Enzyme Kinetics and Pharmacological Characterization of Nucleotidases Released from the Guinea Pig Isolated Vas Deferens during Nerve Stimulation: Evidence for a Soluble Ecto-Nucleoside Triphosphate Diphosphohydrolase-Like ATPase and a Soluble Ecto-5’-Nucleotidase-Like AMPase

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ABSTRACT

Previously, we have demonstrated that stimulation of the sympathetic nerves of the guinea pig vas deferens evokes release not only of the cotransmitters ATP and norepinephrine but also of soluble nucleotidases that break down extracellular ATP, ADP, and AMP into adenosine. In this study we show that the apparent $K_m$ values of the releasable enzyme activity vary depending on which of these adenine nucleotides is used as initial substrate. The $K_m$ value for ATP was 33.6 $\pm$ 2.3 $\mu$M, 21.0 $\pm$ 2.3 $\mu$M for ADP, and 10.0 $\pm$ 1.1 $\mu$M for AMP. The ratios of the $V_{max}$ values for each enzyme reaction were 4:2:3. We have also found a different sensitivity of the metabolism of ATP and AMP by releasable nucleotidases to known nucleotidase inhibitors. Suramin inhibited the breakdown of ATP by releasable nucleotidases in a noncompetitive manner and with a $K_i$ value of 53 $\mu$M, but had no effect on the breakdown of AMP. The 5’-nucleotidase inhibitor $\alpha,\beta$-methylene ADP inhibited the breakdown of AMP but not that of ATP. Concanavalin A inhibited the breakdown of AMP but had neither inhibitory nor facilitatory effects on the breakdown of ATP. 6-N,N-Diethyl-$\beta$-$\gamma$-dibromomethylene-$\gamma$-ATP (ARL67156), an ecto-ATPase inhibitor, suppressed ATPase and AMPase activities, whereas NaN$_3$ (10 mM) affected neither reaction, but inhibited the ADP metabolism. Phosphatase- and phosphodiesterase inhibitors did not affect the activity of the releasable nucleotidases. This evidence suggests that the soluble nucleotidases released during neurogenic stimulation of the guinea pig vas deferens combine an ecto-5’-nucleotidase-like and an ecto-nucleoside triphosphate diphosphohydrolase-like activity.

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ATP is released as a cotransmitter from cholinergic, adrenergic, and GABAergic neurons (Silinsky et al., 1998; Burnstock, 1999; Jo and Schlichter, 1999). Traditionally, the activation of neurotransmitter ATP in both the central and the peripheral nervous systems has been attributed to its breakdown by cell membrane-bound enzymes, classified as ecto-ATPases, ecto-apyrases, and ecto-5’-nucleotidases (Zimmermann, 1992; Plesner, 1995).

We have demonstrated, however, that neurogenic stimulation of the guinea pig vas deferens dramatically accelerates the degradation of exogenous ATP (Todorov et al., 1996). The difference between the rate of degradation of extracellular ATP by tissue preparations under resting conditions and that during nerve stimulation appears to be associated with a release of enzymes that break down ATP as well as ADP and AMP into adenosine. Inhibition of the propagation of neuronal action potentials with tetrodotoxin, suppression of adrenergic neurotransmission with guanethidine, or inhibition of exocytosis by omission of extracellular Ca$^{2+}$ all prevented the release of nucleotidase activity, implying that sympathetic nerves are the source of the enzyme(s) (Todorov et al., 1997). Interestingly, the nucleotidase activity appears to be coreleased with neurotransmitter ATP and not with the sympathetic cotransmitter NE (Mihaylova-Todorova et al., 2001), suggesting that the proteins carrying...
the enzyme activity originate from a putative “ATP storage vesicle” rather than from a catecholamine storage vesicle.

We investigated the possibility that known ATPases, activated during the process of exocytosis, may be involved as releasable ATPases. The vacuolar H+-transporting ATPase, the Na+/K+-ATPase, the multidrug-resistance channel, and the cytosolic N-ethylmaleimide-sensitive fusion protein were rejected as possible candidates based on the evidence that their specific antagonists did not inhibit the releasable ATPase activity. We have found, however, that suramin and their specific antagonists did not inhibit the releasable AT-rejected as possible candidates based on the evidence that the cytosolic H1-nucleotidases, or phosphatases may be responsible the possibility that ENTPDases, ENPPases, phosphodiester-
ficinity of the releasable neuronal nucleotidases we explored et al., 1990; Lehto and Sharom, 1998).

in soluble form, while retaining its catalytic activity (Misumi phospholipase C, the GPI-anchored ecto-protein could be released 1992). Upon activation of phosphatidylinositol-specific phospho-
enosine- or inosine-specific phosphatase (Zimmermann, 3.1.3.5) or CD73 (Resta et al., 1993) is a 5′/H11032 glycosylphosphatidylinositol (GPI)-anchored and soluble polyphosphates into monophosphates. Although ENPPases have a short membrane-spanning domain, the large extracellular domain containing the catalytic center could be cleaved from the membrane and released in soluble form (for review, see Bollen et al., 2000). However, ENPPases do not metabolize AMP to adenosine. Alkaline- and tissue-nonspecific acid phosphatases are able to hydrolyze ATP, ADP, and AMP and exist in both glycosylphosphatidylinositol (GPI)-anchored and soluble forms (Ohkubo et al., 2000). Ecto-5′-nucleotidase (E.C. 3.1.3.5) or CD73 (Resta et al., 1993) is a 5′-monophospha-
denosine- or inosine-specific phosphatase (Zimmermann, 1992). Upon activation of phosphatidylinositol-specific phos-
pholipase C, the GPI-anchored ecto-protein could be released in soluble form, while retaining its catalytic activity (Misumi et al., 1990; Lehto and Sharom, 1998).

Given the broad, mono-, di-, or triphophonucleotide speci-
licity of the releasable neuronal nucleotidases we explored the possibility that ENTPDases, ENPPases, phosphodiester-
as, 5′-nucleotidases, or phosphatases may be responsible for the releasable nucleotidase activity. Our results suggest that the sympathetic nerves of the guinea pig vas deferens release either a soluble, heretofore unidentified enzyme that combines separate phosphohydrolase and 5′-monophosphate-diesterase catalytic activities or a mixture of separate enzyme entities, including a soluble ENTPDase-like ATPase and a soluble ecto-5′-nucleotidase-like AMPase.

Materials and Methods

Tissue Preparation. Male albino guinea pigs (350–400 g) were killed by decapitation. The vasa deferentia were removed, cleaned of connective tissue, and the lumen exposed by a section along the longitudinal axis. Three tissues, each from a different animal, were loaded in a superfusion chamber (inner volume of 200 μl; Brandel Inc., Gaithersburg, MD). Whatman (Maidstone, UK) 541 filters were cut to fit both ends of the chamber, which was then inserted vertically into a thermostatic block (36°C) with platinum “screen” electrodes at each end. The tissues were superfused from bottom to top (2 ml/min) with modified Krebs-HEPES buffer, pH 7.4, of the following composition: 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2, 5 mM HEPES, and 11 mM glucose. The buffer was constantly bubbled with 100% O2.

Nerve Stimulation and Sample Collection Protocols. The sympathetic nerves of the guinea pig vasa deferentia were stimulated for 30 s by electrical field stimulation (EFS) at 16 Hz, pulse duration of 0.2 ms, and supramaximal voltage. Three sessions of EFS were applied to the tissues at 30-min intervals. Samples of the Krebs-HEPES buffer superfusing the tissue preparations were collected for 30 s before and for 30 s during each of the stimulations in ice-cold test tubes containing the protease inhibitor leupeptin (1 μM). The samples were combined in two pools, one designated as prestimulation or P (collections before stimulation) and the other designated as S (collections during stimulation). If not used the same day, the pooled samples were frozen in liquid nitrogen and then stored at −86°C.

HPLC-Based Assay for Nucleotidase Activity. To study the properties of the releasable neuronal nucleotidases, the fluorescent 1,N6-etheno analogs of adenine nucleotides were used as substrates (Fig. 1). The rationale of the assay is based on the fact that the sequential dephosphorylation of 1,N6-etheno ATP (eATP) results in formation of 1,N6-etheno ADP (eADP) followed by formation of 1,N6-
etheno AMP (eAMP), and finally formation of 1,N6-etheno adenosine (eADO). Each of the substrates and the resulting metabolites were quantified after separation by HPLC coupled with fluorescent detec-

With this method the rate of hydrolysis of the substrate, as well as the type and rate of generation of the products were evaluated in a single chromatogram. eATP was used as initial substrate to study ATPase activity (Fig. 1A), whereas eADP was used to study ADPase activity (Fig. 1B), and eAMP was used to study AMPase activity (Fig. 1C)

HPLC-Based Assays for Adenine Nucleotides and ADO. ATP, ADP, AMP, and ADO were analyzed as described previously (Todorov et al., 1996). The etheno-adenine purines were separated on a gradient HPLC system equipped with a Resolve radial pack cartridge (8NVPh 4 μm; 8 × 10 mm) (Waters, Milford, MA). The amount of each adenine purine was quantified using an RF 535 fluorescent monitor (Shimadzu, Columbia, MD) at an excitation wavelength of 230 nm and an emission wavelength of 420 nm. Buffer solutions consisted of 0.1 M phosphate (KH2PO4, pH 6.0) (buffer A) and 75% 0.1 M phosphate and 25% methanol (buffer B). The adenine nucleotides and ADO were separated using a gradient in which the concentration of buffer B was increased from 0 to 100% in 8 min according to Waters gradient profile 7. The HPLC equipment was controlled by, and data collected by, a Pentium II computer equipped with an LAC/E card and Millenium 2010 Chromatography Manager software (Waters). Identification of individual peaks in chromato-
grams was by comparison with the retention times of known etheno-adenine purine standards, and the concentration was determined by peak area per picomole relationship compared with standards. Standards were run with each set of samples.

Preparation of 1,N\textsuperscript{6}-Etheno Derivatives of ATP, ADP, AMP, and ADO. Stock solutions of eATP, eADP, and eAMP were prepared by incubation of ATP, ADP, and AMP (1.5 \times 10^{−2} \text{ mol/l}) in citric phosphate buffer, pH 4, in the presence of 2-Cl-acetaldehyde for 40 min at 80°C. Furthermore, serial dilutions (3 \mu M−10 mM) of the stock solutions were prepared using deionized water (18 MΩ) and stored at −20°C. The etheno-derivatives of adenine nucleotides are stable at −20°C for years and at room temperature for several days.

Hydrolysis of ATP, ADP, and AMP and Their Etheno-Derivatives by Commercially Available Enzymes. To test whether the modification of the molecules of ATP, ADP, or AMP by the etheno-group addition affects the enzymatic degradation of adenine nucleotides we have compared the rate of breakdown of native ATP and ADP with the rate of breakdown of their etheno-derivatives (eATP and eADP) by apyrase VI and VII purchased from Sigma-Aldrich (St. Louis, MO). The hydrolysis of AMP and that of eAMP by 5′-nucleotidase from Crotalus atrox venom (Sigma-Aldrich) was also evaluated. The etheno-analogs were metabolized by the commercially obtained enzymes with rates closely comparable with the rates for the nonmodified substrates (data not shown).

Time Course of Product Formation. eATP, eADP, or eAMP (1, 10, and 100 \mu M) was incubated with superfusate S or P for 0, 0.5, 1, 2, and 4 h at 37°C. At the end of the incubation periods the reactions were stopped with acidification with ice-cold citrate phosphate buffer

Fig. 1. Chromatographic image of etheno-purines before (Aa, Ba, and Ca) and after (Ab, Bb, andCb) exposure to releasable nucleotidases. Structural formulae and representative chromatograms of eATP (Aa), eADP (Ba), and eAMP (Ca) in Krebs-HEPES buffer. The structural formula of eADO is shown as an inset in Cb. The etheno-modification, consisting of insertion of acyl group between nitrogen 1 and 6 of the adenine moiety, renders the molecule fluorescent but leaves unaltered the phosphate chain. Note that an invariable with time nonenzymatic dephosphorylation product (less than 5% for eATP, less than 8% for eADP, and less than 2% for eAMP) is always detected in the solutions. eATP incubated for 60 min at 37°C with superfusate S collected during nerve stimulation of the guinea pig isolated vas deferens was metabolized to eADP, eAMP, and eADO (Ab). Likewise, in presence of superfusate S, eADP was metabolized to eAMP and eADO (Bb), whereas eAMP was broken down to eADO (Cb). Metabolism of etheno-adenine nucleosides did not occur in presence of superfusate P, collected before the onset of the nerve stimulation of the guinea pig isolated vas deferens (data not shown).
buffer, pH 4, and the samples analyzed for ATP, ADP, AMP, and ADO by HPLC.

Increase in Enzyme Activity with Protein Concentration. Samples of superfusate diluted 2-, 4-, or 8-fold with Krebs-HEPES buffer were tested for ATPase and AMPase activity. Concentration of enzyme(s) was achieved by reduction of the volume of the superfusate by filtration through Centricon centrifugal filters (Millipore Corporation, Bedford, MA) with membrane pore size cutoff of 30 kDa. Typically, a 2-ml sample was reduced to 40 μl. Enzyme activity was tested using 5, 10, and 20 μl of the concentrated sample, corresponding to theoretical 12.5-, 25-, and 50-fold concentration of proteins with molecular size higher than 30 kDa.

Kinetic Constants of the Releasable Nucleotidase Activity. Under the standard protocol used to study the enzyme activity of releasable nucleotidase, 5 μl of stock solution of eATP, eADP, or eAMP at a given concentration (3 μM–10 mM) was added to a mixture of 20 μl of superfusate collected during neurogenic stimulation of the guinea pig vas deferens (source of enzyme activity) and 25 μl of Krebs-HEPES buffer, pH 7.4. A similar sample was run using superfusate P. After incubation for 60 min at 37°C, the reaction was stopped by addition of 100 μl of ice-cold citrate phosphate buffer, pH 4. Aliquots (100 μl) were injected into the HPLC system for evaluation of the adenine nucleotides and adenosine present in the sample. The enzyme activity was estimated from the difference between the sum of products generated in the presence of superfusate collected during nerve stimulation (S) and the sum of products generated in the presence of superfusate collected during resting conditions (P). This difference (net product) was expressed in picomoles per minute per microliter of superfusate (pmol/min/μl). Normalization by microliters of superfusate was used to replace the conventionally used normalization by protein weight. Kinetic constants (K_m and V_max) were derived from Michaelis-Menten plots using the nonlinear regression analysis of GraphPad Prism software, version 3 (GraphPad Software, San Diego, CA).

Calcium and Magnesium Dependence of Releasable Nucleotidases. Nucleotidases were released in Krebs-HEPES buffer as described above. The collected samples were desalted by centrifugation through a Centricon filter, with a membrane pore cutoff of 30 kDa. The collected samples were desalted by centrifugation through a Centricon filter, with a membrane pore cutoff of 30 kDa. The collected samples were desalted by centrifugation through a Centricon filter, with a membrane pore cutoff of 30 kDa. The collected samples were desalted by centrifugation through a Centricon filter, with a membrane pore cutoff of 30 kDa. The collected samples were desalted by centrifugation through a Centricon filter, with a membrane pore cutoff of 30 kDa.

Results

Substrate Specificity of Releasable Nucleotidases. eATP, eADP, and eAMP were all metabolized in the presence of superfusate from nerve-stimulated guinea pig vas deferens tissue preparations (S) (Fig. 1b) but remained unaffected by superfusate collected under resting conditions (P) (data not shown). The hydrolysis was time-dependent, sequential, and unidirectional from ATP to ADO. eADP was the first product formed from eATP, whereas eAMP and eADO appeared later in the time course of the reaction. When eADP was used as substrate, eAMP appeared first, and eADO was formed later (Fig. 1b). E-ADO was the first and only product during the time course of the metabolism of AMP (Fig. 1c). If sufficient time was allowed, eADO was the end product of the reaction regardless of whether eATP, eADP, or eAMP was used as initial substrate.

Increase in Enzyme Activity after Sample Concentration. The volume of samples of superfusate was reduced by filtration through Centricon filters with a membrane pore size cutoff of 30 kDa. The ATPase, ADPase, and AMPase activities were retained above the filter. There was no enzyme activity in the filtrate. In addition, we have observed an increase in specific activity (determined as activity per microliter of superfusate) consistent with concentration of enzyme(s) with size larger than 30 kDa. Dilution of the superfusate, on the other hand, led to a proportional decrease in specific enzyme activity.

Kinetic Constants of ATPase, ADPase, and AMPase Activity. The Michaelis-Menten plots of substrate concentration versus product velocity for all three activities and the Lineweaver-Burk reciprocal plots are shown in Fig. 2. The product velocity increased with the increase of the substrate concentrations (0.3–300 μM) according to a rectangular hyperbola. The K_m value for the ATPase activity, calculated from 28 separate experiments, was 33.6 ± 3.2 μM and the V_max value was 0.204 ± 0.003 pmol/min/μl of superfusate (inserted table in Fig. 2). The ADPase activity had a K_m value of 21.0 ± 2.3 μM and V_max value of 0.111 ± 0.002 pmol/min/μl of superfusate (n = 4). The K_m value of the AMPase activity was 10.0 ± 1.1 μM, and the maximal velocity was 0.168 ± 0.003 pmol/min/μl of superfusate (n = 12). These data suggest that the AMPase operates at a slightly slower rate than the ATPase. The ADPase was the slowest of the three, having only one-half and two-thirds of the maximal velocities of the ATPase and the AMPase, respectively. The V_max/K_m ratio revealed that the AMPase had a three-fold higher efficiency than the ATPase or the ADPase. The ratio of the maximal velocities of the ATPase/ADPase was 2.1. ATP is therefore preferred 2-fold over ADP, which is consistent with the possibility that a single ENTPDase-like enzyme is responsible for the metabolism of both ATP and ADP (Plesner, 1995).

Calcium and Magnesium Dependence of Nucleotidase Activity. Previously, we have demonstrated that omission of Ca^{2+} from the superfusing solution or addition of cadmium (Cd^{2+}) abolishes both the nerve stimulation-evoked release of neurotransmitters and the release of nucleotidase.
activity from the guinea pig vas deferens. These data have suggested that neuronal nucleotidases are released by a calcium-dependent mechanism (Todorov et al., 1997). To support the release of enzymes we had to maintain calcium in the superfusing solution. Magnesium, however, was not required for neuronal release of ATP, norepinephrine, or soluble enzymes. We therefore carried out release experiments using buffer containing calcium (1.5 mM) but not magnesium ions. The enzyme activity of these samples increased following a rectangular hyperbola when Mg^{2+} was increased from 0.6 to 4.8 mM. Maximal increase of the ADPase activity (12%), ATPase activity (8%), and AMPase activity (4%) was achieved when the Mg^{2+} concentration was increased to 2.4 mM. Further increase of Mg^{2+} concentration to 9.6 mM led to a decline in enzyme activity. Half-maximal activation of the ADPase, ATPase, and AMPase was achieved with 0.7, 1.3, and 0.85 mM Mg^{2+}, respectively.

Treatment of samples of superfusate S with EGTA (2 mM, pH 7.4) abolished the ATPase, ADPase, and AMPase activities. Samples of superfusate S desalted by reduction of the fluid volume and restored back to the initial volume with Ca^{2+} and Mg^{2+}-free buffer showed a 75% decrease of their initial activities. When supplemented with Ca^{2+} or Mg^{2+} the enzyme activities of the samples of superfusate increased according to a rectangular hyperbola. Half-maximal activation of the ATPase was achieved with 2.6 ± 0.2 mM Ca^{2+} and with 2.4 ± 0.1 mM Mg^{2+}. Half-maximal activation of the ADPase was achieved with 2.5 ± 0.3 mM Ca^{2+} and with 2.3 ± 0.2 mM Mg^{2+}. These data demonstrate that each of these cations is sufficient to fully activate the ATPase or AMPase activities of the releasable nucleotidase.

**Pharmacological Characteristics of Releasable Neuronal Nucleotidases.** The effects of ecto-ATPase inhibitors on the ATPase and AMPase activities are shown in Fig. 3. Suramin inhibited the releasable ATPase activity in a dose-dependent and noncompetitive manner (Fig. 3A) with a $K_i$ value of 53 μM, derived from Dixon plot. On the other hand, suramin had no effect on the AMPase activity (Fig. 3B).

ARL67156, previously known as FPL67156, is an ATP analog devoid of purinergic receptor activity that has been promoted as a selective ecto-ATPases inhibitor with a $K_i$ value of 5.2 (Crack et al., 1995). In addition, it has been shown that ARL67156 increases the ATP-induced contractile responses of smooth muscle tissue preparations, presumably by protecting extracellular ATP from degradation by ecto-ATPases (Westfall et al., 1996). We tested the ability of ARL67156 to inhibit the breakdown of eATP (Fig. 3C) and eAMP (Fig. 3D) by releasable nucleotidases. ARL67156 inhibited ATPase activity in a noncompetitive manner with a concentration-dependent linear decrease of $V_{max(app)}$ and a linear increase of $K_{m(app)}$. The $K_i$, calculated from replots of the slope [$K_{m(app)}/V_{max(app)}$], derived from reciprocal transforms versus inhibitor concentrations (Plowman, 1972), was 55.8 μM (Fig. 3E). ARL67156 also inhibited the rate of the AMPase activity (Fig. 3D). The mode of this inhibition was complex and seemed consistent with the possibility that ARL67156 binds to more than one enzyme or more than one binding site. Analysis of the changes in $V_{max(app)}$ with increase in inhibitor concentrations revealed that the maximal decrease in $V_{max}$ (about 30%) occurred when ARL67156 concentration was increased from 0.1 to 3 μM. Further increase (10–100 μM) had less effect on $V_{max}$. There was also an increase in $K_{m(app)}$ parallel to the increase in concentration of this inhibitor. Within the range from 0.1 to 3 μM the $K_i$ value almost doubled. With further increase of inhibitor (10–100 μM), $K_{m(app)}$ continued to increase, whereas $V_{max}$ remained constant. It seems possible, therefore, that ARL67156 binds to a “high-affinity” binding site, leading to a noncompetitive inhibition of the AMPase activity. This binding site appears to saturate at low micromolar inhibitor concentrations. At higher concentrations ARL67156 acts as a competitive inhibitor, because $V_{max}$ value does not change.
whereas $K_m$ value increases. Slopes of the reciprocal curves within the above-defined inhibitor ranges were linear. The abscissa intercept of the replots was used to determine the $K_i$ value for the high-affinity binding site $[K_{i(1)} = 1.7 \mu M]$ and for the “low-affinity” binding site $[K_{i(2)} = 75 \mu M]$ (Fig. 3F). The effects of NaN₃ (5 and 10 mM) on the kinetic constants

![Graphs and diagrams showing inhibitory profiles of suramin (A and B) and ARL67156 (C and D) on releasable ATPase (A, C, and E) and AMPase (B, D, and F) activities.](image-url)
of the releasable ATPase and the releasable AMPase are presented in Fig. 4, A and B, respectively. Sodium azide had no effect on the rate of hydrolysis of ATP and AMP by the releasable enzymes. However, when eADP (100 μM) was used as substrate, a 30% inhibition of the ADP hydrolysis in presence of 10 mM NaN₃ was observed (data not shown).

We tested the effect of α,β-mADP, an ecto-5'-nucleotidase antagonist (Knofel and Strater, 2001), on the releasable nucleotidase activities. α,β-mADP inhibited the releasable AMPase activity in a competitive manner (Fig. 4D) with a hyperbolic increase in $K_{m(app)}$ value and no change in $V_{max}$ value as inhibitor increased. The concentration of inhibitor that doubled the $K_m$ value of the AMPase reaction was estimated to be 0.0125 μM. However, α,β-mADP failed to produce any inhibitory effect on the ATPase activity (Fig. 4C).

Levamisole (10 mM) (Fig. 4, E and F) and phosphatase inhibitor cocktail II (data not shown), inhibitors of phosphatases, and IBMX (10, 100, and 1000 μM), a phosphodiesterase inhibitor, did not affect either releasable ATPase or AMPase activity (data not shown).

We tested the effect of para-nitrophenyl tymidine 5'-monophosphate (100 μM), a specific substrate for ecto-nucleotide pyrophosphatases/phosphodiesterases, on the releasable ATPase activity using eATP (100 μM) as substrate. para-Nitrophenyl tymidine 5'-monophosphate did not affect the metabolism of eATP, suggesting that it does not compete for the

**Fig. 4.** Inhibitory profile of sodium azide, α,β-mADP, and levamisole on releasable ATPase (A, C, and E) and AMPase (B, D, and F) activity. Superfusate was collected and enzyme reactions were carried out as described in Fig. 3. Sodium azide (5 and 10 mM) does not affect the releasable ATPase (A) or AMPase (B) activities. α,β-mADP (0.1, 1, and 10 μM), a specific inhibitor of ecto-5'-nucleotidase inhibited competitively releasable AMPase activity (D), but had no effect on releasable ATPase activity (C). Levamisole (10 mM), a noncompetitive inhibitor of alkaline phosphatase, had no effect on releasable ATPase (E) or releasable AMPase (F).
ATP binding site of the releasable enzyme. It seems, therefore, that ENPPases do not contribute for the releasable ATPase activity.

It has been shown that the mannose-binding lectin Con A inhibits the activity of 5'-nucleotidases by a noncompetitive mechanism (Zimmermann, 1992). In our experiments Con A, at 0.1 μM, completely abolished the releasable AMPase activity. However, Con A (0.1, 0.5, and 1 μM) had neither inhibitory nor stimulatory effects on the releasable neuronal ATPase activity.

**Discussion**

The results reported herein confirm our previous findings that the breakdown of extracellular ATP and consequently the level of extracellular ADO are regulated by soluble nucleotidases, released upon nerve stimulation of the sympathetic nerves of the guinea pig vas deferens (Todorov et al., 1996, 1997; Mihaylova-Todorova et al., 2001). Concentration of superfusate collected during nerve stimulation leads to an increase in nucleotidase activity and suggests that soluble proteins with a size greater than 30 kDa are involved.

In an attempt to more completely understand the nature of the releasable nucleotidases, we have examined the effects of several pharmacological agents that are known to inhibit ecto-phosphatases and ecto-phosphodiesterases. These families of adenine nucleotide-metabolizing enzymes exhibit broad substrate specificity. There is also evidence that ecto-phosphatases may be released from the cell membrane upon activation of endogenous phospholipases and cleavage of the anchoring GPI (Hooper, 1997). We have found, however, that the activity of releasable nucleotidases is not affected by either levamisole, a specific alkaline phosphatase antagonist, or by the phosphatase inhibitor cocktail II (Sigma-Aldrich), which is designed to block the actions of a wide range of phosphatases. These results suggest that alkaline-, acid-, neutral-, or protein-tyrosine phosphatases are not involved in the nerve stimulation-triggered metabolism of adenine nucleotides in the guinea pig vas deferens. Likewise, IBMX, a nonselective phosphodiesterase antagonist, failed to inhibit the ATPase and AMPase activities, thereby excluding the possibility that releasable nucleotidases may share catalytic properties with ecto-phosphodiesterases. Additionally, paranitrophenyl thimidine monophosphate, a preferred substrate of ENPPases, had no influence on the eATP metabolism by releasable nucleotidases, suggesting that ENPPases do not contribute to the soluble ATPase activity.

Several lines of evidence presented in this study support our previous hypothesis that releasable nucleotidases may share catalytic properties with ecto-ATPases (Kennedy et al., 1997; Todorov et al., 1997; Westfall et al., 2000b). Our results show that like most ecto-ATPases the releasable nucleotidases depend on either Ca²⁺ or Mg²⁺ for activity. Removal of divalent cations by chelation or buffer exchange abolishes the ATPase, ADPase, and AMPase activities. Addition of Ca²⁺ or Mg²⁺, on the other hand, restored these activities. The effects of Ca²⁺ and Mg²⁺ were additive, suggesting that both cations use the same mechanism of activation. Mg²⁺ activated ADPase more than it activated ATPase or AMPase, suggesting that small differences in the metal ion coordination for ATP and ADP may exist. Recently, Chen and Guidotti (2001b) demonstrated that the metal-ATP enzyme complex of the ENDPase1 exists in a single form, whereas the metal-ADP-enzyme complex has two states, one corresponding to the intermediate complex formed during ATP hydrolysis and the second corresponding to the ADP binding as substrate for further hydrolysis.

Knowles and Nagy (1999) have shown a nucleotide-substrate-dependent inhibition effect of sodium azide on the chicken oviduct ecto-ATPase. The effect of azide was prominent on the ADP hydrolysis, whereas the ATP hydrolysis was less influenced. Herein, we show that the soluble ATPase activity was not affected by sodium azide, but the rate of the ADPase activity was decreased. This is consistent with the hypothesis that the enzymes released upon nerve stimulation of the guinea pig vas deferens include an ATPDase.

The $K_m$ value of the releasable ATPase (33 μM) determined in this work seems similar to the $K_m$ values of ecto-ATPases purified from synaptosomes of the rat and mouse cortex ($K_m = 39–53$ μM; Nagy et al., 1986), but was lower than that of ATPase from rat striatal cholinergic synapse ($K_m = 131$ μM; James and Richardson, 1993). The expressed recombinant ENTDPases, however, show $K_m$ values for ATP ranging from 75 to 400 μM that are in general higher than the ones found in tissues. ENTDase1 and 3 use ADP as well as ATP as substrates, whereas ENTDase2 is mostly an ATPase because it prefers ATP 10 times more than it prefers ADP. ENTDase1 metabolizes ATP directly to AMP, and ADP does not appear as free product in the reaction. The guinea pig vas deferens neuronal nucleotidases generate ADP as a free product resulting from the ATP hydrolysis. ADP is detected in the reaction before it is hydrolyzed further to AMP. The velocity of ATP hydrolysis is 2-fold higher than that of ADP. The soluble ATPase released from the sympathetic nerves of the guinea pig vas deferens is therefore a triphosphatase-diphosphohydrolase-like enzyme. Because ADP accumulates as a product of the metabolism of ATP, it seems that the releasable ATPase is more similar to ENTDase3, which prefers ATP 3 to 4 times more than ADP (Smith and Kirley, 1998) than it is to ENTDase1, which does not discriminate between ATP and ADP (Wang and Guidotti, 1996). On the other hand, recent work of Chen and Guidotti (2001a) demonstrated that the velocity of the ATP hydrolysis and the preference for ADP binding are functions of the oligomerization state of the ENTDase monomers. Disruption of the tetrameric organization of the membrane-bound rat ENTDase1 by either detergent solubilization or truncation of the N- and C-terminal segments of the recombinant protein decreased its preference for ADP 3.5 times and ADP appeared in solution during the metabolism of ATP. The $K_m$ value for ATP was also decreased into the low micromolar range. It seems, therefore, that the kinetics of the releasable ATPase from the guinea pig vas deferens reported in the current work approximates the kinetics of the C-terminal- and N-terminal-truncated rat ENTDases. This opens the possibility that the releasable ATPases could in fact represent a proteolytic cleavage form of the membrane-bound ENTDases.

The activity of ecto-ATPase in neuronal (Marti et al., 1996) and non-neuronal tissues (Bultmann et al., 1996) is inhibited by suramin. Suramin has also been shown to inhibit the ATPase activity of the Chinese hamster ovary cells transfected with the rat brain ecto-ATPase gene, but not that of Chinese hamster ovary cells transfected with the ecto-apyrase gene (Heine et al., 1999). Herein, we show that the
neuronal ATPase activity released from the guinea pig vas deferens was inhibited by suramin in a noncompetitive manner and with a $K_i$ value of 53 μM. At the same time, the breakdown of AMP remained unaffected.

Our results demonstrate that unlike suramin, which inhibits only the ATPase, α,β-mADP and Con A inhibit only the metabolism of AMP and have no effect on the metabolism of ATP by releasable nucleotidases. These data suggest that the binding and hydrolysis of triphosphonucleotides is independent of the binding and catalytic hydrolysis of 5'-monophosphonucleotides. One possible explanation for these findings is that the sympathetic nerves of the guinea pig vas deferens release a separate enzyme that is pharmacologically similar to the members of the family of ecto-5'-nucleotidases. The apparent $K_m$ value of the releasable AMPase activity reported herein (10 μM) is comparable with the $K_m$ value of ecto-5'-nucleotidase isolated from electric organ of Torpedo (25 μM) and bovine cerebral cortex (46 μM) (Vogel et al., 1992). Ecto-5'-nucleotidase, a GPI-anchored protein, may become soluble upon activation of phospholipases. Previously, we have shown that the AMPase and the ATPase activities are coreleased with neurotransmitter ATP (Mihaylova-Todorova et al., 2001). It is not known at this time whether the events leading to exocytosis and release of neurotransmitters may concomitantly release or cleave ecto-enzymes from cell membranes.

ARL67156 inhibits the activity of ecto-ATPases expressed by blood (Crack et al., 1995) and smooth muscle cells (Westfall et al., 1996) as well as the ATPase activity of the releasable nucleotidases from guinea pig (Kennedy et al., 1997; Todorov et al., 1997; Westfall et al., 2000b) and rabbit vas deferens (Westfall et al., 2000a). Herein, we confirm our previous results that ARL67156 inhibits the ATPase activity and determined that the mode of inhibition is noncompetitive with a $K_i$ value of 55.8 μM. In addition, we show that ARL67156 inhibits AMPase activity in a complex manner. Binding of ARL67156 to a high-affinity binding site ($K_i = 7.4$ μM) contributes to a noncompetitive inhibition of AMPase activity. At a second, low-affinity binding site ($K_i = 75$ μM), ARL67156 seems to inhibit AMPase activity in a competitive manner. It has been previously reported that ARL67156 inhibits purified human placental 5'-nucleotidase (Chayet et al., 1997) but the mode of inhibition was not tested. It will be interesting to determine whether inhibition of ecto-5'-nucleotidase activity is a common property of ARL67156 or whether this mode of inhibition is specific for the releasable AMPase. Based on the evidence provided by this study we favor the notion that at least two enzymes, an ATPDase and an AMPase that work cooperatively to break down extracellular ATP to ADO, are coreleased from the sympathetic nerves of the guinea pig vas deferens. Pharmacological analysis suggests that the ATPDase behaves as an ENTPDase (possibly C- and N-terminal truncated), whereas the AMPase closely resembles ecto-5'-nucleotidase. However, an alternative hypothesis that a single protein carries both ATPDase and AMPase activity cannot be rejected at this time. Enzymes showing functional characteristics of apyrases and amino acid sequence similarity with 5'-nucleotidases have been identified. For example, a soluble apyrase secreted from the salivary gland of adult female mosquito Aedes aegypti seems to share sequence similarity with vertebrate and bacterial 5'-nucleotidases (Champagne et al., 1995). It has to be noted, however, that this enzyme does not metabolize AMP. If the releasable nucleotidase described herein is a single enzyme then it should have at least two separate active centers that may be independently modulated by ecto-ATPase- and ecto-5'-nucleotidase inhibitors.

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References


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