fluoride is an inhibitor of Ap_nA hydrolase, although the IC_{50} value (Table 2) indicates low inhibition of ϵ -(Ap_4A) hydrolysis.

ATP analogues and PPADS as inhibitors of ϵ -(Ap_nA) hydrolysis

The ATP analogues Ap_4 , ATP[S] and $\text{p[CH}_2\text{]pA}$, together with the P2-receptor-antagonist PPADS, inhibited the Ap_nA hydrolase from *Torpedo* synaptic terminals. As some of these compounds have already been described as inhibitors of other components of the ecto-nucleotidase cascade, Ap_nA hydrolysis was measured using the HPLC method. The disappearance of ϵ -(Ap_nA) and the appearance of the progressive degradation products until reaching ϵ -Ado were then quantifiable, yielding more complete information. The HPLC chromatograms for the

Figure 3 Inhibition by ATP analogues and PPADS of ϵ -(Ap_3A) hydrolysis

Suspensions of plasma membranes containing 100 μg of protein/ml were incubated at 37 $^{\circ}\text{C}$ under continuous stirring in standard medium containing 1 μM ϵ -(Ap_3A) in control experiments and 1 μM ϵ -(Ap_3A) plus 25 μM ATP[S] ($\text{ATP}\gamma\text{S}$) (a), 25 μM Ap_4 (b), 25 μM $\text{p[CH}_2\text{]pA}$ (α, β -MeADP) (c) or 25 μM PPADS (d) in test experiments; the inhibitors were added to the reaction mixture 1 min before the addition of substrate. Aliquots were removed from the reaction medium after incubation for 0 and 10 min, filtered (see the Materials and methods section) and 20 μl samples injected into the chromatographic system to analyse substrate and degradation products. Under these experimental conditions Ap_4 , ATP[S] , $\text{p[CH}_2\text{]pA}$ and PPADS inhibited ϵ -(Ap_3A) cleavage by 97, 95, 44 and 28% respectively. These results are traces from one representative experiment of three. A.U., arbitrary units.

hydrolysis of ϵ -(Ap_3A) in the presence of the inhibitors are shown in Figure 3. ϵ -(Ap_3A) was chosen as the substrate due to the lower number of degradation products.

Ap_4 and ATP[S] proved to be very good inhibitors of polyphosphate hydrolysis; both exhibited competitive behaviour, with K_i values of 0.29 ± 0.03 and 0.43 ± 0.05 μM respectively. Figures 3(a) and 3(b) show that inhibition was almost complete at a concentration of 25 μM for both compounds, with no appearance of degradation products. The other nucleotide analogue tested, $\text{p[CH}_2\text{]pA}$, was also an inhibitor of Ap_nA hydrolase, showing competitive behaviour with a K_i value of 7.18 ± 0.8 μM (more than one order of magnitude higher than the other inhibitors). It is noteworthy from the chromatogram shown in Figure 3(c) that this compound also inhibited the action of ecto-5'-nucleotidase, since, in spite of the lowest proportion of ϵ -AMP, this product accumulated to a greater extent than with the other inhibitors.

PPADS inhibited Ap_nA hydrolase in a non-competitive manner, with a K_i value of 29.7 ± 3.1 μM . The HPLC chromatograms in Figure 3(d) show the effect of PPADS at 25 μM . The accumulation of ϵ -ADP, but not ϵ -AMP, was the main differential characteristic. This result contrasts with the action of the other

inhibitors. With Ap_4 and ATP[S] , there was no accumulation of intermediate compounds, and when $\text{p[CH}_2\text{]pA}$ (an inhibitor of ecto-5'-nucleotidase) was employed, ϵ -AMP was the nucleotide that accumulated. So, with PPADS, the accumulation of ϵ -ADP but not ϵ -AMP indicates that this compound is an inhibitor of apyrase as well as of Ap_nA hydrolase.

Influence of pH on kinetic parameters, and inhibition by DEPC

The effects of pH on V_{max} and K_m are represented in Figure 4 as Dixon-Webb plots [27]. The range of pH values studied was limited between pH 6 and 9; within this interval, there were no changes in the fluorescence emission of ϵ -nucleotides. This unavoidable experimental situation limits the range of pH values under study and therefore does not allow the detection of ionizable groups outside this range. The residual ionizable groups of the enzyme relevant to catalysis and affinity were determined from the pK_a values obtained from the experimental curves. From the representation of $\log V_{\text{max}}$ against pH, an ionizable group with a pK_a value of 7.3 necessary for the catalytic action of the enzyme-substrate complex was revealed (Figure 4a). The graphical representation of $\log(V_{\text{max}}/K_m)$ is shown in Figure

Figure 4 Effects of pH and DEPC on the activity of the enzyme hydrolysing ϵ -(Ap₃A)

(a) Dixon-Webb representation of $\log V_{\max}$ against pH. The value of $V_{\max}$ at each pH was calculated after incubation of membranes (100 μ g of protein/ml) in the fluorimeter cuvette at 37 °C with continuous stirring in the appropriate buffer (Pipes or Tris; see the Materials and methods section for details) containing 0.5 μ M or 2 μ M ϵ -(Ap₃A). The intersection between the dashed lines indicates the pK_a of the group implicated in catalysis in the enzyme-substrate complex, within the range of pH values studied. (b) Dixon-Webb representation of $\log(V_{\max}/K_m)$ against pH. The kinetic parameters at each pH were calculated as described in the Materials and methods section, using the same experimental conditions as above. The intersections between the dashed lines indicate the pK values of the groups implicated in the affinity of the enzyme for the substrate. (c) Dose-dependent inhibition of ϵ -(Ap₃A) hydrolysis by DEPC. Membranes (100 μ g of protein/ml) were incubated in standard medium containing 2 μ M ϵ -(Ap₃A) at 37 °C with continuous stirring, and the ϵ -(Ap₃A)-hydrolysing activity was quantified by measuring the percentage decrease in the velocity of consumption of the substrate at increasing concentrations of DEPC. Values are means $\pm$ S.D. of three experiments performed in duplicate.

4(b). Two different ionizable groups necessary for the affinity of the free enzyme were apparent, one with a pK_a value of 7.6 and the other with a pK_a value of 8.5.

The pK_a values around 7.3 (enzyme-substrate complex) to 7.6 (enzyme) indicated the presence of functional residual groups

able to deprotonate within this pH range. The role of the thiol groups of cysteine residues and the imidazolium groups of histidine residues was analysed. The inhibitory action of DEPC, with an IC₅₀ value of 1 mM, strongly suggests the existence of histidine residues essential for catalysis and substrate binding (Figure 4c). A functional role for cysteine residues was excluded, as *N*-ethylmaleimide did not inhibit the reaction (results not shown).

DISCUSSION

The experimental work reported here demonstrates the presence of Ap_nA hydrolase activity in the membranes of the *Torpedo* electric organ. This enzyme degrades ϵ -(Ap_nA) dinucleotides, producing ϵ -AMP and the corresponding ϵ -Ap_(n-1). The products are further degraded by the ecto-ATP diphosphohydrolase (apyrase) and ecto-5'-nucleotidase which are present in these synaptic membranes [2,23,24]. ϵ -Ado is the terminal product that accumulates at the end of the enzymic cascade.

The presence of ectoenzymic Ap_nA hydrolase activity has been reported previously in vascular endothelial cells and in adreno-medullary Ap_nA-secreting chromaffin cells; these activities initiate the degradation of extracellular Ap_nA to Ado [11-14]. These observations, and results demonstrating the ability of *Torpedo* synaptosomes to exocytotically release vesicular Ap_nA that may bind to high-affinity receptors [21,22], indicate strongly that the membrane-bound enzyme investigated here is a synaptosomal ectoenzyme involved in the extracellular degradation of Ap_nA compounds that have been released from synaptic vesicles.

Ap₄A and ϵ -(Ap₄A) exhibited very similar K_m and $V_{\max}$ values, with an almost identical $V_{\max}/K_m$ ratio in the neural model of cultured chromaffin cells and their isolated plasma membranes [13,14]. Thus the etheno-derivatives can be employed as good analogues of the natural substrates.

The high affinity exhibited by the *Torpedo* enzyme, with $K_m < 1 \mu$ M, could be related to the low concentrations of these compounds present after release at the synaptic space. Although there are no experimental measurements of extracellular Ap_nA concentrations after *Torpedo* electric organ stimulation, the secretory model of chromaffin cells can provide some indications. In this model, after massive induced release, the extracellular levels of Ap_nA can reach micromolar concentrations in the space surrounding the secretory cell [30]. The affinity of the ecto-Ap_nA hydrolase in chromaffin cells for the substrates is in the low micromolar range, and appears to be adapted to the extracellular concentrations of these compounds [13,14]. The higher affinity of the *Torpedo* enzyme could be the result of an adaptation to lower levels of these compounds at the synaptic terminal of *Torpedo* after release.

The ecto-Ap_nA hydrolase activities reported in vascular endothelial cells show large variability with respect to substrate affinity, and also differ from those of neural origin [11,12,15]. In spite of the different methodological approaches used, differences of one or two orders of magnitude in the K_m parameter should be considered significant.

Ion-dependence of Ap_nA hydrolase

Although all of the Ap_nA hydrolases so far reported are inhibited by chelating agents and activated by Mg²⁺, they exhibit significant differences with regard to other ions. This leads us to propose the existence of at least two types of enzyme with different biochemical properties: neural and endothelial. The neural ecto-Ap_nA hydrolase is activated by Ca²⁺, Mg²⁺ and Mn²⁺ to a similar extent [14,15]. In contrast, the endothelial ecto-enzyme is

inhibited by Ca^{2+} , and Mn^{2+} is a more powerful activator than Mg^{2+} [12,15]. Concerning the fluoride ion, it is significant that the cytosolic enzyme specific for Ap_4A hydrolysis, diadenosine tetraphosphate hydrolase, is inhibited by this ion in the low micromolar range, with an IC_{50} of approx. $30 \mu\text{M}$ [17,31]. Much higher concentrations are required to inhibit the ecto-enzymes, each one of which exhibits striking differences. The most sensitive is the enzyme of endothelial origin (IC_{50} $500 \mu\text{M}$), followed by the *Torpedo* enzyme (IC_{50} 1.5 mM) and finally the chromaffin cell enzyme, which is highly resistant to inhibition by fluoride [14].

Ionizing groups involved in substrate recognition and catalysis

The Dixon–Webb representation clearly indicates that the enzyme is more active in alkaline conditions, reaching a plateau of maximal activity from pH 8. A diprotic model for the enzyme can be postulated, in which the free enzyme binds the substrate via residual groups with pK_a values of 7.6 and 8.5 [obtained from the $\log(V_{\text{max}}/K_m)$ representation]. Once the substrate is bound, only the group of pK_a 7.3 in the enzyme–substrate complex, corresponding to that of pK_a 7.6 in the free enzyme, is catalytically active, in agreement with the representation of $\log V_{\text{max}}$ [32]. Inhibition by DEPC strongly suggests the presence of catalytically active histidine residues. The involvement of cysteine groups is ruled out, since *N*-ethylmaleimide is not inhibitory. The pK_a of 8.5 corresponding to the free enzyme could be the residual amino group of lysine, necessary for interaction with the substrate. Studies with specific blockers of the ϵ -amine group should be performed to test this possibility. A representative example of a catalytic centre for phosphodiester bond hydrolysis, i.e. that in RNase A, contains catalytic histidine and lysine residues that bind the substrate [33].

Purinergic pharmacology of the enzyme

The inhibitory action of ATP analogues on Ap_nA hydrolase from *Torpedo* membranes required use of the HPLC technique to demonstrate clearly at what level or levels of the cascade each compound was acting. Ap_4 and ATP[S] were the best inhibitors, with K_i values of $< 1 \mu\text{M}$. Although ATP[S] is a synthetic compound, this is not the case for Ap_4 , which is produced on hydrolysis of exocytotically released Ap_5A [3,14,30]. This increases the physiological significance of the data reporting a better agonistic effect of Ap_4 compared with ATP on guinea pig vas deferens P2X receptors [34]. $\text{p[CH}_2\text{]pA}$ is also an inhibitor of the enzyme, although with a K_i value more than one order of magnitude higher than those of Ap_4 and ATP[S] . In this case, the significant inhibitory effect on ecto-5'-nucleotidase, described by other authors in different models, is observable in the HPLC chromatograms as the accumulation of ϵ -AMP. It is important that $\text{p[CH}_2\text{]pA}$ is two or three orders of magnitude less effective in inhibiting Ap_nA hydrolysis compared with its effect on AMP hydrolysis [9,35,36].

The inhibitory pattern exhibited by PPADS, resulting in the accumulation of ϵ -ADP, confirms previous reports in which this compound inhibited the ecto-ATPase and ecto-ADPase activities from *Xenopus* oocytes [37]. In synaptic plasma membranes from *Torpedo* the ecto-ATPase and ecto-ADPase activities appear to be on the same enzyme, known as apyrase [24]. Thus PPADS behaves as inhibitor of apyrase as well as of Ap_nA hydrolase. Suramin, which has been described as an inhibitor of ecto-apyrase and ecto-ATPase in a large variety of systems, is also a much more powerful inhibitor than PPADS of ecto- Ap_nA hydrolase (K_i $1.8 \mu\text{M}$) in *Torpedo* synaptic terminals [24,37–40].

Nucleotidase cascade: from Ap_nA to Ado

In the context of ectoenzymes that degrade nucleotides, the Ap_nA hydrolase should be considered as the first enzyme of the extracellular cascade. The activity reported for ecto- Ap_nA hydrolase in the neural models studied is about two or three orders of magnitude lower than that reported for ecto-ATPase, ecto-ADPase or apyrase; compared with ecto-5'-nucleotidase, the activity is about two orders of magnitude lower [23,24,41]. The enzymic activities corresponding to ecto- Ap_nA hydrolase, apyrase and ecto-5'-nucleotidase appear to lie in different proteins, because when cultured chromaffin cells are submitted to the action of cycloheximide the disappearance of each activity follows a distinct pattern with different half-life values [42]. The presence of Ap_4A and Ap_5A , but not ATP or ADP, in the perfused brain after amphetamine stimulation, together with the increases in AMP and adenosine levels, can be explained if a similar pattern exists for the ecto-nucleotidase activities in the brain [43]. The longer half-life of Ap_nA when compared with ATP in extracellular media suggests a role in cellular signalling other than at the synaptic level. In this regard, an effect of Ap_nA on the growth of rat renal mesangial cells has been reported [44,45]. In future studies it would be interesting to characterize fully the enzymes that hydrolyse Ap_nA from diverse origins, and to investigate their involvement in the control of purinergic signalling.

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REFERENCES

- Winkler, H. and Carmichael, S. W. (1982) in *The Secretory Granule* (Poisner, A. M. and Triflaró, J. M., eds.), pp. 3–79, Elsevier–North Holland Biomedical Press, New York
- Zimmermann, H. (1994) *Trends Neurosci.* **17**, 420–426
- Rodríguez del Castillo, A., Torres, M., Delicado, E. G. and Miras-Portugal, M. T. (1988) *J. Neurochem.* **51**, 1696–1703
- Pintor, J., Rotllán, P., Torres, M. and Miras-Portugal, M. T. (1992) *Anal. Biochem.* **200**, 296–300
- Gualix, J., Abal, M., Pintor, J., García-Carmona, F. and Miras-Portugal, M. T. (1996) *J. Biol. Chem.* **271**, 1957–1965
- Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Daly, J. W., Harden, T. K., Jacobson, K. A., Leff, P. and Williams, M. (1994) *Pharmacol. Rev.* **46**, 143–156
- Harden, T. K., Boyer, J. L. and Nicholas, R. A. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35**, 541–579
- Plesner, L. (1995) *Int. Rev. Cytol.* **158**, 141–214
- Zimmermann, H. (1992) *Biochem. J.* **285**, 345–365
- Komoszynski, M. and Wojtczak, A. (1996) *Biochim. Biophys. Acta* **1310**, 233–241
- Goldman, S. J., Gordon, E. L. and Slakey, L. L. (1986) *Circ. Res.* **59**, 362–366
- Ogilvie, A., Lüthje, J., Pohl, U. and Busse, R. (1989) *Biochem. J.* **259**, 97–103
- Rodríguez-Pascual, F., Torres, M., Rotllán, P. and Miras-Portugal, M. T. (1992) *Arch. Biochem. Biophys.* **297**, 176–183
- Ramos, A., Pintor, J., Miras-Portugal, M. T. and Rotllán, P. (1995) *Anal. Biochem.* **228**, 74–82
- Miras-Portugal, M. T., Rotllán, P. and Mateo, J. (1995) *FASEB J.* **9**, 687 (abstract)
- Guranowski, A. and Sillero, A. (1992) in *Ap_nA and Other Dinucleoside Polyphosphates* (McLennan, A. G., ed.), pp. 81–133, CRC Press, Boca Raton
- Ramos, A. and Rotllán, P. (1995) *Biochim. Biophys. Acta* **1253**, 103–111
- Grummt, F., Waltz, G., Jantzen, H. M., Hamprecht, K., Huebscher, U. and Kuenzle, C. C. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6081–6085
- Rotllán, P. and Miras-Portugal, M. T. (1985) *Eur. J. Biochem.* **151**, 365–371
- Lienhard, G. E. and Secemski, I. I. (1973) *J. Biol. Chem.* **248**, 1121–1123
- Pintor, J., Kowalewski, H. J., Torres, M., Miras-Portugal, M. T. and Zimmermann, H. (1992) *Neurosci. Res. Commun.* **10**, 9–15

- 22 Pintor, J., Kowalewski, H. J., Zimmermann, H. and Miras-Portugal, M. T. (1994) *Neurosci. Res. Commun.* **15**, 167–175
- 23 Grondal, E. J. M. and Zimmermann, H. (1986) *J. Neurochem.* **47**, 871–881
- 24 Martí, E., Canti, C., Gómez de Aranda, I., Miralles, F. and Solsona, C. (1996) *Br. J. Pharmacol.* **118**, 1232–1236
- 25 Morel, N., Marsal, J., Manaranche, R., Lazareg, S., Maize, J. C. and Israel, M. (1985) *J. Cell Biol.* **101**, 1757–1762
- 26 Wierchowski, J., Sierakowska, H. and Shugar, D. (1985) *Biochim. Biophys. Acta* **828**, 109–115
- 27 Dixon, M. and Webb, E. C. (1979) in *The Enzymes*, pp. 116–166, Academic Press, New York
- 28 Ramos, A., Guerra, M. and Rotllán, P. (1991) *Chromatographia* **32**, 350–356
- 29 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 30 Pintor, J., Torres, M. and Miras-Portugal, M. T. (1991) *Life Sci.* **48**, 2317–2324
- 31 Guranowski, A. (1990) *FEBS Lett.* **262**, 205–208
- 32 Segel, I. H. (1993) *Enzyme Kinetics*, John Wiley & Sons, New York
- 33 Fersht, A. (1984) *Enzyme Structure and Mechanism*, W. H. Freeman and Co., New York
- 34 Bailey, S. J. and Hourani, S. M. O. (1995) *Br. J. Pharmacol.* **114**, 1125–1132
- 35 Pearson, J. D., Coade, S. B. and Cusack, N. J. (1985) *Biochem. J.* **230**, 503–507
- 36 Torres, M., Pintor, J. and Miras-Portugal, M. T. (1990) *Arch. Biochem. Biophys.* **279**, 37–44
- 37 Ziganshin, A. U., Ziganshina, L. E., King, B. F., Pintor, J. and Burnstock, G. (1996) *Biochem. Pharmacol.* **51**, 897–901
- 38 Beukers, M. W., Kerkhof, C. J. M., Van Rhee, A. M., Ardanuy, U., Gurgel, C., Widjaja, H., Nickel, P., Ijzerman, A. P. and Soudijn, W. (1995) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **351**, 523–528
- 39 Leff, P., Wood, B. E. and O'Connor, S. E. (1990) *Br. J. Pharmacol.* **101**, 645–649
- 40 Mateo, J., Rotllán, P. and Miras-Portugal, M. T. (1996) *Br. J. Pharmacol.* **119**, 1–2
- 41 Miras-Portugal, M. T., Pintor, J., Rotllán, P. and Torres, M. (1990) *Ann. N. Y. Acad. Sci.* **603**, 523–526
- 42 Rodríguez-Pascual, F., Torres, M. and Miras-Portugal, M. T. (1992) *Neurosci. Res. Commun.* **11**, 101–107
- 43 Pintor, J., Porras, A., Mora, F. and Miras-Portugal, M. T. (1995) *J. Neurochem.* **64**, 670–676
- 44 Schulze-Lohoff, E., Zanner, S., Ogilvie, A. and Sterzel, R. B. (1995) *Hypertension* **26**, 899–904
- 45 Heidenreich, S., Tepel, M., Schlüter, H., Harrach, B. and Zidek, W. (1995) *J. Clin. Invest.* **95**, 2862–2867