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Characterisation of Ca^{2+} or Mg^{2+}-dependent nucleoside triphosphatase from rat mesenteric small arteries

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When isolated rat mesenteric small arteries were submitted to 2 s of sonication, a nucleoside triphosphatase activity was released to the medium, mainly from the plasma membrane of the vascular smooth muscle cells. The activity was kinetically characterized: It hydrolysed ATP, UTP and GTP with the same substrate affinity and the same specific activity. CaATP, as well as MgATP were substrates for the enzyme with an apparent K_m in the micromolar range. ATPase inhibitors: ouabain, vanadate, AlF_4^-, oligomycin and *N*-ethylmaleimide were without effect on the hydrolytic activity. Among other modifiers tested only *N,N'*-dicyclohexylcarbodiimide caused significant (> 30%) inhibition. In the presence of micromolecular concentrations of Ca^{2+} and Mg^{2+}, small (< 20 mM) concentrations of Na^+, K^+, Rb^+, Cs^+ and choline$^+$, irrespective of the nature of the anion, activated the hydrolysis with an equilibrium ordered pattern, but concentrations of monovalent cation salts above 20 mM decreased the hydrolysis rate. No activation by monovalent cation salts was seen at millimolar concentrations of divalent cations and substrate. On the basis of the results a standard mixture is proposed, which allows a sensitive assay of the specific enzyme activity.

Introduction

In recent years a Ca^{2+}- or Mg^{2+}-stimulated ATPase, which is distinct from the plasma membrane Ca^{2+}-pump, has been described in plasma membrane preparations from a series of tissues: liver [1–8], granulocytes [9], kidney basal membrane [10], vascular smooth muscle cells [11], placenta [12], corpus luteum [13], myometrium [14], parotis [15], heart [16] and brain synaptosomes [17].

The major characteristics of the enzyme, as described in these studies, are:

(1) The enzyme is activated by submicromolar concentrations of Ca^{2+} or Mg^{2+} [1,3]. It is active with only endogenous Mg^{2+} present [8–11,17], millimolar concentrations of Mg^{2+} being inhibitory [3,8,10,11,13].

(2) Inhibitors of P-type ATPases (ouabain, vanadate, AlF_4^-), of the V-type ATPases (NEM, KNO_3, KSCN, NBD-Cl) and of the F-type ATPases (oligomycin, azide and mercurials) are without significant effect on the enzyme activity [3,7,8,13–15,17]. No specific inhibitor of the enzyme has yet been found.

(3) The enzyme shows a certain lack of substrate specificity, i.e. it hydrolyses ATP, UTP, GTP, CTP and the corresponding dinucleotides, but it does not hydrolyse mononucleotides and *p*NPP [3,7–10,15].

The name '(Ca^{2+}-Mg^{2+})-nucleotidase' was proposed [3], however, as the enzyme does not hydrolyse mononucleotides, a more appropriate name would probably be nucleoside triphosphatase (EC 3.6.1.15) and in the following the enzyme will be referred to as Ca^{2+} or Mg^{2+}-dependent NTPase.

In various tissues an *ecto*-ATPase activity has been described with characteristics quite similar to the char-

Abbreviations: CDTA, *trans*-1,2-diaminocyclohexanetetraacetic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; EDTA, ethylenedinitrilotetraacetic acid; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; NTPase, nucleoside triphosphatase (EC 3.6.1.15); PCMB, *p*-chloromercuribenzoate; *p*NPP, *para*-nitrophenyl phosphate.

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acteristics of the Ca^{2+} or Mg^{2+}-dependent NTPase [18,19], and lately strong evidence has been presented that the liver enzyme is an ecto-enzyme [4].

With the increasing interest in the physiological transmitter or co-transmitter function of nucleotides (particularly ATP, ADP, AMP) and adenosine [20–23], it was suggested that the function of the Ca^{2+} or Mg^{2+}-dependent NTPase would be to hydrolyze extracellular ATP and ADP, which are ligands for P_2-purinoceptors, to terminate the stimulation, or to act with 5'-nucleotidase in the formation of adenosine, which may either interact with its receptor (P_1) on the cell surface or be recaptured into the cell by nucleoside transporters [6]. Other proposed functions are: proton transport [7], and protection from the cytolytic effects of extracellular ATP [24].

The amino acid sequence of Ca^{2+} or Mg^{2+}-dependent ecto-NTPase from rat liver plasma membrane has been deduced from the cloning and expression of a cDNA coding of the system [6]. A comparison of the primary structure of rat liver ecto-NTPase with that of human biliary glycoprotein 1 (BGP1) [25] showed high homology, with 65% of the amino acids being identical [6].

Based on the published amino acid sequence [6] the protein was found to be a substrate for the tyrosine kinase activity of the insulin receptor [26], and identical to the cell adhesion molecule Cell-CAM 105, which is a number of the immunoglobulin superfamily [27]. This suggests yet another function for this system which may play a role in ATP-regulated cell-adhesion [27].

We have characterized the effect of extracellular nucleotides in rat mesenteric small arteries and found P_2-receptors mediating a depolarisation and a contraction (Juul, B., Plesner, L. and Aalkjær, C. (1991), submitted). Rat mesenteric small arteries are well characterized electrophysiologically, and it has been suggested that the force response to nerve stimulation could in part be mediated by ATP-receptors in the vessels [28]. These results are in agreement with perfusion experiments in the rat mesenteric bed [22].

On this background it was of interest to study the fate of extracellular nucleotides in this tissue, and in the subsequent paper [29] a study of the nucleotide hydrolysing activity of the intact isolated mesenteric small artery is presented.

In the present paper a nucleoside triphosphatase activity is characterized which is released to the medium upon sonication of the vessel for 2 s. The aim of this investigation was (1) to clarify the origin of this enzyme, (2) to elucidate if the enzyme showed the characteristic features described above for the Ca^{2+} or Mg^{2+}-dependent ecto-NTPase, (3) to define non-saturating conditions with respect to substrates and ligands for inhibitor studies, and (4) to deduce optimal conditions for a sensitive standard assay that would yield near maximal activity and be specific for this particular enzyme.

Materials and Methods

Enzyme source. Male Wistar rats (200–400 g) of our own colony were killed by a blow on the head. The mesenterium was immediately taken out and placed in modified Krebs-Ringer-bicarbonate solution [29], where 2nd to 4th order branches of the superior mesenteric artery with an average internal diameter of 0.25 mm were dissected out [30]. After careful removal of fat and connective tissue the vessels were cut into segments of 5 to 15 mm. Each segment was measured and transferred to a tube containing 0.3 ml of histidine buffer (30 mM, pH 7.4). The tube was immersed in an ice bath, and the content submitted to 2 s of sonication in a 150 watt ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., London U.K.) at maximal setting (> 30 μm). This treatment left the vessels macroscopically undamaged. The vessel was discarded, and the remaining solution, containing < 10% of the total protein of the vessel, was used as the enzyme source after dilution of at least 10 times.

Enzyme activity. Hydrolysis of [γ-^{32}P]ATP, [γ-^{32}P]UTP and [γ-^{32}P]GTP was measured in a final volume of 500 μl in a histidine buffer, 30 mM, pH 7.4 at 37 °C. Assay temperature was 37 °C and assay time 10 or 30 min. The assay was started by adding either 50 μl of the diluted enzyme source or (for the inhibitor studies) by adding 50 μl of substrate solution. The protein concentration of the assay was between 0.01 and 0.3 μg per ml. It was confirmed that the nucleotide hydrolysis rate was constant within assay time and the amount of ATP hydrolysed was always less than 10% of total ATP. The specific activity of the nucleotides was between 2000 and 20000 Bq per nanomole. [γ-^{32}P]P_i release was measured according to Ref. 31. Hydrolysis of ADP was determined under the same conditions, but P_i-release was measured according to Ref. 32. A blank was run for each assay tube, as the spontaneous hydrolysis of the nucleotides was found to depend significantly on the concentration of monovalent cation salts. In some experiments a range of Ca^{2+} and Mg^{2+} concentrations were established using CDTA as described in Refs. 1 and 33 based on experimental values determined by Ref. 34.

Protein. Protein was measured with the Coomasie brillant blue technique [35]. As standard we used bovine albumine (Sigma).

Functional removal of endothelium. Functional removal of endothelium was done by carefully rubbing the lumen of the vessel with a 40 μm stainless steel wire [36]. After this procedure the relaxation response of a preconstricted vessel to 1 μM acetylcholine which is dependent on an intact endothelium [37] disappears.

Electron microscopy. Untreated as well as sonicated vessels kept in either modified Krebs-Ringer-bicarbonate solution [29] or in histidine buffer (30 mM, pH 7.4) were immersion-fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at least overnight at 4 °C. After postfixation in 1% osmium tetraoxide in the same buffer (pH 7.4) for 0.5 h, the specimens were stained en bloc for 1 h with 0.5% uranyl acetate in 0.05 M maleate buffer at pH 5.2, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and analyzed in a Jeol 100 CX electron microscope at 80 kV.

Chemicals. Sodium vanadate was boiled for 10 min before use to avoid polymerisation. $NaVO_3$, KNO_3, NaN_3, and theophylline were from Merck, oligomycin A, NEM, ethacrynic acid, furosemide, PCMB, DCCD, from Sigma, NBD-Cl and ouabain from Serva, amiloride from MSD, $HgSO_4$ from Baker, and trinucleotides as well as ADP and AMP were from Boehringer. Radionucleotides [γ-^{32}P]ATP, [γ-^{32}P]UTP and [γ-^{32}P]GTP were from Amersham.

Results and Discussion

1. Enzyme source

5–15 mm segments of branches of rat mesenteric artery were found to have an average wet-weight of 67 ± 9 (S.E.) ($n = 5$) μg/mm. After sonication for 2 s the vessels were macroscopically intact. Electron microscopy (Fig. 1) revealed that the endothelial cells of tunica intima were severely disintegrated in sonicated vessels (Fig. 1b), but the smooth muscle cells of tunica media and the structure of the well-defined internal elastic lamina seemed not be be damaged by sonication. The thickness of the tunica adventitia varied from preparation to preparation without a clear correlation between the amount of connective tissue and sonication.

After sonication the vessel was discarded. The enzyme source was the remaining dilute solution of substances liberated from the tissue by the sonication. It contained an NTPase with a specific activity of 1.32 ± 0.02 (S.E.) ($n = 23$) μmol/mg protein per min, when measured at 37 °C as [γ-^{32}P]ATP hydrolysis in a 30 mM histidine buffer (pH 7.4), with NaCl = 20 mM and with (μM concentrations) [ATP] = 50, [$CaCl_2$] = 50, [$MgCl_2$] = 2. The amount of protein liberated amounted to an average of 0.63 ± 0.03 (S.E.) ($n = 23$) μg/mm of vessel. The same specific activity was obtained under these conditions in the simultaneous presence of 1 mM ouabain, 50 μM vanadate, 6 μM oligomycin, and 500 μM NEM (Fig. 7). This is the standard assay mixture proposed at the end of the Results section.

As the endothelial cells were found to be totally disrupted by the treatment with ultrasound (Fig. 1), it seemed possible that the NTPase originated mainly from this tissue. This suggestion was, however, not supported by the result of the following experiment: When the endothelium was removed (by careful rubbing of the inside of the lumen, see Methods) prior to sonication of the vessel, the specific activity of the protein, liberated by sonication, was found to be 1.49 ± 0.22 (S.E.) ($n = 10$) μmol/mg per min. This result does not differ significantly from the value obtained for vessels with intact endothelium (see above), and the NTPase activity therefore does not seem to be concentrated in the endothelium.

The specific activity of the enzyme source (1.32 μmol/mg per min, see above) is comparable to the specific activity reported for membrane fractions of a series of tissues [8,9–12,14,15,17]. Though 15 times less pure than what is considered to be a pure preparation of the Ca^{2+} or Mg^{2+}-dependent NTPase of the hepatocyte plasma membrane [1], it was found to be satisfactory for characterisation of the system. Based (1) on the electron-microscopic information (Fig. 1), and (2) on the endothelium-independent nucleoside triphosphate hydrolysing activity of the intact vessel described in the subsequent paper [29], it is suggested that the NTPase studied in the enzyme source is derived mainly from the plasma membrane of the vascular smooth muscle cells of the vessel.

2. Effect of Ca^{2+} and Mg^{2+}

Divalent cations are necessary for hydrolytic activity: In experiments where the concentrations of ATP and monovalent cations were varied, no ATP hydrolysis could be measured in the presence of 82 μm EDTA (results not shown). This concentration of EDTA was chosen as it titrates the histidine buffer to pH 7.4, (i.e., assay pH was obtained without the addition of monovalent ions), and it was sufficient to prevent ATP-hydrolysis by the system.

The ability of Ca^{2+} to initiate activity in the virtual absence of Mg^{2+} and vice versa, was investigated in the experiments shown in Fig. 2, where the chelator CDTA, which has a high affinity for both ions, was used to control their concentrations [33,1]. In the presence of 1 mM of CDTA (total) the results indicated by squares were obtained by varying [ATP_{tot}] and [Mg^{2+}_{tot}] to given the indicated concentrations of MgATP and a free Mg^{2+} concentration of 10 μM. The line indicated by circles was obtained by varying [ATP_{tot}] and [Ca^{2+}_{tot}] to give the indicated concentrations of CaATP and a free Ca^{2+} concentration of 10 μM. Apparently CaATP and MgATP may both serve as substrates. It should be noted, however, that the Mg^{2+}- and the Ca^{2+}-stimulated hydrolytic activities are not comparable in experiments like those shown in Fig. 2, where the necessary total concentration of ATP in the presence of Ca^{2+} is 4 times larger than the necessary total concentration of

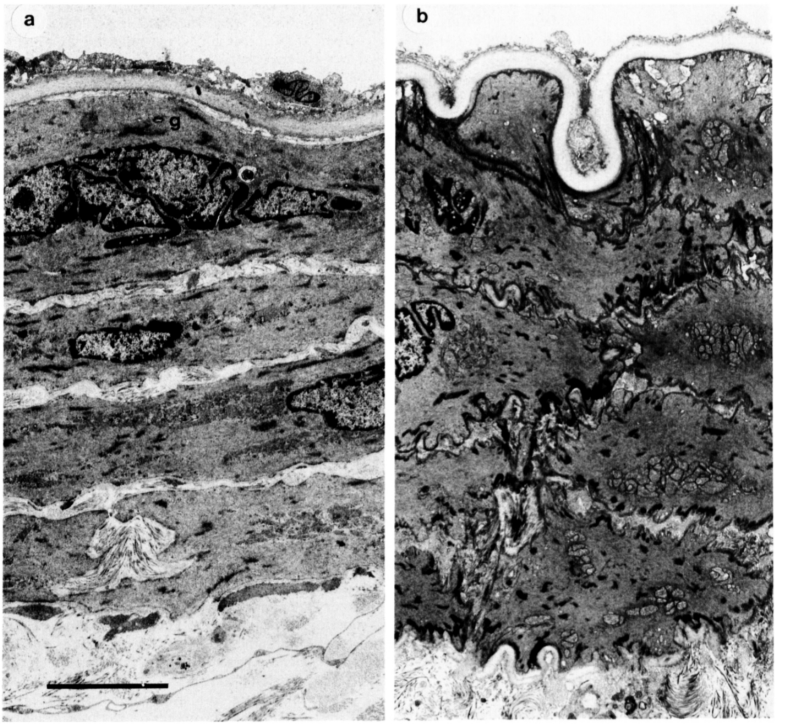


Fig. 1. Transmission electron micrographs of mesenteric small arteries in PSS. (a) Nonsonicated and (b) sonicated. The endothelial cells in tunica intima are severely damaged in the sonicated vessel. A well defined internal elastic lamina is present in both arteries and due to more contracted smooth muscle cells it appears more folded in (b). Tunica media and tunica adventitia were not changed significantly by sonication. Bar indicates 5 μm.

ATP in the presence of Mg^{2+}. The apparent Michaelis constants (2.5 μM for CaATP and 10 μM for MgATP) and the apparent maximal rates, therefore, are not comparable either.

An estimate of maximal velocities under comparable conditions would elucidate whether the enzyme source contains 2 ATPases, one which has CaATP, and one that has MgATP as a substrate. If this were the case, then in the presence of both ions, Ca^{2+} and Mg^{2+}, the sum of the activities of two enzymes should be seen, i.e., the maximal velocity obtained with both ions present should exceed the maximal velocity obtained with only one ion present. In Fig. 3 are shown the results of an experiment where no chelator was present, and where the concentration of free divalent cations was low, i.e., the concentrations of MgATP or CaATP were small compared to the free ATP-concentrations, and therefore $[ATP_{total}] \approx [ATP_{free}]$. The reciprocal free ATP concentration was used as abscissa and four substrate curves were compared, one with only endogenous divalent cation present, one with $[Mg^{2+}_{free}] = 2$ μM, one with $[Ca^{2+}_{free}] = 2$ μM and one with $[Mg^{2+}_{free}] =$

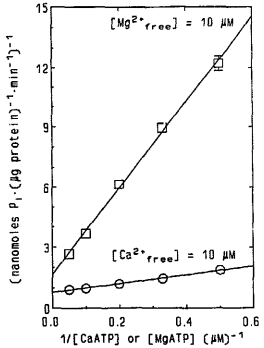


Fig. 2. Double-reciprocal plots of $1/v$ vs. $[CaATP]^{-1}$ (○) and $[MgATP]^{-1}$ (□). The ATP-hydrolysis rate, v, is given as nanomoles of [γ-^{32}P]ATP hydrolysed (= $[^{32}P]P_i$ released) per min per μg of protein in the enzyme source. $[CDTA_{tot}]$ was 1 mM. $[ATP_{tot}]$, $[Ca^{2+}_{tot}]$ and $[Mg^{2+}_{tot}]$ were varied to yield the concentrations given in the figure. For calculations were used the following dissociation constants (see Methods): K_{diss} (CaCDTA) = 0.132 μM, K_{diss} (CaATP) = 125 μM, K_{diss} (MgCDTA) = 0.445 μM and K_{diss} (MgATP) = 26 μM. The points represent the mean of three experiments. The length of the bars indicates 2 S.E. The lines are least-squares regression lines. Assay conditions, buffer, temperature and assay time, see Methods.

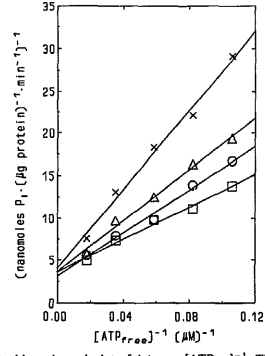


Fig. 3. Double-reciprocal plot of $1/v$ vs. $[ATP_{free}]^{-1}$. The results were obtained in an experiment with no chelator and $[Ca^{2+}_{free}] = 2$ μM (○ and □) and $[Mg^{2+}_{free}] = 2$ μM (△ and □). No cations added (×). The lines are least-squares regression lines.

$[Ca^{2+}_{free}] = 2$ μM. Though the MgATP concentrations are 5 times higher than the CaATP concentrations, in the double-reciprocal plots the intersection on the ordinate still represents the reciprocal of comparable maximal velocities. Thus, the fact that double reciprocal plots of the substrate curves obtained seem (within the experimental error) to intersect in the same point on the ordinate, indicate that most probably the hydrolysis of CaATP and of MgATP are catalyzed by the same enzyme.

The interpretation, that Ca^{2+} and Mg^{2+} ions both activate the same enzyme is further supported by the results presented in Table I and Table II: In the presence of inhibitors and various anions, the proportional activation obtained by Ca^{2+}, Mg^{2+} and Ca^{2+} + Mg^{2+}, respectively, remains constant.

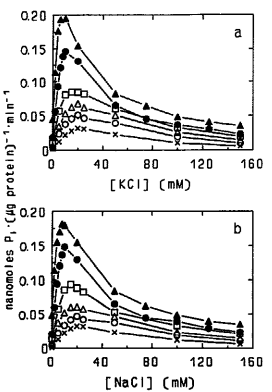
It is noteworthy that in the absence (Fig. 3) and in the presence (Fig. 7) of monovalent cation salts, the activation obtained by increasing the concentration of Ca^{2+} was different: In Fig. 3 only the slope, and in Fig. 7 only the ordinate intercept of the double-reciprocal plot was affected, indicating that divalent cations are necessary for substrate binding in the absence of monovalent cation salts (Fig. 3), whereas in the presence of monovalent cations salts (Fig. 7) Ca^{2+}-activation was caused by acceleration of a step subsequent to substrate addition [41].

3. Effect of monovalent cations and of various anions

In the presence of only endogenous divalent cations, increasing NaCl (Fig. 4a) and KCl (Fig. 4b) caused an increase in the ATP hydrolysis rate with maximum at all substrate concentrations between 10 and 20 mM concentration of the salts. Further increasing the concentration of the salts decreased the activity.

A double-reciprocal presentation ($1/v$ vs. $1/$substrate) of the results is given in Fig. 4c and 4d. The equilibrium ordered pattern [38] obtained for the activation caused by small concentrations of the salts (Fig. 4c) is identical to the pattern obtained with divalent cations in the absence of monovalent cation salts. It indicates that the activator has to bind to the enzyme prior to the binding of substrate, and that it is trapped on the enzyme until the reaction cycle is complete [38]. Increasing the salt concentration from 50 mM to 150 mM caused inhibition, the effect on the slope (substrate binding [38]) being much larger than the effect on the apparent V_{max} (Fig. 4d).

It is evident from the data presented in Fig. 4 that the effects of NaCl and KCl were identical. In Fig. 5 the effect of these salts was compared to the effects of the chloride salts of rubidium (Fig. 5a) and of choline, cesium and lithium (Fig. 5b) in the concentration range below 10 mM. The activation obtained with LiCl was different from and weaker than the activation obtained



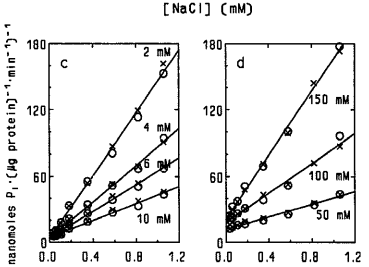


Fig. 4. ATP hydrolysis rate vs. the concentration of KCl (a) and NaCl (b). ATP concentrations were 0.94 μM (×), 1.70 μM (○), 2.83 μM (△), 5.67 μM (□), 9.44 μM (●), and 28.3 μM (▲). No Ca^{2+} and Mg^{2+} was added. No chelator was present. The points represent individual measurements, and the lines are drawn to connect successive points. In (c) and (d) the data given in (a) and (b) are replotted in double-reciprocal form. ○, KCl; ×, NaCl. (c) represents the lower NaCl and KCl concentrations, (d) represents the higher NaCl and KCl concentrations.

with the chloride salts of the other monovalent cations, which seemed to give rise to identical activation.

With the aim of quantitating the effect of the chloride salts of the monovalent cations (except lithium) the Michaelis constant (K_m) was derived from a series of substrate curves like those shown in Fig. 5a and 5b. The results are presented in Fig. 6.

Based on the equilibrium ordered activation pattern, an activation constant (K_a), may be derived for

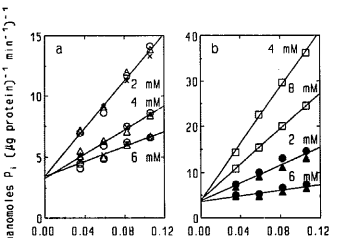


Fig. 5. Double-reciprocal plots of $1/v$ vs. $1/ATP$ in the presence of monovalent cation salts. (a): NaCl (x), KCl (○), RbCl (△) and (b): CsCl (▲), choline Cl (●), LiCl (□) in the concentrations indicated in the figure. Note: different ordinates in (a) and (b). Other conditions (except the substrate concentration range) were the same as in Fig. 4. The points represent individual measurements and the lines are least-squares regression lines.

the activation caused by the chloride salts of the monovalent cations [38], using the linear regression analysis of the data in Fig. 6 (see legend)

$$K'_m = K^0_m\left(1+\frac{K_a}{[\text{salt}]}\right) = 1.16+46.5\cdot[\text{salt}]^{-1}$$

$K_a \approx 40$ mM, $K^0_m \approx 1.16$ μM

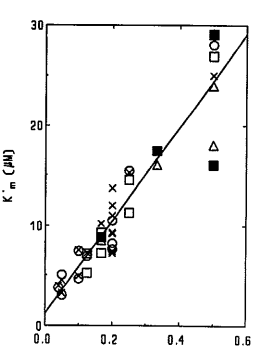


Fig. 6. K'_m vs. reciprocal concentration of monovalent cation salt. Each point represents the calculated value for K'_m obtained from an individual substrate curve (v vs. ATP) in the presence of NaCl (x), KCl (○), choline Cl (△), RbCl (□) and CsCl (■). The least-squares regression line is given by $K'_m = 1.16+46.5\cdot[\text{salt}]^{-1}$, $r = 0.941$.

This large activation constant is consistent with the strong activation obtained with low salt concentrations under these conditions.

The apparent lack of specificity towards monovalent cations could be explained if the active ligand was not the cation, but the anion. But the lithium results are not in accordance with this explanation, and in Table II the results of a series of experiments are given where the effect of changing the anions was tested. It is evident that, if the anions have an effect, it is no more specific than is the effect of the monovalent cations. Increasing ionic strength may be responsible for the inhibitory action of high concentrations of salts, but ionic strength effects can not account for the strong activation exerted by low concentrations.

It should be emphasized, as noted above, that the activity was dependent on endogenous divalent ions, as the presence in the assay of 82 μM of EDTA would completely prevent ATP hydrolysis at all salt concentrations. Also, in the presence of 1 mM of $MgCl_2$, the monovalent cation salts inhibit the activity at micromolar concentrations of ATP, whereas they do not affect the activity in the presence of millimolar concentrations of both $MgCl_2$ and ATP (results not shown). Insensitivity to NaCl and KCl under the latter conditions was reported previously [15–17,19], but to our knowledge, the observed strong activation by low concentrations of salts when the concentration of divalent cations are in the micromolar range has not been reported before. The activation seems to have important implications for the planning and interpretation of certain experiments (see Table II and Table III and Section 5 below) but the importance for the elucidation of the function of the system is more difficult to evaluate.

4. Substrates

UTP and GTP were hydrolyzed by the enzyme source, and measuring the hydrolysis rate as a function of UTP and GTP concentrations at varying concentrations of NaCl, substrate curves were obtained that could be compared to those obtained with ATP, Figs. 4c and 5a + 5b. There was no significant difference in the maximal velocity obtained with, and the affinity towards, the three substrates, ATP, UTP and GTP. Also the affinity for the three substrates showed the same dependence on the concentration of monovalent cation salt (Fig. 6).

Dinucleotide and mononucleotide hydrolysis by the enzyme source could be determined only by colorimetric measurements of released inorganic phosphate, therefore hydrolysis was not measurable in the micromolar range where trinucleotide substrate kinetics was studied. However, at 1 mM of ATP, UTP, GTP, ADP and AMP, the hydrolysis rates of the trinucleotides, dinucleotide and mononucleotide were 0.811 ± 0.018 (S.E.) ($n = 6$), 0.899 ± 0.049 (S.E.) ($n = 4$) and 0.034 ± 0.001 (S.E.) ($n = 3$) nmol of P_i liberated per min per μg of protein, respectively, when measured in the standard assay mixture proposed at the end of this paper.

5. Inhibitors

On the basis of the results reported above it was possible to choose non saturating assay conditions for experiments designed to reveal whether certain inhibitors of established specificity (see Introduction and Ref. 39) would influence the NTPase activity: [ATP] < 10 μM, [Na^+] < 20 mM, [Mg^{2+}] and [Ca^{2+}] < 50 μM.

The results of these experiments are shown in Tables I, II and III.

TABLE I

Activation by Ca^{2+} and Mg^{2+} in the presence of vanadate and oligomycin

The ATP hydrolysis rate (nmol P_i per μg protein per min) was measured as described in Methods. The reaction was started with ATP, final concentration 9.4 μM, after 10 min of preincubation of the enzyme with the ligands at room temperature. Assay time was 30 min. With vanadate and oligomycin the results represent the average of duplicate and triplicate determinations, respectively.

Ca^{2+} (μM)	Mg^{2+} (μM)	NaCl (mM)	ATP hydrolysis rate				
			No additions			Vanadate (50 μM)	Oligomycin (6 μM)
			x	S.E.	n		
0	0	0	0.018	0.002	7	0.022	0.022
2	0	0	0.031	0.003	7	0.033	0.035
0	2	0	0.027	0.004	7	0.031	0.031
2	2	0	0.038	0.005	7	0.042	0.039
0	0	5	0.125	0.016	6	0.143	0.120
2	0	5	0.155	0.017	6	0.156	0.151
0	2	5	0.140	0.016	6	0.144	0.140
2	2	5	0.163	0.017	6	0.165	0.159

TABLE II

Activation by monovalent cation salts

Assay conditions were the same as in Table I. The ATP hydrolysis rate measured in the absence of monovalent cation salts under each set of conditions ($Ca^{2+} = Mg^{2+} = 0$ or $Ca^{2+} = 2$ μM or $Mg^{2+} = 2$ μM or $Ca^{2+} = Mg^{2+} = 2$ μM) was set to 100%. The results are presented as percentage activation obtained adding the salts in the concentrations given below. With Na_2SO_4, NaN_3, KSCN and KNO_3 the results represent the average of duplicate determinations.

Ca^{2+} (μM)	Mg^{2+} (μM)	NaCl (5 mM)			Na_2SO_4 (2.5 mM)	NaN_3 (5 mM)	KSCN (5 mM)	KNO_3 (5 mM)
		x	S.E.	*n*				
0	0	650	14	9	619	636	677	688
2	0	466	11	10	453	495	483	488
0	2	517	26	6	523	502		
2	2	413	9	6	427	420		

Vanadate and oligomycin did not inhibit the enzyme (Table I). The effects of NaN_3, KSCN and KNO_3 (Table II) were identical to that of NaCl and Na_2SO_4, i.e., activation. Testing the effect of inhibitors in the absence of NaCl (Table III, columns 1 and 2) was found to be impossible, as the addition of several inhibitors caused activation, due to the salt effect. Only when a 'salt buffer' was included in the assay ([NaCl] = 5 mM, Table III, columns 3 and 4) the activation disappeared. Of the modifiers tested, however, only the unspecific modifier DCCD (0.5 mM) caused significant inhibition. NEM (0.5 mM), theophylline (2.5 mM) and bumetanide (0.1 mM) were without effect, whereas furosemide (2.0 mM), ethacrynic acid (1.5 mM), PCMB (0.1 mM), $HgSO_4$ (0.1 mM) and NBD-Cl (0.1 mM) probably decreased the hydrolysis rate, but in all cases the inhibition was less than 30%.

The effect of fluoroaluminate [40] was tested in the presence of $[Na^+] = 20$ mM, $[Mg^{2+}] = 2$ μM and $[Ca^{2+}] = 2$ or 20 μM. Substrate curves were run under these conditions adding 10 mM of NaF, or 10 μM of $AlCl_3$ or 10 mM of NaF + 10 μM of $AlCl_3$. 10 mM of NaF caused a slight inhibition corresponding to the inhibition that would be obtained by increasing the concentration of NaCl by 10 mM, see Figs. 7b and 7c. In the presence and in the absence of NaF, the addition of $AlCl_3$ was without effect (results not shown).

With an enzyme source that is not pure, inhibitor studies are of two kinds. One kind is described above: Non-saturated conditions with respect to substrate and activators are defined, as an effect observed under these conditions is most likely to be an effect of the modifier on the enzyme studied. The other kind of inhibitor studies are performed with the purpose to reveal contamination of the enzyme preparation with other ATPases. To provide this kind of information the effect of modifiers should be tested under conditions that will allow these other enzymes to work. To establish for instance the presence of contaminating Na,K-ATPase, ouabain inhibition should be studied under assay conditions that meet the requirements of this enzyme which exhibit maximal velocity at $[Na^+] = 150$

TABLE III

Effect of inhibitors

Assay conditions were the same as in Table I and II. The ATP hydrolysis rate measured in the absence of inhibitors under each set of conditions ($Ca^{2+} = NaCl = 0$ or $Ca^{2+} = 2$ μM or NaCl = 5 mM or NaCl = 5 mM + $Ca^{2+} = 2$ μM) was set to 100%. The results are given in percentage and represent the average of duplicate determinations.

		ATP hydrolysis rate (relative percentage)			
		NaCl = 0		NaCl = 5 mM	
		$Ca^{2+} = 0$	$Ca^{2+} = 2$ μM	$Ca^{2+} = 0$	$Ca^{2+} = 2$ μM
NEM	0.5 mM	111	93	101	100
Theophylline	2.5 mM	137	118	92	89
Bumetanide	0.1 mM	119	103	97	98
Furosemide	2.0 mM	268	202	92	85
Ethacrynic acid	1.5 mM	216	142	73	71
PCMB	0.1 mM	113	73	80	71
$HgSO_4$	0.1 mM	100	85	89	83
HBD-Cl	0.1 mM	87	70	72	70
DCCD	0.5 mM	78	67	32	30

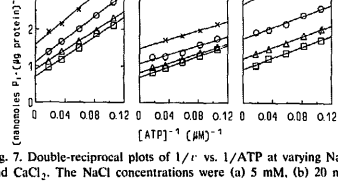


Fig. 7. Double-reciprocal plots of $1/r$ vs. $1/ATP$ at varying NaCl and $CaCl_2$. The NaCl concentrations were (a) 5 mM, (b) 20 mM and (c) 100 mM, and the $CaCl_2$ concentrations were 2 μM (x), 10 μM (○), 20 μM (△) and 50 μM (□). The assay medium contained the buffer used throughout (histidine 30 mM, pH 7.4 at 37 °C) and in addition $MgCl_2$ 2 μM, vanadate 50 μM, oligomycin 6 μM, NEM 500 μM and ouabain 1 mM. The lines are least-squares regression lines.

mM, $[K^+] = 20$ mM, $[ATP] = [Mg^{2+}] = 3$ mM. But under these conditions the NTPase activity is inhibited. Therefore, it is not possible to obtain useful information on the percentage composition of an impure enzyme preparation in this way. However, testing the effect of ouabain (1 mM) under a series of different assay conditions, the maximal inhibition obtained was 7% (result not shown).

6. Optimal assay conditions

In order to establish conditions that would allow a measurement of the ATP-hydrolysis rate to be a quantitative measurement of the Ca^{2+} or Mg^{2+}-dependent NTPase, the experiments presented in Fig. 7 were performed. In the presence of ouabain (1 mM), NEM (0.5 mM), vanadate (50 μM) and oligomycin (6 μM) to exclude interference from other ATPases, the concerted effect on the substrate curve of [NaCl] and $[Ca^{2+}]$ was studied. The concentration of added Mg^{2+} was 2 μM throughout. As mentioned above, the kinetic pattern of the effects of Ca^{2+} in the presence of NaCl is quite different from that obtained in the absence of salts (Fig. 3).

From experiments like those shown in Fig. 7, the standard conditions for assay of the Ca^{2+} or Mg^{2+}-dependent NTPase was chosen to be $[ATP] = 50$ μM, $[Ca^{2+}] = 50$ μM, $[Mg^{2+}] = 2$ μM and $[NaCl] = 20$ mM (total concentrations). The inhibitors, in the concentrations given above, were included in the standard assay. Their presence did not affect the activity of the enzyme source at hand, as discussed above in the beginning of the Results section, but with the inhibitors present in the standard assay, it may safely be used also in other preparations. It is estimated that the assay measures 80% of the maximal velocity (Fig. 7b), and it did not seem necessary to bring this figure closer to 100% by raising the ATP concentration, particularly because in many cases it is preferable to work with a rather high specific activity of the substrate. If one unit of enzyme (U) is defined as the quantity that will (at 37 °C) hydrolyze 1 μmol of nucleotide per min, then the assay suggested will measure 10^{-6} U using $4 \cdot 10^4$ Bq or 1.0 μCi of radioactive nucleotide per assay, assay time 15 min, this probably is at least 3 orders of magnitude less than may be measured in the conventional colorimetric assay.

Conclusion

This paper reports the release of a nucleoside triphosphatase activity following sonication of small arteries for 2 s, and evidence is presented that the enzyme (1) originates mainly from the plasma membranes of the smooth muscle cells of the tissue, and (2) exhibits the characteristic properties of the Ca^{2+} or Mg^{2+}-dependent NTPase. When the concentration of divalent cations was in the micromolar range, (3) a strong influence of monovalent cation salts was revealed and taken into account to establish (4) optimal conditions for the assay of potential inhibitors and (5) optimal conditions for a sensitive assay of the specific activity of the enzyme.

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