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Characterization of the ATPase released during sympathetic nerve stimulation of the guinea-pig isolated vas deferens

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1 The release of ATPase activity evoked by electrical field stimulation (EFS) (8 Hz, 25 s) was investigated in several tissues in which adenosine 5′-triphosphate (ATP) acts as a neurotransmitter.

2 Superfusate collected during EFS of sympathetic nerves of the guinea-pig, rat and mouse isolated vas deferens and parasympathetic nerves of the guinea-pig isolated urinary bladder contained ATPase activity. ATP breakdown was fastest in superfusate collected from the guinea-pig isolated vas deferens. However, EFS of the enteric nerves of the guinea-pig isolated taenia coli did not release any detectable ATPase.

3 The ATPase released from the guinea-pig isolated vas deferens metabolized ATP at similar rates at incubation temperatures of 37°C and 20°C. Lineweaver–Burke analysis of the initial rates of ATP hydrolysis gave a K_M of 39 μM and a V_{max} of 1039 pmol ATP metabolized min^{-1} ml^{-1} superfusate.

4 6-N,N-diethyl-D-β,γ-dibromomethyleneATP (ARL 67156), pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and pyridoxal-5′-phosphate (P-5-P) all inhibited the ATPase activity in a concentration-dependent manner with a potency order of ARL 67156 = PPADS > P-5-P.

5 In conclusion, EFS of several tissues in which ATP is a neurotransmitter causes the release of an ATPase and activity is greatest in the guinea-pig vas deferens. The enzyme has pharmacological and kinetic characteristics that are similar to ectonucleoside triphosphate diphosphohydrolases.
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Abbreviations: ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ARL 67156, 6-N,N-diethyl-D-β,γ-dibromomethyleneATP; ATP, adenosine 5′-triphosphate; EFS, electrical field stimulation; eNTDPase, ectonucleoside triphosphate diphosphohydrolase; P-5-P, pyridoxal-5′-phosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid

Introduction

Electrical field stimulation (EFS) of the sympathetic nerves of the guinea-pig vas deferens results in the corelease of the neurotransmitters adenosine 5′-triphosphate (ATP) and noradrenaline, which act on postjunctional $P2X_1$ receptors and α_1-adrenoceptors respectively to evoke a biphasic contraction of the smooth muscle. The contraction consists of a rapid, transient component which is predominantly purinergic and a slower, more maintained component which is largely noradrenergic (see Sneddon *et al*., 1996 for review).

As for all neurotransmitters, ATP and noradrenaline once released into the extracellular space, must be inactivated. Noradrenaline, like the amino acid and other amine neurotransmitters, is taken back up into the nerve by a specific transporter (Amara & Kuhar, 1993) and degraded by monoamine oxidase or repackaged into synaptic vesicles. In contrast, ATP, like acetylcholine, is degraded in the extracellular space. Whereas acetylcholine is broken down in one step (acetylcholinesterase forms a complex with acetylcholine and reacts to release choline, which is taken up by the nerve), ATP is degraded by a three-step process to adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP) and adenosine, which can then be taken up by surrounding tissues.

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The initial dephosphorylation of ATP to ADP is the most important step in this metabolic cascade in the guinea-pig vas deferens. ADP is much less potent than ATP at $P2X_1$ receptors (Evans *et al*., 1995) and so makes little or no contribution to purinergic neurotransmission. Extracellular breakdown of ATP (as well as of ADP and AMP) can be mediated by membrane-bound ectonucleotidases, which have been characterized in many tissues (Burnstock, 1972; Plesner, 1995). These enyzmes are insensitive to inhibitors of intracellular ATP-dependent ion pumps, such as Na^+/K^+ ATPase, Ca^{2+}-transport ATPase, vesicular H^+ ATPase and mitochondrial ATPase, but are inhibited by 6-N,N-diethyl-D-β,γ-dibromomethyleneATP (ARL 67156), pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and pyridoxal-5′-phosphate (P-5-P) (Crack *et al*., 1995; Khakh *et al*., 1995; Ziganshin *et al*., 1995; 1996; Bültmann *et al*., 1996; 1999; Chen *et al*., 1996). PPADS (Lambrecht *et al*., 1992; McLaren *et al*., 1994) and P-5-P (Trezise *et al*., 1994; Westfall *et al*., 1997b) were introduced as antagonists at P2X receptors, but were subsequently shown to inhibit ATP breakdown at higher concentrations.

Several ectonucleotidases have been cloned and renamed as ectonucleoside triphosphate diphosphohydrolases (eNTDPases) (see Zimmermann, 1999; Zimmermann & Braun, 1999 for reviews). They have a putative structure of two transmembrane domains, short intracellular N- and C-terminals and a large extracellular loop. Their sequences are

unrelated to those of intracellular ATPases and they are unaffected by inhibitors of these enzymes.

Todorov and colleagues recently reported another mechanism by which extracellular ATP can be broken down, in that sympathetic nerve stimulation in the guinea-pig vas deferens leads to the release of nucleotidases that sequentially degrade ATP to adenosine (Todorov *et al*., 1997; Kennedy *et al*., 1997). Nucleotidase release is Ca^{2+}-dependent and inhibited by tetrodotoxin. The enzyme activity is stable for at least 3 h at room temperature and is inactivated by acidic pH or heating to 80°C. Like eNTPDases, the releasable ATPase is unaffected by inhibitors of intracellular ATPases, but is sensitive to ARL 67156. Thus, the releasable enzyme may be related to the eNTPDases and may play a role in the termination of the neurotransmitter actions of ATP.

Other than these basic properties, little is known about the ATPase released in the guinea-pig vas deferens. It is not known if release is restricted to this tissue or if it is more widespread. Therefore, the aims of this study were (a) to investigate if similar activity is released from the vas deferens of the rat and mouse and from the guinea-pig urinary bladder and taenia coli, tissues in which ATP is also a neurotransmitter and (b) to characterize further the biochemical properties of the releasable ATPase by determining characteristics such as its kinetic parameters and sensitivity to inhibitors of eNTDPases.

Methods

Tissue preparation

Male albino guinea-pigs (400–500 g), rats (250–300 g) and mice (25–30 g) were killed by asphyxiation with CO_2 and subsequent cervical dislocation. The vas deferens of all three species and the urinary bladder and taenia coli of the guinea-pig were removed and cleaned of connective tissue. Each vas deferens was bisected and only the prostatic half (approximately 1 cm) used. In addition, they were cut open along the longitudinal axis, exposing the lumen.

Approximately 150 mg (wet weight) of tissue was loaded into a Brandel perfusion chamber (total volume, 200 μl). This was equivalent to three vas deferens from the rat or guinea-pig, eight vas deferens from the mouse, one urinary bladder (cut into 3–4 strips) and one taenia coli. Whatman 541 filters were cut to fit both ends of the chamber, which was then inserted vertically into a thermostatic block with two platinum screen electrodes at either end. The tissues were perfused from bottom to top at 2 ml min^{-1} with Krebs solution of the following composition (mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11; heated to 37°C and bubbled with 95% O_2 5% CO_2

Collection and measurement of ATPase activity

EFS was applied to the tissues *via* the screen electrodes at 8 Hz, 0.1 ms pulse width, supramaximal voltage for 25 s with a Grass S88 stimulator, connected to a Grass SIU5F stimulus isolation unit. The superfusate was collected throughout the stimulation period (approximately 800 μl) and divided into 80 μl aliquots. Ten μl of a stock ATP solution was added to each aliquot, along with 10 μl of H_2O, ARL 67156, PPADS or P-5-P, giving a total incubation volume of 100 μl. Unless stated otherwise, all assays were performed at room temperature (20°C).

To determine the ATP content of the assay samples at the end of the incubation, the total sample volume (100 μl) was then added to 100 μl of luciferin-luciferase assay mix and the light emitted recorded on a Chrono-log Lumivette luminometer for 20 s. A standard curve using known amounts of ATP was constructed before each experiment and from this the amount of ATP in the test samples was calculated.

In each experiment at least two identical samples were prepared. The ATP content of one was assayed immediately (time = zero) and the others were assayed at various intervals of up to 100 min. The amount of ATP metabolized at each time point was calculated by subtracting the amount present in that sample from the value at time = zero. As this value represents the amount of ATP metabolized by 80 μl of superfusate, it was multiplied by 12.5 to give the amount of ATP metabolized per ml of superfusate. The effect of ARL 67156, PPADS and P-5-P on ATPase activity was investigated by incubating superfusate samples with ATP (10 μM) for 30 min in the absence or presence of a range of concentrations of the putative inhibitors.

Drugs

ATP (disodium salt), P-5-P (both Sigma), ARL 67156 (provided by Astra Charnwood) and PPADS (Tocris Cookson) were dissolved in distilled water and stored as 10 mM stocks. The luciferin-luciferase assay (Sigma) contained firefly luciferase, luciferin, $MgSO_4$, DTT, EDTA, bovine serum albumin and tricine buffer salts.

Statistics

Values in the text refer to mean ± s.e.mean. Statistical significance of the results was tested either by Student's paired *t*-test or one way analysis of variance and Tukey's comparison as appropriate. Differences were considered significant when $P < 0.05$.

Results

Release of ATPase activity

Todorov *et al*. (1997) have previously shown that EFS of the sympathetic nerves innervating the guinea-pig vas deferens releases ATPase activity into the superfusate. Here we initially determined if similar activity could be released from the vas deferens of other species and from other guinea-pig tissues in which ATP is a neurotransmitter. Figure 1 shows that superfusate collected during EFS of the sympathetic nerves of the rat and mouse vas deferens and of the parasympathetic nerves of the guinea-pig urinary bladder, also contained ATPase activity, but the rate of breakdown of ATP was slower than when the guinea-pig vas deferens was stimulated. No enzyme activity was seen in samples collected in the absence of EFS (not shown). In contrast to the other tissues, EFS of the enteric nerves innervating the guinea-pig taenia coli did not release any detectable ATPase into the superfusate. Subsequent experiments were all performed using the guinea-pig vas deferens.

Temperature-dependence and kinetic properties

The influence of the incubation temperature on the ATPase activity was then investigated. A slight increase in the rate of ATP breakdown was seen when samples were incubated at 37°C compared with 20°C, but this difference was not statistically significant (Figure 2). Consequently all assays were performed at 20°C.

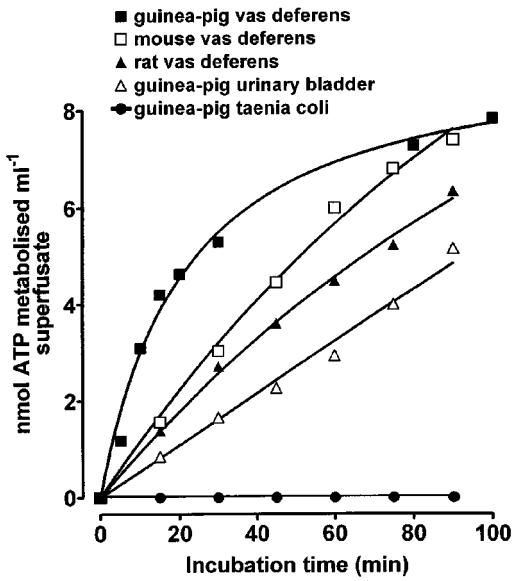

Figure 1 Tissue- and species-dependence of the release of ATPase activity. EFS (8 Hz, 25 s) was applied to the superfused guinea-pig isolated vas deferens, urinary bladder and taenia coli and the superfused rat and mouse isolated vas deferens. The ability of samples of superfusate collected during stimulation to degrade exogenous ATP (10 μM) was then determined. For clarity, each record is from a single experiment and is typical of 3–8 individual experiments.

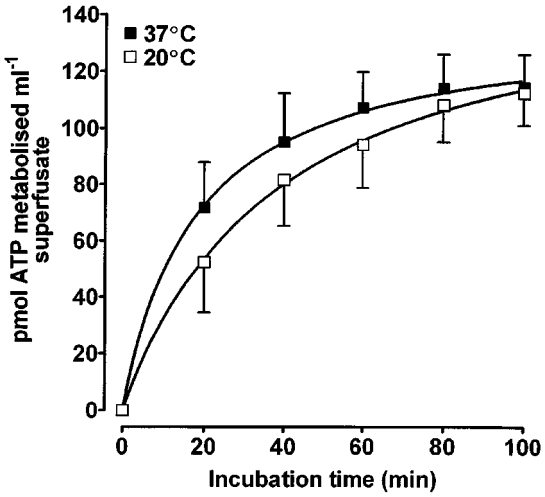

Figure 2 The effect of temperature on the degradation of ATP. EFS (8 Hz, 25 s) was applied to the superfused guinea-pig vas deferens and the superfusate collected. The ability of superfusate samples to degrade exogenous ATP (100 nM) at 37°C and 20°C was then compared. Each point is the mean of four measurements. Vertical bars indicate s.e.mean.

To obtain the apparent kinetic parameters for the hydrolysis of ATP by the releasable ATPase, we measured the initial rates of hydrolysis of ATP (0.3–10 μM). Analysis of the data using the Lineweaver–Burke equation gave a K_M value of 39 μM and a V_{max} of 1039 pmol ATP metabolized min^{-1} ml^{-1} superfusate (Figure 3).

Effect of putative inhibitors on enzyme activity

Finally, the ability of several known inhibitors of eNTPDases to inhibit the activity of the releasable ATPase was determined.

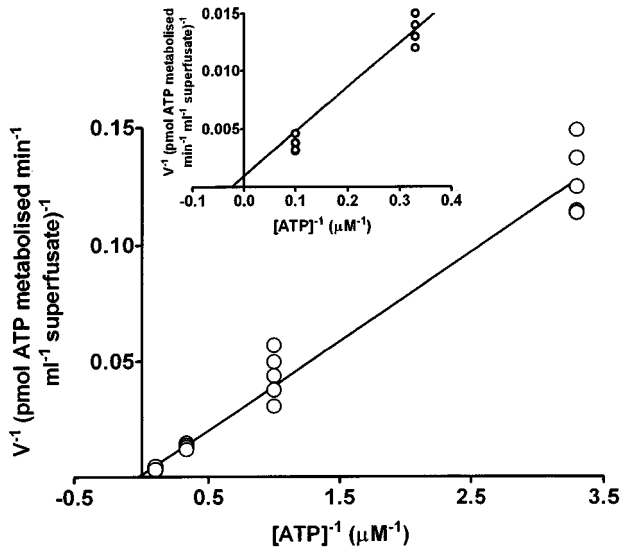

Figure 3 Kinetic analysis of the ATPase activity. EFS (8 Hz, 25 s) was applied to the superfused guinea-pig isolated vas deferens and the superfusate collected. The initial rates of hydrolysis of a range of concentrations of ATP (0.3–10 μM) were determined five or six times and the data analysed using the Lineweaver–Burke equation. Each point is the value obtained from a single assay. The inset shows the lower portion of the plot on an expanded scale to illustrate where the fitted line crosses the axes.

ARL 67156 (10–300 μM) (Figure 4, top panel) and PPADS (30–300 μM) (middle panel) both inhibited the ATPase activity in a concentration-dependent manner and with similar potency. Fifty per cent inhibition was seen at approximately 30 μM of either compound. At the concentrations used, neither compound abolished enzyme activity, but higher concentrations could not be tested as limited amounts of ARL 67156 were available and at 1 mM, PPADS significantly inhibited the luciferin-luciferase assay system.

P-5-P was much less effective than ARL 67156 or PPADS in that significant enzyme inhibition was only seen at 100 and 300 μM and 50% inhibition was not reached at these concentrations of P-5-P (Figure 4, bottom panel).

Discussion

The present study has extended the initial report by Todorov *et al.* (1997) that sympathetic nerve stimulation in the guinea-pig isolated vas deferens causes the release of an ATPase. It shows that similar ATPase activity is released from several other tissues in which ATP is a neurotransmitter. Release is not species-dependent, as it could be evoked in the rat and mouse isolated vas deferens, nor is release specific to sympathetic nerves, as ATPase activity was released by stimulation of the parasympathetic nerves of the guinea-pig isolated urinary bladder. Of these tissues, releasable ATPase activity was greatest from the guinea-pig vas deferens. It is not clear if this reflects differences in the density of innervation of these tissues or if other factors are involved.

In contrast to these tissues, under the conditions of our experiments, no release of ATPase was seen during stimulation of the enteric nerves of the guinea-pig isolated taenia coli, where ATP is an inhibitory neurotransmitter (Burnstock, 1972; Hoyle *et al.*, 1990; Barthó *et al.*, 1998). The reason for this difference is not known, but the data imply that the release of the ATPase is not a general property of all autonomic nerves or smooth muscle tissues.

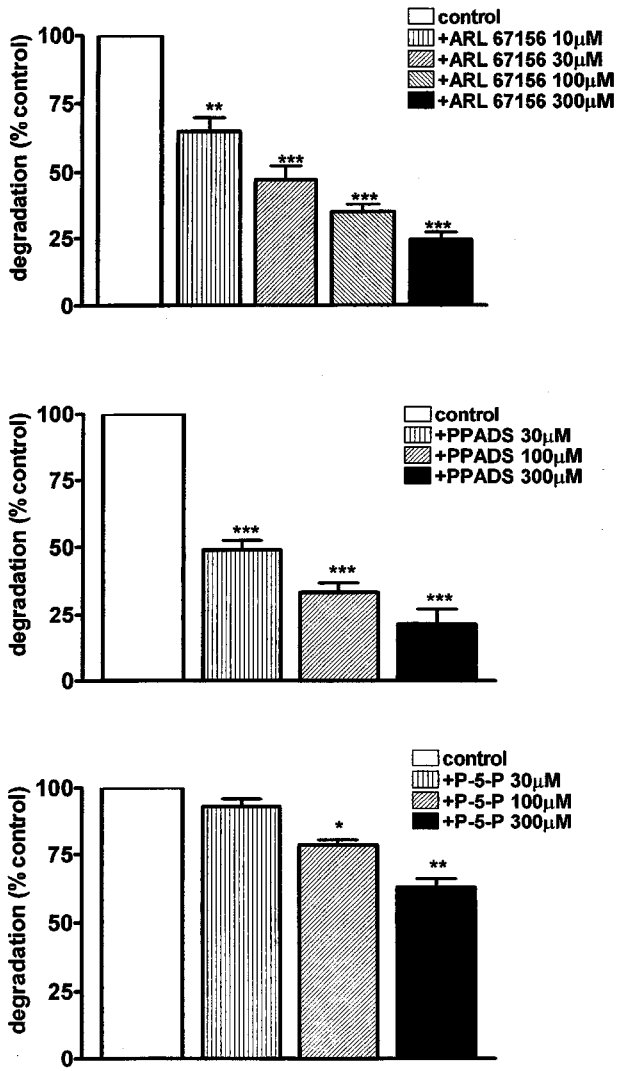

Figure 4 The effect of inhibitors of ATPase activity. EFS (8 Hz, 25 s) was applied to the superfused guinea-pig isolated vas deferens. The superfusate was collected and its ability to degrade exogenous ATP (10 μM) was determined over 30 min in the absence or presence of ARL 67156 (10–300 μM) (top panel), PPADS (30–300 μM) (middle panel) and P-5-P (30–300 μM) (bottom panel). Each point is the mean of 4–7 measurements. Vertical bars indicate s.e.mean. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

In this study we found that decreasing the incubation temperature from 37°C to 20°C had little effect on the time-course of breakdown of ATP by the enzyme released from the guinea-pig vas deferens. This is unusual, but not unique, as the temperature-dependence of enzymes shows great variability, with some being much less sensitive than others (Lehninger, 1975).

We found that the ATPase released by the guinea-pig isolated vas deferens metabolized ATP with a K_M of 39 μM, which is similar to the value reported by Mihaylova-Todorova *et al.* (1998). Three related subtypes of eNTPDase have recently been cloned and functionaly expressed (see Zimmermann, 1999; Zimmermann & Braun, 1999 for reviews) and interestingly each has a K_M for ATP that is only a little higher than this; 75 μM for rat CD39 (also known as ecto-apyrase or eNTPDasel; Wang *et al.*, 1998), 394 μM for human ecto-ATPase (also known as CD39-L1 or eNTPDase2; Mateo *et al.*, 1999) and 128 μM for HB6 (also known as CD39-L3 or eNTPDase3; Smith & Kirley, 1999). A soluble form of recombinant human CD39, created by removing the two transmembrane domains, has a much lower K_M for ATP (2.1 μM; Gayle *et al.*, 1998), although a similar rat CD39 mutant has a K_M of 220 μM (Wang *et al.*, 1998).

This study has also shown that the releasable ATPase is sensitive to inhibitors of eNTPDases. ARL 67156 and PPADS suppressed ATP breakdown in a concentration-dependent manner, with both producing 50% inhibition at approximately 30 μM. This is close to the IC_{50} value for inhibition of ATP breakdown by eNTPDases by ARL 67156 in human blood (25 μM; Crack *et al.*, 1995) and by PPADS in rat vas deferens (40 μM; Khakh *et al.*, 1995), macrophages (16 μM), C6 glioma cells (20 μM) and bovine pulmonary artery endothelial cells (100 μM) (Chen *et al.*, 1996). At similar concentrations ARL 67156 also inhibits ATP metabolism and potentiates the neurotransmitter actions of ATP in the guinea-pig isolated vas deferens and urinary bladder (Westfall *et al.*, 1996; 1997a). A single concentration of PPADS (100 μM) has also been reported to inhibit recombinant rat CD39 and ecto-ATPase to a similar extent (Heine *et al.*, 1999).

In the present study, P-5-P also inhibited the releasable ATPase, but was much less potent than ARL 67156 and PPADS. At 300 μM P-5-P only produced about 40% inhibition, which is close to its IC_{50} value for inhibiting ATP breakdown by eNTPDase in rat vas deferens (300 μM; Khakh *et al.*, 1995).

The molecular identity of the ATPase released by autonomic nerve stimulation is unknown, but the data reported here and previously (Todorov *et al.*, 1997) suggest that it may be related to the eNTPDases: (1) The K_M values for breakdown of ATP are similar for the releasable enzyme and the cloned eNTPDases: (2) Neither is sensitive to inhibitors of intracellular ATPases, such as ouabain, orthovanadate and N-ethyl maleimide (Plesner, 1995; Todorov *et al.*, 1997) and (3) ARL 67156 and PPADS inhibits the releasable ATPase and eNTPDases in several tissues with very similar potency. Two further eNTPDase clones have been identified (CD39-L2 and CD39-L4) and both were predicted to be soluble enzymes (Chadwick & Frischauf, 1998). CD39-L4 was subsequently shown to be a soluble enzyme with high ADPase activity (Mulero *et al.*, 1999). CD39-L2 remains to be functionally expressed and remains a possible candidate for the releasable ATPase reported here.

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