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Unique Ca^{2+}-activated ATPase in the nervous ganglia of *Phyllocaulis soleiformis* (Mollusca)

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Abstract

Nucleotide-metabolizing enzymes play important roles in the regulation of intracellular and extracellular nucleotide levels. We studied ATPase activity in the nervous ganglia of *Phyllocaulis soleiformis*, a terrestrial slug. The ATPase was divalent cation-dependent, with a maximal rate for ATP hydrolysis at pH 6.0 and 7.2 in the presence of Ca^{2+} (5 mM). Mg^{2+}-ATPase activity was only 26% of the activity observed in the presence of Ca^{2+} (5 mM). $ZnCl_2$ (10 mM) produced a significant inhibition of 70%. Ca^{2+}-ATPase activity was insensitive to the classical ATPase inhibitors ouabain, *N*-ethylmaleimide, orthovanadate and sodium azide. Levamisole, an inhibitor of alkaline phosphatase, was ineffective. Among nucleotides, ATP was the best substrate. The apparent $K_{m\ (ATP)}$ for Ca^{2+}-ATPase was 348 ± 84 μM ATP and the V_{max} was 829 ± 114 nmol Pi min^{-1} mg^{-1} protein. The *P. soleiformis* ganglial ATPase does not appear to fit clearly into any of the previously described types of Ca^{2+}-ATPases.

Keywords: Adenosine 5′-triphosphate; ATP; ATPase; Invertebrates; Mollusca; Mollusks; Nervous ganglia; *Phyllocaulis soleiformis*

1. Introduction

Phyllocaulis soleiformis (d'Orbigny, 1835) is a terrestrial slug belonging to the Veronicellidae, a family containing approximately 200 species. *P. soleiformis* is of medical importance since it is an intermediate host of *Angiostrongylus costaricensis*, the nematode responsible for abdominal angiostrongyliasis (Graeff-Teixeira et al., 1993). Veronicellids are also of economic significance, since they represent a principal agriculture and floriculture pest. Many investigators have attempted to develop efficient methods of slug control, but as yet few advances have been made (Bennett and Andrews, 1985).

Besides its energetic function in the intracellular environment, extracellular ATP also has sev-

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eral effects in many biological processes. These include smooth muscle contraction, immune response and neurotransmission (Ralevic and Burnstock, 1998). Since the purinergic nerve hypothesis defining extracellular ATP as a neurotransmitter was proposed (Burnstock, 1972), there has been growing interest in its role in synaptic transmission. ATP has been shown to be stored in nerve terminals and released with several neurotransmitters, including acetylcholine, noradrenaline and serotonin (Di Iorio et al., 1998; Rathbone et al., 1999). ATP exerts potent effects on the central nervous system, where it can act as a neurotransmitter or as a modulator regulating the activity of other transmitter substances (Di Iorio et al., 1998; Rathbone et al., 1999). Extracellular ATP evokes responses through two subclasses of P2-purinoceptors, P2X and P2Y, and the signaling actions induced by extracellular ATP have been correlated to the activity of a group of ectonucleotidases. Neurotransmitter ATP can be hydrolyzed to adenosine, an important neuromodulator, by the conjugated action of this group of ectonucleotidases, that includes an ecto-ATPase (EC 3.6.1.3), an ATP diphosphohydrolase (apyrase, EC 3.6.1.5) and a 5′-nucleotidase (EC 3.1.3.5) (Sarkis et al., 1995). The adenosine formed can act on P1-purinoceptors and is rapidly internalized via uptake systems present in neurons, mediating the salvage of physiological purine for incorporation into cellular nucleotides (Brundege and Dunwiddie, 1997). Except in insects, few studies on these ectonucleotidases exist in other invertebrates (Meyer-Fernandes et al., 1997; Matos et al., 2001).

In gastropods, studies in *Helix aspersa* demonstrated rectum and esophagus responses to purine nucleotides and nucleosides and showed that adenosine, AMP, ADP and ATP elicited dose-dependent contractions (Knight et al., 1992). Lozovaya et al. (1993) suggested that a change in ATP levels modulates the desensitization of acetylcholine receptors in neurons of *Lymnaea stagnalis*.

Since ATP acts as a co-transmitter and neurotransmitter, elucidation of the role of the enzymes involved in the termination of the physiological action of this compound is necessary. These enzymes hydrolyze the nucleotide, controlling its life span and the duration and extent of receptor activation. Thus, the purpose of the study is to explore the role of an ATPase in the nervous ganglia of *P. soleiformis* starting with enzyme characterization.

2. Material and methods

2.1. Phyllocaulis soleiformis

P. soleiformis (Mollusca, Gastropoda, Veronicellidae) were collected from the metropolitan region of Porto Alegre, RS, Brazil. Animals were maintained in plastic boxes at 25 ± 5°C. The specimens, weighing 4–8 g, were fed on a mixture of vegetables and maintained in a room for at least 7 days before experiments.

2.2. Isolation and homogenization of the nervous ganglia

The nervous system of *P. soleiformis* is composed of ganglia and their connectives fused into a circumoesophageal ring (South, 1992). The ring was isolated with a single razor blow in the anterior region of the animal. Under the stereomicroscope, the nervous ring was separated. Ganglia were then weighed and gently homogenized in 100 volumes of 0.65% (w/v) saline with a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged for 3 min at $4000 \times g$ and the supernatant was used in enzyme assays.

2.3. Enzyme assays

Enzyme activity was assayed in standard reaction medium containing 50 mM Tris–HCl, pH 7.2, 5 mM $CaCl_2$ (or other cation, as indicated) in a final volume of 200 μl. Fractions of nervous ganglia of *P. soleiformis* (10–20 μg protein) were added to the reaction medium and pre-incubated for 10 min at 30°C. The reaction was initiated by the addition of substrate (ATP or other, as indicated) to a final concentration of 1 mM and stopped by adding 200 μl 10% trichloroacetic acid. The samples were chilled on ice for 10 min before inorganic phosphate (Pi) released was measured (Chan et al., 1986). Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of substrates. Specific activity is expressed as nmol of Pi released ·

$min^{-1} \cdot mg^{-1}$ of protein. All enzyme assays were run in triplicate.

2.4. Protein determination

Protein was determined by a Coomassie Blue method using bovine serum albumin as a standard (Bradford, 1976).

2.5. Statistical analysis

Data were analyzed using Student's *t*-test and one-way analysis of variance (ANOVA), considering a level of significance of 5%.

3. Results

Previous experiments demonstrated that the time course for ATP hydrolysis from nervous ganglia of *P. soleiformis* was linear up to 15 min and that the protein concentration was 30 μg.

In order to determine the optimum pH for the enzyme, Ca^{2+}-ATP was used as substrates in a medium consisting of 50 mM sodium acetate (pH 4.0, 5.0 and 6.0) and 50 mM Tris–HCl (pH 7.2, 8.0 and 9.0) (Fig. 1). The maximal rate for ATP hydrolysis was observed at pH 6.0 and 7.2.

To further optimize assay conditions, the effect of varying the Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} (chloride salts) concentrations was investigated (Fig. 2). The patterns for ATPase activity in fractions from the nervous ganglia of *P. soleiformis* in the presence of the four divalent cations differed. The most striking differences were: (i) ATPase activation activities in the presence of Ca^{2+} and

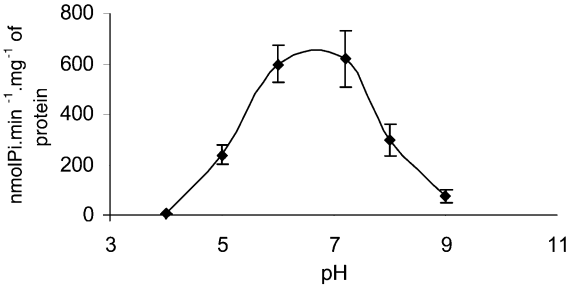

Fig. 1. pH dependence of ATP hydrolysis in nervous ganglia from *P. soleiformis*. Enzyme activity was determined as described in Section 2 containing the following buffers: 50 mM acetate, pH 4.0, 5.0 and 6.0; 50 mM Tris–HCl, pH 7.2, 8.0 and 9.0. Data represent means ± S.D. of three different experiments, each in triplicate.

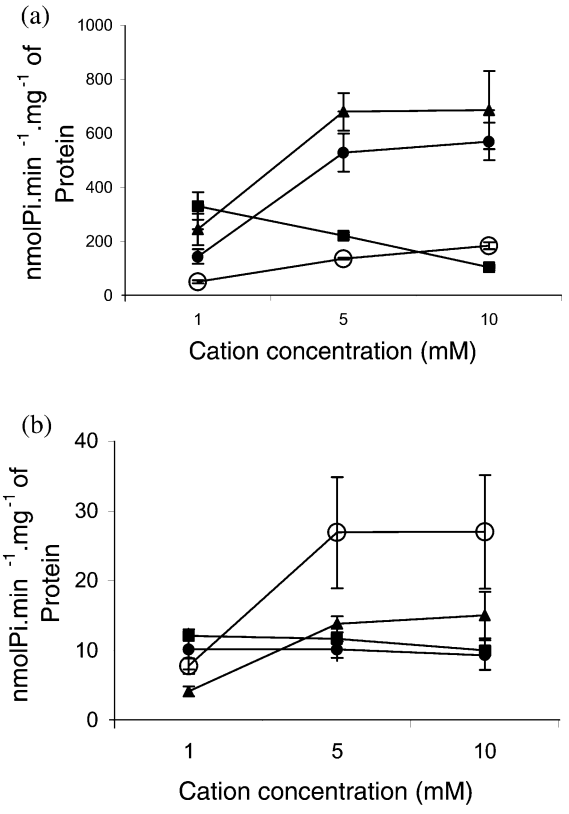

Fig. 2. Requirement of divalent cations for ATP (a) and ADP (b) hydrolysis in nervous ganglia from *P. soleiformis*. Enzyme assays were carried out as described in Section 2 except that $MnCl_2$ (▲), $MgCl_2$ (○), $CaCl_2$ (●), $ZnCl_2$ (■) concentrations were varied between 1 and 10 mM. Data represent means ± S.D. of three different experiments, each in triplicate.

Mn^{2+} were dose-dependent and greater than those obtained with Zn^{2+} and Mg^{2+}, reaching a plateau at 5 mM (Fig. 2a); (ii) ATPase activity exhibited the greatest activation in the presence of 1 mM Zn^{2+}, but a 70% inhibition was observed in the presence of this cation at 10 mM (Fig. 2a); and (iii) Mg^{2+}-ATPase activity was dose-dependent, but was lower than in the presence of the other cations tested, reaching only 26% of that observed in the presence of 5 mM Ca^{2+} (Fig. 2a). ADP hydrolysis in the presence of Mn^{2+}, Ca^{2+} and Zn^{2+} was less than 5% of the corresponding ATPase activity. In the presence of Mg^{2+}, maximal ADPase activity in fractions from nervous ganglia of *P. soleiformis* corresponded to 20% of the ATPase activity (Fig. 2a,b). Calcium (5 mM) was used for the experiments.

The ability to hydrolyze other mono, di and triphosphate nucleosides was also investigated

Table 1
Substrate specificity of ATPase activity in nervous ganglia from *P. soleiformis*

Substrate	% Activity
ATP	100
CTP	74 ± 12
GTP	30 ± 5.8
ADP	2 ± 0.07
CDP	1 ± 0.1
GDP	1.3 ± 0.21
UDP	3 ± 0.23
AMP	ND
Ppi	ND

Results are expressed as mean ± S.D. of at least four experiments. All substrates were used at 1.0 mM (5.0 mM Ca^{2+}). Control Ca^{2+}-ATPase activity was 568 ± 125 nmol Pi min^{-1} mg^{-1} protein. ND, not detectable.

(Table 1). ATP was the best substrate and CTP was the second most hydrolyzed nucleotide (hydrolysis was 74% of that of ATP). Nucleoside 5′-diphosphates, such as ADP, CDP, GDP and UDP produced very low reaction rates and AMP and inorganic pyrophosphate were not hydrolyzed by *P. soleiformis* nervous ganglia fractions.

To determine the kinetic constants of ATPase, Ca^{2+}-ATP hydrolysis was evaluated at concentrations ranging between 100 and 2500 μM (Fig. 3). Enzyme activity increased with increasing substrate concentrations. K_m and V_{max} values for the ATPase activity of *P. soleiformis* nervous ganglia fractions were estimated from the Lineweaver–Burk plot with three different enzyme preparations. The apparent K_m for Ca^{+2}-ATP was 348 ± 84 μM and V_{max} for Ca^{+2}-ATP was 829 ± 114 nmol Pi min^{-1} mg^{-1} protein ($r^2 = 0.98$).

We also investigated the effect of classical ATPase inhibitors upon ATP hydrolysis. There are no significant differences ($P > 0.05$) in ATP hydrolysis in the presence of ATPase and alkaline phosphatase inhibitors (Table 2).

Fig. 4 shows that ATP hydrolysis increased when the temperature was raised from 4 to 30°C. Above this temperature range, enzyme activity progressively decreased and was abolished at 50°C.

Since ADP hydrolysis was extremely low in the nervous ganglia of *P. soleiformis*, we also investigated the hydrolysis of nucleoside diphosphates and triphosphates in the digestive gland of *P. soleiformis*. Similar assay conditions for nucleotide hydrolysis in the digestive gland and nervous tissue were used. Maximal ATP hydrolysis and ADP hydrolysis was obtained in the presence of 5 mM Ca^{2+} and fractions from the digestive gland of *P. soleiformis* demonstrated higher ADP hydrolysis and lower ATP hydrolysis than nervous tissue. The differences between the two tissues is revealed when the ratios of ADPase/ATPase activities are compared. ADPase activity in the nervous ganglia was less than 3% of the ATPase activity, but in the digestive gland was 13% in the presence of 5 mM Ca^{2+}. Fractions from digestive gland promoted ATP and ADP hydrolysis with a

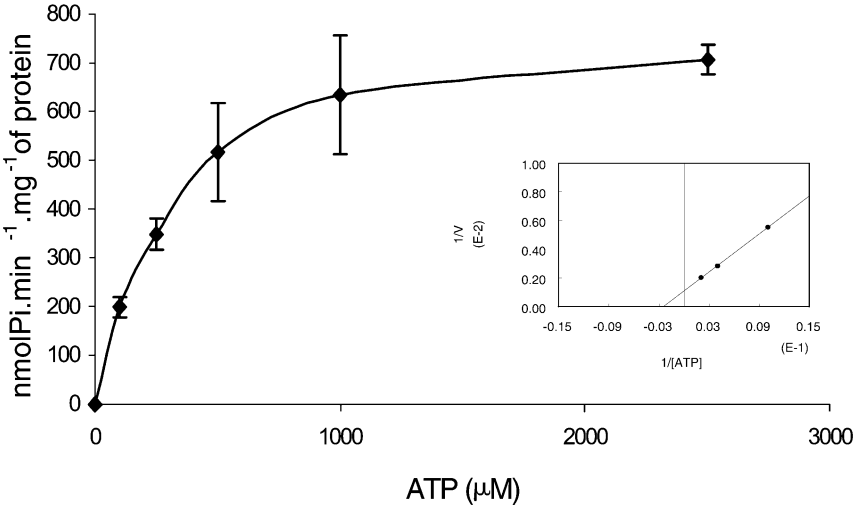

Fig. 3. Effect of Ca^{2+}-ATP concentration upon ATPase activity in nervous ganglia of *P. soleiformis*. Incubation conditions are described in Section 2. Data represent means ± S.D. of three different experiments, each in triplicate. *Inset*: Lineweaver–Burk plot presentation of a typical experiment.

Table 2
Effects of inhibitors of ATPase activity in nervous ganglia from *P. soleiformis*

Compounds	Concentration	% of control enzyme activity
Ouabain	1 mM	84 ± 12
Sodium vanadate	0.1 mM	95 ± 11
Levamizole	1 mM	92 ± 19
N-Ethylmaleimide	10 mM	99 ± 14
Sodium azide	5 mM	96 ± 12
	20 mM	88 ± 20

Control Ca^{2+}-ATPase activity was 641 ± 110 nmol Pi min^{-1} mg^{-1} protein. Results are expressed as percentages of control activity (100%). Data represent mean ± S.D. of at least four experiments. Data were analyzed statistically by one-way analysis of variance or by paired Student's *t*-test.

specific activity of 110 ± 22 nmol Pi min^{-1} mg^{-1} of protein and 14 ± 2 nmol Pi min^{-1} mg^{-1} protein, respectively (cf. Fig. 2a,b).

4. Discussion

The nervous ganglia of *Phyllocaulis soleiformis* demonstrates ATPase activity in the presence of divalent cations. This enzyme showed maximal ATP hydrolysis activity in the presence of 5 mM Ca^{2+} and Mn^{2+} (Fig. 2a). $CaCl_2$ was utilized in the experiments of this study because it is probably the physiological activator of the synaptic cleft (Sarkis et al., 1995). Furthermore, the concentration used in the incubation medium (5 mM $CaCl_2$) is similar to the concentration found in the hemolymph of *Ariolimax columbianus* (6.6 mM), indicating that the medium used in our experiments reflects physiological conditions (Deyrup-Olsen et al., 1983). In the literature, the zinc presents opposite effects on the ATPases, activating or inhibiting these enzyme activities (Gandhi and Ross, 1988; Sarkis et al., 1995). Caldwell et al. (1999) observed an inhibitory effect on ATP hydrolysis from chicken gizzard smooth muscle and liver in the presence of 5–10 mM Zn^{2+}. Other investigators have been concerned with this effect and showed zinc as a non-specific inhibitor of Na^+, K^+-ATPase from rat brain (Shafiq-ur-Rehman, 1986). This interesting characteristic could be explored in future studies, since that a better understanding of the metal effects can be a useful tool for toxicological studies.

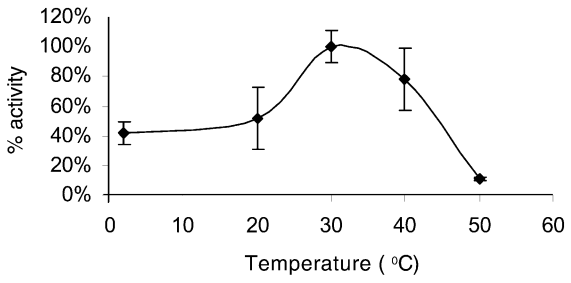

Fig. 4. Thermal inactivation of ATPase of nervous ganglia from *P. soleiformis*. Fractions from nervous ganglia (10–20 μg protein) were pre-incubated at different temperatures for 10 min. After pre-incubation, ATP hydrolysis activities were determined in the presence of 5 mM Ca^{2+} at 30°C. Data are the means of at least three different experiments, each in triplicate.

The maximal rate of ATP hydrolysis was observed at pH 6.0 and 7.2, a range similar to that cited for the hemolymph of some slugs, such as that of *Deroceras reticulatum* which has a pH of 7.72 (South, 1992).

The low ATPase activity observed in the presence of Mg^{2+} excludes some of the Mg^{2+}-dependent enzymes, such as Na^+-ATPase, Na^+, K^+-ATPase and adenylate kinase from being identified as the enzyme responsible. Na^+-ATPase is an ouabain-insensitive enzyme and found in many organisms, including squid and vertebrates (Moretti et al., 1991). Na^+, K^+-ATPase was also excluded because neither ouabain nor sodium vanadate inhibited ATP hydrolysis in *P. soleiformis* nervous ganglia (Table 2). In addition, calcium, used as an activator of ATP hydrolysis in this study, inhibits Na^+, K^+-ATPase activity (Skou, 1998). Adenylate kinase can be also ruled out because the rate of Pi released from ADP was extremely low (Table 1). The possibility that ADP hydrolysis occurs by prior conversion to ATP catalyzed by adenylate kinase and hydrolysis later by an ATP-specific enzyme can also be ruled out (Sarkis et al., 1995).

The insensitivity to classical inhibitors of ATPases and the ability to hydrolyze nucleoside 5′ triphosphates and diphosphates are characteristics of an ATP diphosphohydrolase (apyrase, EC 3.6.1.5). However, this enzyme can be excluded since nucleotide 5′-diphosphate hydrolysis is extremely low in *P. soleiformis* nervous ganglia (Table 1). Furthermore, no significant changes in ATP hydrolysis occurred in the presence of sodium azide (20 mM), a known inhibitor of ATP

diphosphohydrolase at this concentration (Sarkis et al., 1995) (Table 2). Sodium azide, at a low concentration (5 mM), is considered to be a specific mitochondrial ATPase inhibitor and does not promote significant changes in the ATP hydrolysis of *P. soleiformis* nervous ganglia (Table 2). The association of an ATP pyrophosphohydrolase (EC 3.6.1.8) and an inorganic pyrophosphatase (EC 3.6.1.1) was excluded because no Pi was released when 1.0 mM pyrophosphate (PPi) was used as substrate instead of ATP (Table 1). Levamizole, a specific alkaline phosphatase inhibitor, also failed to inhibit ATP hydrolysis.

Wan et al. (1995) analyzed the responses of neurons of *L. stagnalis* to extreme osmomechanical stress and suggested that a plasma membrane-linked contractile machinery (presumably involving myosin ATPase) might contribute to the neuron's mechano-osmotic robustness. For this reason, the authors used *N*-ethylmaleimide (NEM), a specific SH-group inhibitor, to inhibit this activity. NEM, however, did not affect ATP hydrolysis in the fractions of *P. soleiformis* nervous ganglia, thus excluding the participation of sensitive-NEM myosin ATPase and Ca^{2+}, Mg^{2+}-ATPase.

In *H. aspersa* the administration of AMP, ADP and ATP in the isolated rectum and esophagus produced concentration-dependent contractions, suggesting that purinoceptors are important for evoking the release of acetylcholine from these tissues (Knight et al., 1992). We investigated nucleotide hydrolysis in the digestive gland of *P. soleiformis.* The data showed a higher ADPase/ATPase activity ratio than observed in nervous tissue. However, it is possible to suggest that in the digestive gland there are others enzymes contributing for nucleotide hydrolysis. It is possible that the nucleotide hydrolysis in nervous ganglia and digestive gland from *P. soleiformis* could have an important role controlling the availability of ligands (ATP, ADP, AMP) for nucleotide receptors. By the hydrolysis of the nucleotide, they might be involved in controlling activation of the purinoceptors, modulating the action of ATP as a neurotransmitter and/or neuromodulator.

In mammals, ATP is known to be stored at the nerve terminals and co-released with some neurotransmitters, such as acetylcholine (Di Iorio et al., 1998; Ralevic and Burnstock, 1998). After exerting its actions, acetylcholine is hydrolyzed by acetylcholinesterase, an enzyme used as a marker of contamination by pesticides. There are reports of the successful use of acetylcholinesterase inhibition to identify exposure to cholinergic pesticides (Mora et al., 2000). Evidence suggests that the mollusks are more sensitive to anticholinesterases than vertebrates (Mora et al., 2000). Thus, the mollusks constitute an important group for ecotoxicological studies due their specific characteristics, such as low metabolic rate and importance in food chain (Ozretic and Krajnovic-Ozretic, 1992; Mora et al., 2000). Considering that ATP and acetylcholine are co-released in the synaptic cleft and acetylcholinesterase is a marker of contamination by carbamates and organophosphorous pesticides, it is important to investigate the influence of these compounds on ATPase activity in nervous ganglia from *P. soleiformis* to examine the use of this enzyme as a possible toxicological tool.

Extracellular ATP evokes responses via two sub-classes of P2-purinoceptors, P2X and P2Y. Previous studies suggest that invertebrate purinoceptors are heterogeneous, suggesting a diversification of receptor types that would appear to be equivalent to the P2-purinoceptors seen in vertebrate tissues (Knight et al., 1992). The different behavior of the *P. soleiformis* nervous ganglia ATPase activity may be related to differences in the purinergic signal transduction and in the cellular environment of invertebrates. In the present study, we described an enzyme in the nervous ganglia of *P. soleiformis* capable of hydrolyzing ATP with peculiar and different kinetic characteristics than those previously described.

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