

T-tubule membranes from chicken skeletal muscle possess an enzymic cascade for degradation of extracellular ATP

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The chicken T-tubule Mg²⁺-ATPase is an integral membrane glycoprotein that presents properties different from those of other ATPases located in skeletal muscle cells and exhibits ATP-hydrolysing activity on the extracellular side of the transverse tubule (TT) membranes. In this study we demonstrate that TT vesicles purified from chicken skeletal muscle possess ecto-ADPase and ecto-5'-nucleotidase activities that, along with ecto-ATPase, are able to sequentially degrade extracellular ATP to ADP, AMP and adenosine. Characterization studies of these TT ectonucleotidases revealed remarkable differences between ecto-ATPase and ecto-ADPase activities with respect to thermal stability, temperature dependence of the hydrolytic activity, effect of ionic strength, kinetic behaviour, divalent cation preference and responses to azide, *N*-ethylmaleimide, NaSCN, Triton X-100 and concanavalin A. Ecto-ATPase, but not ecto-ADPase, was inhibited by a polyclonal antibody against the

chicken TT ecto-ATPase. On the basis of these results we propose that ATP and ADP hydrolysis are accomplished by two distinct enzymes and therefore the TT ecto-ATPase is not an apyrase. 5'-Nucleotidase activity was inhibited by adenosine 5'-[α,β -methylene]diphosphate and concanavalin A, followed simple Michaelis–Menten kinetics and was released from the membranes by treatment with phosphatidylinositol-specific phospholipase C, indicating that AMP hydrolysis in T-tubules is catalysed by a typical ecto-5'-nucleotidase. Results obtained from electrophoresis experiments under native conditions suggest that ecto-ATPase, ecto-ADPase and 5'-nucleotidase might be associated, forming functional complexes in the T-tubule membranes. The TT ectonucleotidases constitute an enzymic cascade for the degradation of extracellular ATP that might be involved in the regulation of purinergic signalling in the muscle fibre.

INTRODUCTION

Transverse tubule (TT) membranes isolated from skeletal muscle possess a very active Mg²⁺-ATPase (EC 3.6.1.3) that exhibits properties different from those of other divalent-cation-dependent ATPases. These differences include ion and nucleotide selectivity, an extremely high turnover rate, inhibition by detergents and insensitivity to a variety of specific inhibitors of P-type, F-type and V-type ATPases [1,2]. The active site(s) of the Mg²⁺-ATPase have been conclusively demonstrated to be oriented to the lumen of the T-tubules [2–4]. The enzyme therefore belongs to the group of ecto-ATPases that are glycoproteins located on the extracellular face of the cell membrane in a variety of species, tissues and cell types. The identities and possible function of these enzymes have been reviewed recently [5] but their physiological roles have not yet been established. The TT Mg²⁺-ATPase has been proposed to serve as an alternative receptor for phorbol esters and diacylglycerols [6] and to be involved in cellular communication processes [4,7] but no conclusive lines of evidence support any concrete function other than hydrolysis of extracellular ATP.

Extracellular ATP has been shown to induce diverse biological effects in a wide variety of cells and tissues through interaction with distinct P₂ purinoceptor types located at the cell surface membrane [8]. In skeletal muscle, extracellular ATP activates ionic channels via ATP-sensitive receptors [9] and produces considerable increases in the level of inositol phosphates as well as in the intracellular Ca²⁺ concentration [10]. Thus the existence of an enzymic system able to regulate the nucleotide–receptor interaction is conceivable.

In many cellular systems a complete cascade of surface located

ecto-enzymes that sequentially hydrolyse ATP to adenosine have been described. These include chromaffin cells [11], vascular endothelial and smooth-muscle cells [12–14], hepatocytes [15], renal cortex cells [16], ventricular myocytes [17], innervated sartorius muscle [18], blood cells [19,20] and cholinergic synapses from mammalian and non-mammalian sources [21,22]. The first step, the hydrolysis of ATP, is mediated by an ecto-ATPase with properties different from those of the well-characterized ATPases with intracellular active sites but similar to those of the TT Mg²⁺-ATPase. It is controversial at present whether ADP hydrolysis is also performed by the ecto-ATPase as proposed by some authors [12,16,23–28] or whether the ADPase activity is due to an additional enzyme as suggested by others [2,14,17,29,30]. The last step in the hydrolysis cascade is the formation of adenosine from AMP by 5'-nucleotidase.

Here we report that TT membranes isolated from chicken skeletal muscle present ecto-ATPase, ecto-ADPase and 5'-nucleotidase activities able to transform extracellular ATP into adenosine. 5'-Nucleotidase possesses the typical features of this well-known enzyme. Ecto-ATPase and ecto-ADPase activities exhibit important differences in their properties and responses to a variety of compounds, indicating that two distinct enzymes are responsible for the hydrolysis of ATP and ADP in the skeletal muscle TT membranes.

EXPERIMENTAL

Materials

ATP (vanadium-free), adenosine 5'-[α,β -methylene]diphosphate (p[CH₂]pA), diadenosine(5' → P¹)pentaphosphate (A1'p5'A),

Abbreviations used: A1'p5'A, diadenosine(5' → P¹)pentaphosphate; Con A, concanavalin A; PI-PLC, phosphatidylinositol-specific phospholipase C; p[CH₂]pA, adenosine 5'-[α,β -methylene]diphosphate; SR, sarcoplasmic reticulum; TT, transverse tubule.

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concanavalin A (Con A), *N*-ethylmaleimide, ouabain, alamethicin, Malachite Green, cyclopiazonic acid, thapsigargin and pyrophosphate reagent kit were obtained from Sigma. Ionophore A23187 and suramin [8-(3-benzamido-4-methylbenzamido)-naphthalene-1,3,5-trisulphonic acid] were purchased from Calbiochem. [^{14}C]AMP (538 mCi/mmol) was from Amersham. Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was kindly provided by Dr. M. G. Low (Columbia University, New York, U.S.A.). The polyclonal antibody against the chicken T-tubule Mg^{2+} -ATPase was a gift from Dr. R. A. Sabbadini (San Diego State University, San Diego, CA, U.S.A.). All other chemicals were of analytical grade.

Isolation of TT membranes

TT and sarcoplasmic reticulum (SR) membranes were obtained from the breast muscle of 8–12-week-old Warren chickens by a Ca^{2+} -loading technique [31] as indicated elsewhere [3]. Aliquots of fresh membranes were used to determine the integrity and sidedness of the TT vesicles as previously reported [3]. Protein concentration was estimated by the method of Lowry et al. [32].

Enzymic activities

TT Mg^{2+} -ATPase and SR Ca^{2+} -ATPase activities were determined by a coupled-enzyme spectrophotometric ADP-release assay under the conditions previously described [3]. Alternatively, Mg^{2+} -ATPase activity was determined by a colorimetric method by measuring the amount of P_i released. The reaction mixture contained 25 mM Mops, pH 7.3, 0.2 mM EGTA, 5 mM MgCl_2 and 5–8 μg of protein in a final volume of 1 ml. The reaction was started by the addition of a final concentration of 3 mM substrate; after 10 min of incubation at 25 °C it was stopped with 0.5 ml of 10% (w/v) SDS [33] and the P_i produced was determined by the procedure of Taussky and Shorr [34]. ADPase activity was measured by the P_i -release assay as described above. The reaction mixture contained 25 mM Mops, pH 7.3, 0.2 mM EGTA, 5 mM MgCl_2 and 10–20 μg of protein in a final volume of 1 ml. The reaction was initiated by the addition of 2 mM ADP (final concentration) and the mixture was incubated at 37 °C for 30 min. To unmask latent activity, 1 mg of alamethicin/mg of protein was added to the reaction medium. Controls containing 10% (w/v) SDS and the appropriate amount of TT membranes were always included to correct for the non-enzymic hydrolysis of the substrates.

5'-Nucleotidase activity was measured at 37 °C by two methods. In the first procedure the assay mixture contained 20 mM Tris/HCl, pH 7.4, 5 mM MgCl_2 and 20–30 μg of TT membranes in a volume of 1 ml. AMP at a final concentration of 2 mM was added to start the reaction. After incubation at 37 °C for 30 min, the amount of P_i produced was measured as indicated above. Triton X-100 at a final concentration of 0.02% was used to unmask latent activity. In some selected experiments, 5'-nucleotidase activity was determined by a radioisotopic assay as described previously [35]. Both methods gave similar results with a difference of $\pm 13\%$. Controls for the non-enzymic AMP hydrolysis were performed.

Inorganic pyrophosphatase activity was measured by the release of P_i in a medium containing 25 mM Mops, pH 7.3, 0.2 mM EGTA, 5 mM MgCl_2 and 40–50 μg of protein in a final volume of 1 ml. The reaction was started by the addition of PP_i at a final concentration of 2 mM. The mixture was incubated at 37 °C for 60 min and the reaction was stopped with 0.5 ml of 10% (w/v) SDS. Controls for the spontaneous hydrolysis of PP_i were incubated in parallel.

ATP-pyrophosphatase activity was determined in a medium containing 25 mM Mops, pH 7.3, 0.2 mM EGTA, 10 mM MgCl_2 , 5 mM ATP and 25–30 μg of protein in a final volume of 1 ml. After 45 min of incubation at 37 °C, the reaction mixture was immersed for 5 min in a water bath at 85 °C, quickly cooled at 0–4 °C and the PP_i produced was measured with a commercial kit from Sigma (P-7275). Simultaneous controls were performed.

Treatment with antibodies

TT and SR membranes (5 μg for the ATPase assays and 15 μg for the ADPase assay) were incubated at 37 °C for 10 min with a polyclonal antibody raised against the chicken TT Mg^{2+} -ATPase in a medium containing 25 mM Mops, pH 7.3, 0.2 mM EGTA and 5 mM MgCl_2 in a final volume of 25 μl . A ratio of 4.5 μg of antibody/ μg of TT protein was used. After the incubation period the membranes were diluted 1:40 into the same medium and the Mg^{2+} -ATPase, ADPase and SR Ca^{2+} -ATPase activities were determined by measuring the production of P_i .

Treatment with PI-PLC

T-tubule vesicles (0.5–0.7 mg/ml) were treated at 37 °C for 2 h with 1 unit/ml of PI-PLC in a medium containing 25 mM Mops, pH 7.0, and 7% (w/v) sucrose in a final volume of 180 μl . After the incubation period, the mixture was centrifuged at 175000 *g* for 1 h and the pelleted membranes were resuspended in 180 μl of the same incubation medium. Mg^{2+} -ATPase, ADPase and 5'-nucleotidase activities were then determined in the supernatant and the resuspended fraction by measuring the released P_i (Mg^{2+} -ATPase and ADPase activities) or by the radiometric method (5'-nucleotidase).

Solubilization of TT membranes with digitonin

TT membranes (1 mg/ml) were incubated for 30 min at 4 °C in a medium containing 20 mM Mops, pH 7.4, 2 mM MgCl_2 , 10% (w/v) sucrose and 10 mg/ml digitonin. Insoluble material was removed by centrifugation at 175000 *g* for 60 min and the supernatant was further analysed. Before the solubilization treatment a 20 min preincubation at 25 °C with 3 μg of Con A/ μg of TT protein was included for some preparations.

Gel electrophoresis

Electrophoresis under non-denaturing conditions was performed with a 6% (w/v) polyacrylamide resolving gel (0.75 mm thick), a 4% (w/v) polyacrylamide stacking gel and the buffers described by Laemmli [36] except that there was no SDS in any buffer or gel, and digitonin was included in the gel (0.1%) and the running buffer (0.05%). Samples were prepared from TT membranes by treatment with digitonin or PI-PLC. Gels were run for 15 min at 80 V and for 90 min at 180 V at room temperature. Gel sections were then cut vertically and stained for activity as follows. Gels were equilibrated for 10 min in 25 mM Mops (pH 7.3)/5 mM MgCl_2 /0.2 mM EGTA and were incubated with gentle shaking in the same medium containing 2 mM ATP, ADP or AMP for 15 min at 25 °C (ATP) or 37 °C (ADP and AMP). The gels sections were rinsed quickly with water (twice, 10 ml each time) to remove substrate and excess P_i from the gel surface; the P_i liberated into the gel matrix was revealed by the formation of a Malachite Green–phosphomolybdate complex, essentially as described [37].

RESULTS

Enzymic activities of T-tubules involved in the metabolism of extracellular ATP

The chicken TT membranes used in this work have been extensively characterized and their properties are typical of highly purified T-tubules [3]. We determined the activity of the enzymes that could potentially be involved in the metabolism of ATP. As shown in Table 1, chicken TTs were able to hydrolyse ATP, ADP and AMP when used as substrates. Because the spectrophotometric method was used routinely to measure Mg^{2+} -ATPase, we investigated the presence of adenylate kinase in our preparations. Adenylate kinase (EC 2.7.4.3) catalyses the reaction $ATP + AMP \rightleftharpoons 2 ADP$ and therefore could potentially interfere with this assay, which follows ADP production over time. The inhibitor of adenylate kinase, A1'p5'A, [38] at concentrations up to 500 μ M did not inhibit the Mg^{2+} -ATPase activity (results not shown), indicating that the formation of ADP in the coupled-enzyme assay is due only to the activity of Mg^{2+} -ATPase. Furthermore the addition of 0.15 mM exogenous AMP to the standard assay did not produce any increase in the rate of NADH oxidation, either at 25 °C or at 37 °C (results not shown), demonstrating the absence of ecto-adenylate kinase in the TT vesicles.

Another enzymic reaction with ATP as substrate is the hydrolysis of the nucleotide to AMP and PP_i catalysed by ATP pyrophosphatase (EC 3.6.1.8). Table 1 shows that this activity was negligible in the TT membranes. In addition, inorganic pyrophosphatase, which could be involved in the hydrolysis of the released PP_i , was barely detectable.

The TT membranes exhibited ADPase and 5'-nucleotidase activities (Table 1). Because the P_i -release assay was used to measure ADP hydrolysis, there is the possibility that the AMP formed by this reaction can be subsequently hydrolysed by 5'-nucleotidase, leading to an overestimation of ADPase activity. Addition of the ADP analogue p[CH₂]pA at a concentration of 0.5 mM, which totally inhibited the TT 5'-nucleotidase (see below), was without effect on ADPase activity (see Table 4), indicating that in our assay conditions 5'-nucleotidase did not contribute to the formation of P_i . This result could be expected because 5'-nucleotidase is inhibited by ADP as well as by ATP in a variety of cell types [21]. At 25 °C, ADPase activity represents approx. 5% of the Mg^{2+} -ATPase activity (see below); therefore it does not interfere significantly in the determination of Mg^{2+} -ATPase activity by the P_i -release method. In fact, the agreement between the values estimated by spectrophotometric and colorimetric procedures was excellent.

Finally we investigated the presence in the TT of non-specific phosphatases that could act in several steps of the sequential degradation of ATP. Hydrolysis of several phosphate esters was undetectable after 30 min incubation (Table 1). Furthermore the addition of these phosphate esters at 1 or 10 mM to the standard assays did not alter the degradation rates of ATP, ADP and AMP. In contrast, the inhibitors of alkaline phosphatase and non-specific phosphatases levamisole and fluoride at 1 mM were without effect on ATPase, ADPase and 5'-nucleotidase activities. Thus the participation of phosphatases in the metabolism of ATP in chicken TT can be excluded.

Orientation of the catalytic sites

TTs isolated from chicken muscle have been shown to be largely composed of sealed right-side-out vesicles accounting for a 69–76% of the total vesicle population [3]. The fact that ATPase, ADPase and 5'-nucleotidase activities can be measured in the

Table 1 Enzymic activities of chicken TT membranes involved in the metabolism of extracellular ATP

Results are expressed as means \pm S.D. for the number of preparations in parentheses. Within each experiment duplicate or triplicate samples were assayed. ATPase activity was determined by the coupled-enzyme spectrophotometric assay (1 mM ATP) or by the colorimetric method (3 mM ATP). 5'-Nucleotidase activity was measured by the colorimetric assay or by the radioisotope method. For the phosphatase assays the phosphate esters used as substrate were phosphoenolpyruvate, β -glycerophosphate, glucose 6-phosphate and sodium phenylphosphate. The reaction was performed for 30 min at either 25 or 37 °C in the same reaction mixture as for the ATPase assay with 20–40 μ g of TT membranes. Adenylate kinase was determined as described in the text. Abbreviation: n.d., not detected.

Enzymic activity	Substrate	Specific activity (μ mol/h per mg of protein)
ATPase	1 mM ATP	222 \pm 27 (15)
	3 mM ATP	214 \pm 31 (15)
ADPase	2 mM ADP	23 \pm 3 (15)
5'-Nucleotidase	2 mM AMP	10.8 \pm 2.1 (15)
	100 μ M [U- ¹⁴ C]AMP	9.5 \pm 1.8 (5)
ATP-pyrophosphatase	5 mM ATP	0.38 \pm 0.14 (5)
Inorganic pyrophosphatase	2 mM PP_i	< 0.25 (5)
Phosphatases	1 and 10 mM of phosphate esters	n.d. (5)
Adenylate kinase	—	n.d. (5)

native preparations indicates that the substrates added to the external medium have access to the respective catalytic sites, which should be located on the luminal side of the TT membranes because most of the vesicles are right-side-out oriented. To confirm that patent activities represent a major percentage of the total activities, we measured ATPase, ADPase and 5'-nucleotidase in the presence of alamethicin or Triton X-100 as unmasking agents. As shown in Table 2, alamethicin slightly increased Mg^{2+} -ATPase; similar increases were detected in ADPase and 5'-nucleotidase activities. This enhancement of enzymic activities can be attributed to the unmasking of the small fraction of catalytic sites located on the internal side of the sealed inside-out vesicles [3]. From these results it can be concluded that the enzymes involved in the sequential degradation of ATP to adenosine are extracellularly oriented.

Table 2 Effect of unmasking agents on Mg^{2+} -ATPase, ADPase and 5'-nucleotidase activities of T-tubule vesicles

Results are means \pm S.D. for four independent preparations. Duplicate samples were assayed within each experiment. Patent and total activities are enzymic activities determined in the absence or in the presence of unmasking agents. Total ATPase and ADPase activities were measured in the presence of an alamethicin-to-protein ratio of 1:1 (w/v); patent activities were determined in the presence of ethanol in the amount necessary to dissolve alamethicin (0.2% and 0.6% for ATPase and ADPase assays respectively). Total 5'-nucleotidase was measured in the presence of 0.02% Triton X-100. In each case, TT membranes were preincubated for 10 min with the unmasking agent before the reaction was started.

Enzyme	Activity (μ mol/h per mg of protein)		
	Patent	Total	Latent (%)
Mg^{2+} -ATPase	212 \pm 21	256 \pm 14	17
ADPase	24 \pm 3	28 \pm 4	14
5'-Nucleotidase	11 \pm 2	13 \pm 3	15

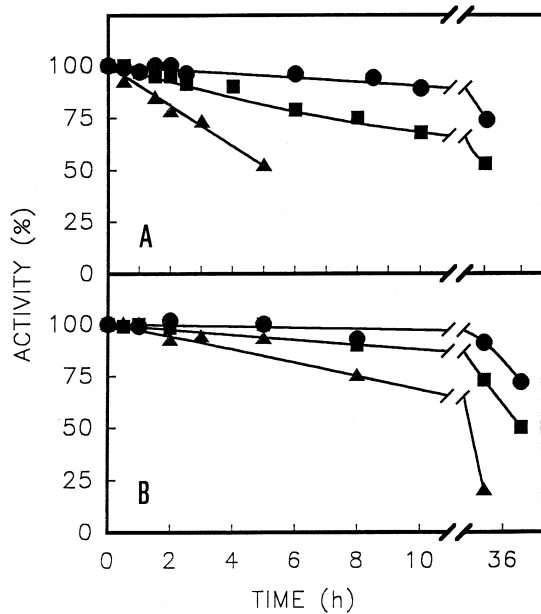


Figure 1 Effect of temperature on stability of Mg^{2+} -ATPase (A) and ADPase (B) activities in TT membranes

T-tubule vesicles (0.3–0.4 mg/ml) were incubated at 0–4 °C (●), 25 °C (■) or 37 °C (▲) for the indicated times. After the incubation period, aliquots were assayed by the spectrophotometric (A) or colorimetric (B) method. Results are means from three independent experiments.

Characterization of ecto-ATPase and ecto-ADPase activities

Effect of temperature on stability and activity

Figure 1 shows that at any of the temperatures employed, ADPase activity was more stable than Mg^{2+} -ATPase activity. The temperature dependence of both enzymic activities is

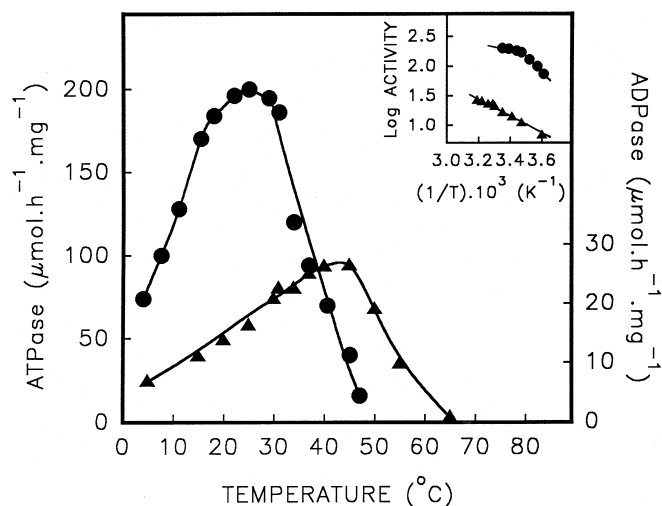


Figure 2 Temperature dependence of Mg^{2+} -ATPase (●) and ADPase (▲) activities in TT vesicles

The enzymic activities were determined by the colorimetric assay as described in the Experimental section. Arrhenius plots from the data are shown in the inset. Results are means from three experiments.

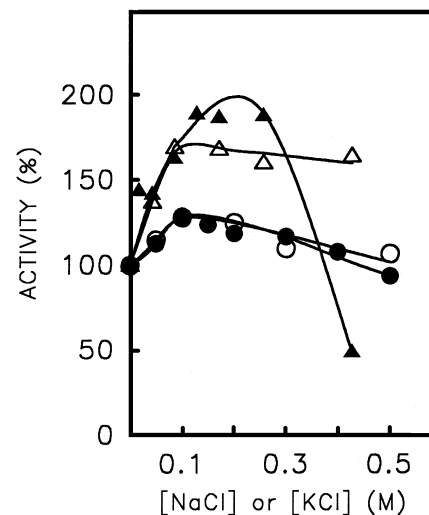


Figure 3 Effect of ionic strength on Mg^{2+} -ATPase (○, ●) and ADPase (△, ▲) activities

The indicated concentrations of NaCl (○, △) and KCl (●, ▲) were added to the reaction mixture and the enzymic activities were determined by the colorimetric assay. Each value is an average from three different preparations.

illustrated in Figure 2. Mg^{2+} -ATPase activity increased progressively from 5 °C to a maximum at 25 °C, decreasing at temperatures above this value. The Arrhenius plot up to 25 °C shows a break at 15 °C and the activation energies calculated above and below the transition temperature were 2.7 and 11.6 kcal/mol (11.3 and 48.5 kJ/mol) respectively. The decrease in enzymic activity cannot be explained, at least in the range 25–37 °C, assuming irreversible denaturation of the Mg^{2+} -ATPase, because stability studies indicated that at 37 °C the enzyme was stable for 30 min (Figure 1A). The anomalous temperature dependence exhibited by the chicken TT Mg^{2+} -ATPase has been also described by Moulton et al. [39], although they did not report on the existence of a transition temperature below 25 °C. In rabbit and rat preparations, the Mg^{2+} -ATPase activity increased progressively from 5 °C to approx. 40 °C [33,41] and analogous results have been obtained in our laboratory with rabbit TT membranes, suggesting a difference between species.

In contrast, the rate of ADP hydrolysis increased from 4 to 43 °C, decreasing at higher temperatures, probably due to thermal denaturation of the enzyme (Figure 2). The Arrhenius plot gives a straight line [E_a = 6.84 kcal/mol (28.6 kJ/mol)].

Effect of ionic strength and pH

Increasing the NaCl or KCl concentration caused an increase in the ATPase activity, with a maximum at a salt concentration of 100 mM as shown in Figure 3. This figure demonstrates that the response of ADPase activity was different. Stimulation of the enzymic activity at low salt concentrations was higher than that observed for Mg^{2+} -ATPase, suggesting a greater sensitivity of the active site to the ionic environment. The different response of ADPase to high concentrations of NaCl and KCl might indicate some specific effect of monovalent cations on this activity.

Mg^{2+} -ATPase was active in the alkaline pH region and exhibited a broad pH optimum between 7.5 and 8.5 (Figure 4). A similar profile was obtained when ADP was used as substrate. When 3 mM levamisole was included in the reaction medium, the

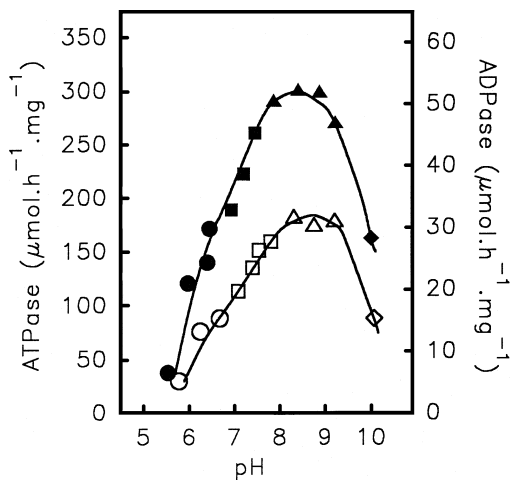


Figure 4 pH dependence of T-tubule ecto-ATPase (●,▲,■,◆) and ADPase (○,△,□,◇) activities

The enzymic activities were determined by the colorimetric assay. The following buffers, at 25 mM, were used: Mes (●,○), Mops (■,□), Tris (▲,△) and potassium carbonate (◆,◇). Results are mean values from four independent preparations.

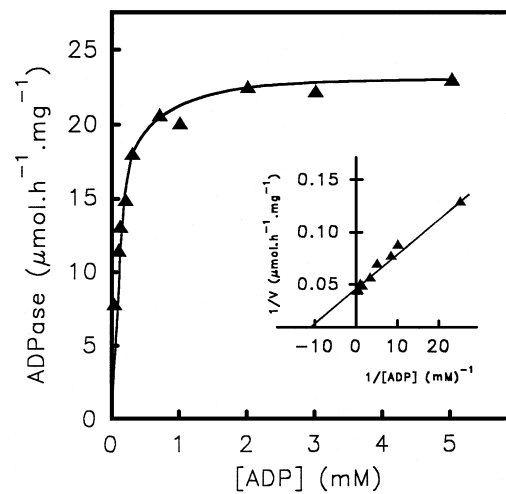


Figure 5 Substrate concentration dependence of the rate of ADP hydrolysis by the T-tubule ecto-ADPase

ADP concentrations were varied with $MgCl_2$ at a 5 mM excess over ADP. The enzymic activity was measured at 37 °C. Inset: Lineweaver–Burk plot of the same data. The values are averages of four experiments with different preparations.

Table 3 Effect of different divalent cations on the ecto-ATPase and ADPase activities in TT membranes

Data are means \pm S.D. for three independent preparations with assays performed in triplicate. The enzymic activities were determined by the P_i -release assay in the presence of 3 mM ATP or 2 mM ADP, and the indicated divalent cations (5 mM) in the form of the chloride salts. Assays were also performed in the absence of 5 mM $MgCl_2$ with or without 1 mM EDTA and 1 mM EGTA. Results are expressed as percentages of the ecto-ATPase or ecto-ADPase activities determined in the presence of 5 mM $MgCl_2$.

Divalent cation	Relative activity (%)	
	ATPase	ADPase
None	0	6 \pm 2
1 mM EGTA + 1 mM EDTA	0	0
Mg^{2+}	100 \pm 8	100 \pm 6
Ca^{2+}	140 \pm 6	60 \pm 4
Mn^{2+}	33 \pm 1	31 \pm 1
Co^{2+}	16 \pm 2	16 \pm 6
Zn^{2+}	30 \pm 6	6 \pm 2
Ni^{2+}	8 \pm 1	3 \pm 2

patterns of ATPase and ADPase activities were unmodified, confirming the absence of alkaline phosphatase in our preparation (Table 1). It has previously been reported that the chicken TT Mg^{2+} -ATPase exhibits optimal activity at pH 7.3 [31] and the rabbit and rat enzymes display maximal activities in the pH ranges 6.0–7.5 and 5.5–8.5 respectively [33,42]. Our results are in agreement with those reported for most ecto-ATPases so far characterized that exhibit a broad pH optimum between 7.5–8.5 [22,27,43–45]. Ecto-ADPase activity has not been characterized in T-tubules from other species but in microsomal fractions of bovine lung and smooth-muscle cells of bovine trachea the optimum pH for ADPase is close to 7 [25,26].

Cation requirements

Table 3 shows that ecto-ATPase and ecto-ADPase have an

absolute requirement for divalent cations. In the absence of exogenous cations, both activities were negligible and they became undetectable when the metal chelators EDTA and EGTA were added. ATPase and ADPase activities were predominantly activated by Ca^{2+} and Mg^{2+} respectively but other divalent cations were also able to activate ATP and ADP hydrolysis, although to a smaller extent. With both Mg^{2+} and Ca^{2+} in the reaction medium, no additive effects on the ATPase activity were observed, indicating that the same enzyme was responsible for the Ca^{2+} - and Mg^{2+} -dependent ATP hydrolysis. The same was true of ADPase activity. Maximal rates of catalysis were obtained with 3 mM Ca^{2+} or Mg^{2+} and with 1.5 mM Ca^{2+} or Mg^{2+} when ATP or ADP respectively was used as substrate; an excess of either Ca^{2+} or Mg^{2+} over ATP or ADP did not cause inhibition of ATPase and ADPase activities (results not shown).

The TT Mg^{2+} -ATPases from rabbit and rat also require divalent cations but differences were observed in their preferences for divalent cations [33,41]. In general, cation specificity studies show that ecto-ATPases from different tissues require divalent cations and are maximally activated by Mg^{2+} and Ca^{2+} and to a smaller extent by Mn^{2+} and Co^{2+} [5,16,22,38,43–45]. The cation specificity of ADPase activity has been poorly characterized; in rabbit T-tubules ADP hydrolysis was more active with Ca^{2+} than with Mg^{2+} [33], whereas the opposite was found for ADPase activity of bovine brain synaptosomes [22] and rat renal brush border membranes [16].

Dependence of ecto-ATPase and ecto-ADPase activities on substrate concentration

Chicken TT Mg^{2+} -ATPase exhibits a complex dependence on ATP concentration that has been interpreted as a combination of negative co-operativity and substrate inhibition [39]. This behaviour seems to be unique to the chicken enzyme because the rat and rabbit Mg^{2+} -ATPases manifest simple apparent Michaelis–Menten kinetics [33,42] as found for the Mg^{2+} - or Ca^{2+} -dependent ecto-ATPases from a variety of tissues and cell types [11,20,25,27,30,41,44,46]. The ADPase activity of TT membranes exhibit in contrast a Michaelis–Menten kinetic ($K_m = 89 \pm 6 \mu M$ and

Table 4 Substrate specificity of ecto-ATPase and ecto-ADPase activities from chicken TT membranes

Data are means \pm S.D. for four independent preparations with assays performed in triplicate. Hydrolysis rates of nucleoside triphosphates and diphosphates are expressed as percentages of the hydrolysis rates of ATP ($217 \pm 19 \mu\text{mol/h}$ per mg of protein) and ADP ($23.5 \pm 2.7 \mu\text{mol/h}$ per mg of protein) respectively. ATPase and ADPase activities were measured in the presence of either 5 mM MgCl_2 or 5 mM CaCl_2 by the P_i -release method. Nucleoside triphosphates were used at 3 mM and nucleoside diphosphates at 2 mM.

Substrate	Relative activity (%)	
	With Mg^{2+}	With Ca^{2+}
ATP	100	137 \pm 13
CTP	132 \pm 11	237 \pm 22
GTP	134 \pm 17	181 \pm 15
UTP	136 \pm 18	201 \pm 25
ADP	100	66 \pm 8
CDP	68 \pm 10	126 \pm 14
GDP	60 \pm 13	97 \pm 17
UDP	62 \pm 15	151 \pm 23

$V_{\text{max}} = 24.1 \pm 2.8 \mu\text{mol/h}$ per mg of protein; mean \pm S.D.) as shown in Figure 5. ADPase activities of renal brush border membranes, cultured pig endothelial and smooth-muscle cells and chromaffin cells also display Michaelis–Menten kinetics [16,47,29,11].

Substrate specificity

Table 4 shows that ecto-ATPase activity had a broad substrate specificity because UTP, CTP and GTP were hydrolysed at an even higher rate than ATP. All the nucleotides were more efficiently hydrolysed with Ca^{2+} as activating cation than with Mg^{2+} . ADPase activity also showed a broad substrate specificity. The hydrolysis rate of ADP was higher than that of other nucleoside diphosphates in the presence of Mg^{2+} but the opposite was found when the activating cation was Ca^{2+} . A broad substrate specificity is a common characteristic of all the ecto-ATPases, irrespective of whether they are membrane-bound enzymes [1,5,38,43] or the purified forms [22,23,44,48–50]. Although preferential activity towards ATP is usual, there are numerous exceptions [15,16,19,22,27,33]. Concerning the ecto-ADPase activity, rat mesenteric small arteries and bovine lung microsomal fractions hydrolysed ADP more actively than other nucleoside diphosphates [13,25], whereas rat platelets exhibit a preference for UDP [27].

The possibility that ATP and ADP are hydrolysed by the same enzyme has been also examined. When TT membranes were incubated at 37 °C for 20 min in the presence of 3 mM ATP alone, 2 mM ADP alone or both nucleotides at these concentrations, the rate of P_i production was respectively 101 ± 8 , 26 ± 2 and $83 \pm 6 \mu\text{mol/h}$ per mg of protein. The value obtained in the simultaneous presence of ATP and ADP indicated no additive effects on P_i release, additive effects would be expected if totally independent active sites were responsible for both hydrolysing activities. This experimental result could be interpreted as being due to the existence of an ecto-ATP diphosphohydrolase able to hydrolyse ATP and ADP. However, the fact that the rate of P_i release in the presence of ATP plus ADP is lower than that observed with ATP alone suggests the possibility that ADP might act as an inhibitor of the ecto-ATPase, as found for the ecto-ATPases of several cell types [19,20,30,46].

Table 5 Effect of various compounds on ecto-ATPase and ecto-ADPase activities of chicken TT membranes

Results are means \pm S.D. for three experiments performed with different TT preparations. Enzymic activities were measured by the P_i -release assay and are expressed as percentages of the control activities determined under the standard conditions. The various compounds at the indicated concentrations were added to the assay mixture and preincubated with the TT membranes for 10 min before the reaction was started. For oligomycin and *N*-ethylmaleimide, where 0.5% ethanol was present in the assay medium, control samples were treated with the same concentration of ethanol in the reaction mixture. Abbreviation: ρ -HMB, ρ -hydroxy-mercuribenzoate.

Addition	Relative nucleotidase activity (%)	
	ATPase	ADPase
None	100	100
Ouabain (1 mM)	93 \pm 5	98 \pm 7
Vanadate (0.1 mM)	95 \pm 8	89 \pm 4
Oligomycin (50 $\mu\text{g/ml}$)	107 \pm 10	72 \pm 12
Azide		
5 mM	95 \pm 6	63 \pm 8
20 mM	43 \pm 10	48 \pm 11
ρ -HMB (0.1 mM)	58 \pm 7	30 \pm 15
<i>N</i> -ethylmaleimide (5 mM)	92 \pm 6	55 \pm 7
NaNO_3 (5 mM)	68 \pm 14	97 \pm 3
NaSCN (5 mM)	45 \pm 9	89 \pm 12
HgCl_2 (50 μM)	41 \pm 17	11 \pm 7
KF (10 mM)	27 \pm 6	5 \pm 2
pp[CH_2]pA (0.5 mM)	100 \pm 5	99 \pm 3
Suramin (5 mM)	15 \pm 6	13 \pm 7
Thapsigargin (0.2 μM)	93 \pm 5	97 \pm 6
Cyclopiazonic acid (0.1 mM)	78 \pm 9	85 \pm 6
Triton X-100 (0.1 mg/ml)	0	40 \pm 5
Con A (100 $\mu\text{g/ml}$)	268 \pm 29	110 \pm 11

Effect of different compounds on ATPase and ADPase activities

As shown in Table 5, the chicken TT Mg^{2+} -ATPase was insensitive to ouabain, vanadate, oligomycin and 5 mM sodium azide, as reported previously [1]. In contrast, ADPase activity was inhibited by oligomycin and 5 mM azide. Higher concentrations (20 mM) of this mitochondrial ATPase inhibitor also proved inhibitory for ecto-ATPase. Inhibition by azide has been considered a characteristic property that distinguishes apyrases from other nucleotide-hydrolysing enzymes [43,50]. However, our results support the proposal that the inhibitory effect of azide is better correlated with the concentration employed than the capacity to hydrolyse ADP [5]. *N*-Ethylmaleimide was without effect on ATPase but the ADPase activity was decreased to 55%, suggesting the involvement of thiol groups in the ADP hydrolysis. The P_2 -purinergic antagonist suramin markedly inhibited the chicken TT ecto-ATPase as previously described for other ecto-ATPases from several sources [38]; a similar effect on ADPase activity was observed. The fact that suramin is also able to inhibit the SR Ca^{2+} -ATPase from chicken skeletal muscle and rat heart (J. Delgado, unpublished work) suggests that ATPases, ADPases and P_2 purinoceptors might have common structural elements at the nucleotide-binding domain to which suramin binds. Triton X-100 inhibited the hydrolysis of both ATP and ADP in a concentration-dependent manner (results not shown). At 0.1 mg/ml detergent, ATPase was totally inactivated, whereas ADPase was inhibited by only 60%. Higher concentrations of Triton X-100 did not cause a further decrease in the rate of ADP breakdown. Preincubation of the TT vesicles with Con A resulted in stimulation of ecto-ATPase, in agreement with previous data [39]. In contrast, no effect was observed on ecto-ADPase activity.

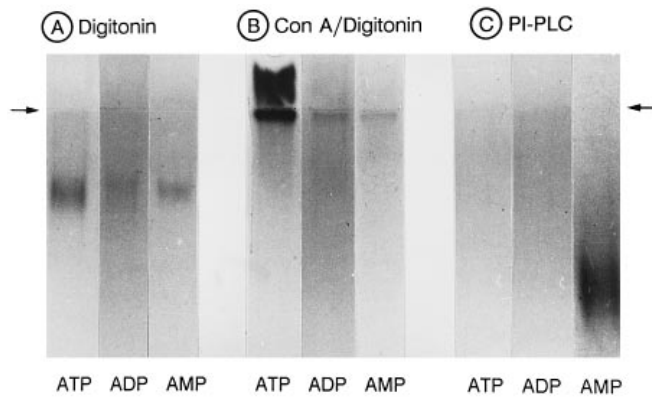


Figure 6 PAGE under non-denaturing conditions and activity staining of ATPase, ADPase and 5'-nucleotidase

Supernatants obtained after the treatment of TTs with digitonin (A), Con A/digitonin (B) or PI-PLC (C) were subjected to non-denaturing electrophoresis. A 30 μ l sample was loaded into each well and was separated on a 6% (w/v) polyacrylamide gel. The gel was cut into three sections, each section was incubated for 15 min with ATP, ADP or AMP and the enzymic release of Pi was assayed as described in the Experimental section. The arrows indicate the beginning of the resolving gel.

The ADP-hydrolysing activity of rat pancreatic cells and renal brush-border membranes have been also shown to be insensitive to Con A [51,16].

Treatment of TT membranes with antibodies

We have used an antibody raised against the chicken TT Mg^{2+} -ATPase that exhibits no cross-reactivity with other skeletal muscle proteins on immunoblots, inhibits the TT Mg^{2+} -ATPase activity in a concentration-dependent manner and recognizes a polypeptide of 85 kDa that has been tentatively identified as an essential component of the Mg^{2+} -ATPase [4]. At an antibody-to-TT protein ratio of 4.5:1, a 56% inhibition of ecto-ATPase activity was observed. Under the same conditions, neither SR Ca^{2+} -ATPase nor TT ADPase activity was significantly affected (97% and 86% of the activity of control values).

Characterization of 5'-nucleotidase in TT membranes

5'-Nucleotidase activity in TT membranes exhibited features typical of this well-characterized enzyme. AMP hydrolysis follows simple Michaelis-Menten kinetics ($K_m = 27 \pm 5 \mu M$ and $V_{max} = 15.1 \pm 4.3 \mu mol/h$ per mg of protein; results not shown). $p[CH_2]pA$ at concentrations 0.5 mM inhibited 92% of the enzymic activity. Con A also decreased the rate of AMP hydrolysis in a concentration-dependent manner; at 100 $\mu g/ml$ this activity was only 15% of the control value. $pp[CH_2]pA$ and Con A have been described as inhibitors of membrane-anchored 5'-nucleotidases (or ecto-5'-nucleotidases) in a variety of animal cells [21]. Thus the hydrolysis of AMP in TT membranes seems to be catalysed by an enzyme of this group. The response of 5'-nucleotidase to these compounds is clearly different from those of ATPase and ADPase activities (Table 5), suggesting that catalytic sites responsible for the hydrolysis of ATP, ADP and AMP reside on distinct proteins. This was confirmed by analysing the mode of insertion of the ectonucleotidases in the T-tubules. Treatment of TT vesicles with PI-PLC released 84% of the 5'-nucleotidase activity from the membranes, indicating that the enzyme is anchored to the T-tubules through a glycosyl phosphatidylinositol group as described for other ecto-5'-nucleotidases

[21]. Under the same experimental conditions, no significant release of ecto-ATPase and ecto-ADPase activities were observed. Our results are in accord with observations on chromaffin cells in culture, where PI-PLC releases 5'-nucleotidase, but not ecto-ATPase or ecto-ADPase, activities from the cell surface [11]. Similarly, ecto-ATPase from bovine brain synaptic membranes is not a glycosyl phosphatidylinositol-anchored protein [22].

Native PAGE and activity staining

Digitonin-solubilized proteins from TT membranes contained three activities that hydrolysed ATP, ADP and AMP (Figure 6A). The staining was due to enzyme activities because the bands were not observed when incubation of the gels with the substrates was performed in the absence of Mg^{2+} . Similarly, the addition of 500 μM $p[CH_2]pA$ to the incubation medium for the detection of 5'-nucleotidase activity prevented the hydrolysis of 1 mM AMP and subsequent staining (results not shown). ATP- and ADP-hydrolysing activities migrated to the same position in the gel and this could be considered as evidence that the same enzyme is responsible for the hydrolysis of both substrates. However, the unexpected observation that the band corresponding to 5'-nucleotidase, an enzyme clearly distinct from ATPase and ADPase, was localized at an identical position raises doubts about this interpretation and suggests the possibility that ecto-ATPase, ecto-ADPase and 5'-nucleotidase might be associated in the TT membrane through interactions not disrupted by digitonin. When solubilization of T-tubules was performed in the presence of Con A, the enzymic activities were unable to penetrate into the resolving gel remaining at the same position (Figure 6B). It is conceivable that Con A induces cross-linking of the proteins, probably through binding to their carbohydrates, leading to the formation of high-molecular-mass aggregates. As would be expected, when TT membranes were treated with PI-PLC and the soluble proteins were subjected to electrophoresis under native conditions, only one band corresponding to 5'-nucleotidase was observed after staining for activity (Figure 6C). Interestingly, the mobility of the band was higher than that of digitonin-solubilized 5'-nucleotidase activity, probably reflecting the change in charge-to-mass ratio owing to separation of this enzyme from ATPase and ADPase.

DISCUSSION

In this study we demonstrate that TT vesicles purified from chicken skeletal muscle possess ectonucleotidase activities capable of hydrolysing ATP, ADP and AMP. The possibility that the nucleotides were degraded by the combined action of different enzymes has been also excluded because ATP pyrophosphatase, adenylate kinase, inorganic pyrophosphatase, alkaline phosphatase and non-specific phosphatase activities were negligible in the TT preparations under our assay conditions.

T-tubule ecto-ATPase activity shares the typical biochemical features reported for ecto-ATPases from a variety of cells and tissues [5,38,43]. These include (1) an alkaline pH optimum between 7.5 and 8.5, (2) activation by either Mg^{2+} or Ca^{2+} , (3) broad substrate specificity, (4) insensitivity to specific inhibitors of F-, P- and V-type ATPases, (5) sensitivity to detergents and (6) stimulation by Con A. The nomenclature 'E-type ATPase' has been proposed to describe the ecto-enzymes with properties similar to those exhibited by the TT ecto-ATPase [5]. The possibility that the ecto-ATPase also catalyses ADP hydrolysis seems unlikely considering the results obtained from the comparative study of the properties of ATPase and ADPase activities. Remarkable differences were found with respect to (1) thermal stability, (2) temperature dependence of hydrolytic activity, (3)

kinetic behaviour, (4) divalent cation preference, (5) effect of various inhibitors and activators and (6) response to a polyclonal antibody against the chicken TT Mg^{2+} -ATPase.

The mechanism of stimulation of the chicken TT ecto-ATPase by Con A is unclear but it has been suggested that this effect could be due to interaction of the lectin with a regulatory site of the ATPase [39] or to the formation of a stabilizing and activating TT ecto-ATPase-Con A cross-linked protein lattice [52]. Evidence supporting the latter suggestion comes from PAGE experiments under native conditions. The position and intensity of the band corresponding to the ecto-ATPase activity present in digitonin-solubilized proteins varied if TT membranes were incubated with Con A before solubilization. The heavier staining of the band, as well as its inability to migrate into the resolving gel, would be consistent with the formation of such activating lattices in which ADPase and 5'-nucleotidase could be trapped or included. Surprisingly, ecto-ADPase and 5'-nucleotidase activities were localized also at a position identical with that of ecto-ATPase although TT vesicles were not treated with Con A before solubilization. The possibility that the three enzymic activities reside on the same protein seems unreasonable and our results are best explained by assuming that ecto-ATPase, ecto-ADPase and 5'-nucleotidase are in close vicinity in the TT membrane, perhaps forming an oligomeric structure or functional complex that is not dissociated by non-denaturing detergents such as digitonin. Some experimental observations favour this hypothesis: (1) the molecular mass of the native solubilized ecto-ATPases has been estimated to be high by different methods [5], (2) it has been reported that immunoaffinity purification of ecto-ATPases from chicken oviduct and gizzard, rat liver and rat ductus deferens resulted in the co-isolation of other proteins [5,7] suggesting that ecto-ATPases tend to be part of functional complexes, (3) 5'-nucleotidase purified from chicken gizzard has been found to present ATP- and ADP-hydrolysing enzymic activities [53] that could be due to the ecto-ATPase and ecto-ADPase, indicating a tight association between these proteins. If the ecto-nucleotidases form oligomeric structures, caution must be taken in considering that the migration of ATPase and ADPase activities at the same position in gels under native conditions is a criterion indicating the existence of an apyrase in membrane preparations.

Taken together, our results indicate that ATP and ADP are hydrolysed in the chicken T-tubules by two distinct proteins. A number of studies have been performed with membrane preparations from different tissues and with several cell types to establish whether or not ecto-ATPases belong to the group of apyrases. From the results obtained with several experimental approaches, it has been suggested that the same catalytic protein is responsible for the hydrolysis of nucleoside triphosphates and diphosphates in kidney, chromaffin cells, lung, vascular and non-vascular smooth muscle, platelets, brain and pancreas [16,24-27,54,55]. However, in cardiac myocytes and endothelial cells, ecto-ATPase and ecto-ADPase activities seem to be due to separate enzymes, as indicated by kinetic and inhibition studies [14,17,47]. Studies with purified enzymes also provide conflicting results. The ecto-ATPases purified from rabbit T-tubules, bovine brain synaptosomes and rat heart sarcolemma do not hydrolyse ADP significantly [2,22,44], whereas the enzymes isolated from bovine aorta endothelial and smooth-muscle cells, rat liver, pig pancreas, chicken oviduct, human placenta and bovine spleen are reported to be apyrases [12,23,28,48,50,56]. It seems likely that there are two or more families of E-type ATPases as proposed by Plesner [5], one of which, the T-tubule subfamily, would be characterized by its inability to catalyse ADP hydrolysis.

The presence of 5'-nucleotidase in chicken TT vesicles along with ecto-ATPase and ecto-ADPase completes an enzymic cascade able to transform extracellular ATP into adenosine. The physiological role of this system has to be determined but two possibilities seem most likely. A potential source of extracellular ATP is the muscle fibre known to release ATP on electrical stimulation [57]. Because T-tubules constitute the major fraction of the total surface membranes, the enzymic cascade of ecto-nucleotidases could serve as an effective mechanism for protection of the working muscle against perturbations of membrane permeability induced by ATP. In contrast, ATP is also released from motor nerve terminals on stimulation of motor neurons [57] and it has been suggested that ATP could have a role in synaptic transmission at the skeletal neuromuscular junction. Extracellular ATP produces a variety of excitatory effects on ion channels of skeletal muscle fibres [9,10]. We have recently demonstrated the presence of P_2 purinoceptors in chicken TT membranes (G. Moro, unpublished work), which suggests the possible involvement of the TT ecto-nucleotidases in the control of purinergic signalling.

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