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Characterization of an ATP diphosphohydrolase activity (APYRASE, EC 3.6.1.5) in rat blood platelets

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Abstract

In the present report we describe an apyrase (ATP diphosphohydrolase, EC 3.6.1.5) in rat blood platelets. The enzyme hydrolyses almost identically quite different nucleoside di- and triphosphates. The calcium dependence and pH requirement were the same for the hydrolysis of ATP and ADP and the apparent Km values were similar for both Ca^{2+}-ATP and Ca^{2+}-ADP as substrates. Ca^{2+}-ATP and Ca^{2+}-ADP hydrolysis could not be attributed to the combined action of different enzymes because adenylate kinase, inorganic pyrophosphatase and nonspecific phosphatases were not detected under our assay conditions. The Ca^{2+}-ATPase and Ca^{2+}-ADPase activity was insensitive to ATPase, adenylate kinase and alkaline phosphatase classical inhibitors, thus excluding these enzymes as contaminants. The results demonstrate that rat blood platelets contain an ATP diphosphohydrolase involved in the hydrolysis of ATP and ADP which are vasoactive and platelet active adenine nucleotides. (Mol Cell Biochem **129:** 47–55, 1993).

Key words: ATP diphosphohydrolase, apyrase, platelets, rat blood

Introduction

APYRASE (ATP diphosphohydrolase, EC 3.6.1.5) is the name proposed by Meyerhof [1] for enzymes that hydrolyse ATP, ADP and other triphospho- and diphosphonucleosides to their equivalent monophosphonucleosides and inorganic phosphate. The enzyme apyrase has been reported in a) plant tissues [2, 3], b) insects [4–7], c) electric ray [8] and d) in various mammalian tissues [9] such as synaptosomes [10, 11], bovine aorta, endothelial and smooth muscle cells [12], rat placental tissue [13], porcine pancreas [14, 15] and bovine spleen [16].

Adenosine diphosphate (ADP) is a nucleotide known to induce changes in platelet shape and aggregation, to promote the exposure of fibrinogen binding sites and to inhibit stimulated adenylate cyclase [17], while adenosine triphosphate (ATP) competitively inhibits ADP-induced platelet aggregation [18]. Several authors have described the important role of these nucleotides in the process of haemostasis and thrombus formation [13, 18–20]. Adenosine diphosphatase (ADPase) activity has been studied in different types of blood cells and an ADP phosphohydrolase was demonstrated in plasma

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[21]. Neutrophils [22], B lymphocytes [23] and erythrocytes [24] have been shown to possess ADPase activity on the external surface of their plasma membrane. With respect to platelets, an ecto-ATPase has been described in human blood [25] but this enzyme is not a classical ATPase because it hydrolyses substrates other than ATP in the presence of calcium or magnesium. The fact that this enzymatic activity was not inhibited by some ATPase inhibitors and was competitively inhibited by ADP represents preliminary evidence that this platelet 'ecto-ATPase' probably is an ecto-apyrase.

In this paper we report the characterization of an ecto-apyrase activity in intact rat blood platelets and postulate that ATP and ADP degradation to adenosine in the bloodstream is mediated by conjugated activities of an apyrase and a 5′-nucleotidase (EC 3.1.3.5). Burnstock [26] has postulated from pharmacological experiments the concerted action of apyrases and 5′-nucleotidases.

Materials and methods

Chemicals

Nucleotides, ouabain, oligomycin, sodium azide, Ap5A [P_1P_5-di(adenosine-5′)-pentophosphate], DNP (dinitrophenol), orthovanadate, NEM (N-ethylmaleimide), lanthanum, hexokinase and glucose-6-phosphate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sepharose 2B gel was obtained from Pharmacia and was de-aerated in a vacuum flask before packing in a polyethylene column. All other reagents were of analytical grade. Polyethylene or siliconized labware was used for all platelet isolation and incubation procedures.

Platelet isolation

Male Wistar rats from our own breeding stock were maintained on a 12-h light/12-h dark cycle in a constant temperature room.

Platelets were isolated exactly as described by Hantgan [27]. In an effort to obtain preparations of normal, undamaged platelets free of non-adsorbed plasma constituents, we separated intact platelets from plasma by means of gel filtration on a 1.5 × 7 cm Sepharose 2B column [28]. The column was equilibrated with a buffer consisting of 130 mM NaCl, 2.5 mM KCl, 0.42 mM NaH_2PO_4, 10 mM HEPES, 5.5 mM dextrose, 0.2 mM EGTA, 0.05 g% azide and 0.2 g% bovine serum albumin, pH 6.8 (Ca^{2+}-free Tyrode's buffer). Platelets were eluted with the same buffer at room temperature; 0.5 ml fractions were collected and the two tubes containing the maximum platelet count (determined visually) were used for subsequent experiments. The material was prepared fresh daily.

Assay of ATP diphosphohydrolase activity (EC 3.6.1.5)

Unless otherwise stated, the reaction medium used to assay the Ca^{2+}-ATPase and Ca^{2+}-ADPase activity contained 120 mM NaCl, 5.0 mM KCl, 60 mM glucose, 5 mM $CaCl_2$, 50 mM Tris-HCl buffer, pH 7.5, in a final volume of 200 µl. A 20 µl sample of platelet preparation (20–30 µg of protein) was added to the reaction medium and preincubated for 10 min at 37° C. The enzyme reaction was started by the addition of ATP or ADP to a final concentration of 0.5 mM. Incubation times were chosen to ensure the linearity of the reaction with substrate and protein content. The reaction was stopped by the addition of 60 µl of 25% trichloroacetic acid. Samples were chilled on ice for 10 min, centrifuged at 1000 g for 10 min, and 150 µl samples were taken for assay of released inorganic phosphate (Pi) by the method of Lanzetta *et al.* [29]. Controls to correct for nonenzymatic hydrolysis were prepared by adding platelet preparations after the reaction was stopped with trichloroacetic acid. All samples were run in duplicate or triplicate. Enzyme activities were generally expressed as nmol of phosphate (Pi) released/min/mg protein. The Ca^{2+}-nucleotide complex concentrations were calculated using a computer program [30, 31].

The reaction medium used to assay Mg^{2+}-ATPase and Mg^{2+}-ADPase was the same as that used for Ca^{2+}-activiy except that 5 mM $MgCl_2$ was used instead of $CaCl_2$.

Assay of pyrophosphatase (EC 3.6.1.1) and nonspecific phosphatase activities

The procedure was the same as that used for the assay of Ca^{2+}-ATP diphosphohydrolase activity, except that ATP and ADP as substrates were replaced by one of the following compounds (0.5 mM final concentration): PPi, glucose-6-phosphate, p-nitrophenylphosphate, B-glycerophosphate, cyclic AMP, tripolyphosphate, phosphate glass, or AMP.

Protein determination

Protein was assayed by the Coomassie Blue method [32] with bovine serum albumin used as a standard.

Results

ATP and ADP hydrolysis

Intact platelet preparations can promote ATP and ADP hydrolysis with a specific activity in the range of 7.0 ± 1.73 nmol Pi/min/mg and 4.0 ± 0.94 nmol Pi/min/mg (mean ± SD), respectively in the presence of Ca^{2+} (5 mM). In the presence of Mg^{2+} (5 mM), ATP hydrolysis was higher (10%) and ADP hydrolysis was lower (30%) (results not shown) than that found for Ca^{2+}, which may indicate that Mg^{2+} activates other ATPases.

ATP and ADP hydrolysis could be catalysed by an ATP diphosphohydrolase (apyrase) or by the action of combined activities of other enzymes that may mimic apyrase activity.

Exclusion of enzymatic combinations

The participation of an adenylate kinase in the nucleotide hydrolysis was excluded because no ATP was formed when platelets were incubated with ADP (results not shown). The protocol was the same as that used by us [7, 8, 11] to exclude this enzyme as participating in ADP hydrolysis by saliva of insects and by synaptosomes from central and peripheral nervous system. Furthermore, the potent adenylate kinase inhibitor Ap5A [33] did not affect either ATP or ADP hydrolysis in the presence of Ca^{2+} (Table 1) or Mg^{2+} (results not shown), indicating that no ADP is hydrolysed by the association of adenylate kinase with an ATPase.

The possibility of the combined action of an ATP pyrophosphohydrolase and an inorganic pyrophosphatase was excluded because no Pi was released when PPi (pyrophosphate) was incubated at a final concentration of 0.5 mM instead of ATP or ADP (results not shown). The association of an ATPase with an ADPase in ATP and ADP hydrolysis was excluded on the basis of the parallelism that was observed for hydrolytic activities of ATP and ADP under several types of conditions (see below). Furthermore a mixed-substrate kinetic approach [34] was used to investigate this possible enzymatic association. Platelets were incubated in standard reaction mixtures containing 0.5 mM ATP (5 mM Ca^{2+}), or 0.5 mM ADP (5 mM Ca^{2+}) or 0.5 mM ATP + 0.5 mM ADP (10 mM Ca^{2+}). In the simultaneous presence of both substrates the rate of Pi production (5.99 ± 2.16) was close to the arithmetic mean (4.96 ± 1.66) of the activities obtained with ATP (6.47 ± 2.35) and ADP (3.46 ± 1.01)

Table 1. Effects of inhibitors on Ca^{2+}-ATP and Ca^{2+}-ADP hydrolysis by platelets. Oligomycin and DNP were added from a concentrated ethanol solution (1% final ethanol concentration). 1% ethanol itself did not promote inhibition of the enzyme activity. Control Ca^{2+}-ATPase and Ca^{2+}-ADPase activities (100%) were 7.2 ± 0.68 and 4.3 ± 0.51 nmol Pi/min/mg (mean ± SD), respectively. Results are expressed as percentage of the control activity. Data represent mean ± SD, with the numbers of experiments given in parentheses. Data were analysed statistically by one-way analysis of variance. * indicates significantly different from control activity (100%) and from activity with 1 mM azide ($P < 0.01$)

Compounds	mM	% of control enzyme activity	
		Ca^{2+}-ATPase	Ca^{2+}-ADPase
Orthovanadate	0.1	93.4 ± 9.28 (4)	95.7 ± 3.88 (4)
Ouabain	1.0	99.3 ± 16.50 (4)	100.7 ± 6.00 (4)
NEM	1.0	93.9 ± 3.71 (3)	91.8 ± 1.61 (3)
Lanthanum	0.1	107.4 ± 3.96 (3)	104.3 ± 6.16 (3)
DNP	0.5	98.3 ± 3.27 (3)	93.9 ± 4.17 (3)
Oligomycin	2.0 µg/ml	100.3 ± 1.45 (3)	100.5 ± 10.80 (3)
Efrapeptin	10.0 µg/ml	93.6 ± 1.19 (3)	99.3 ± 3.16 (3)
NBD-Cl	0.05	98.5 ± 12.09 (4)	100.5 ± 9.83 (4)
Azide	1.0	93.2 ± 4.46 (3)	99.6 ± 4.57 (3)
	5.0	82.8 ± 1.97 (3)*	83.0 ± 4.32 (3)*
	10.0	71.6 ± 4.89 (3)*	63.6 ± 1.59 (3)*
Levamisole	1.0	109.6 ± 5.77 (3)	97.9 ± 4.84 (3)
Ap5A	0.01	112.6 ± 12.90 (4)	102.4 ± 4.74 (4)
	0.5	105.7 ± 15.30 (4)	92.5 ± 5.70 (4)

when incubated individually; all results (nmol Pi/min/mg) are expressed as mean ± SD, n = 6 in each condition. If two enzymes were involved the rate for the mixed-substrate reaction would be the sum of the values found for individual substrates. Nonspecific phosphatases were also excluded because the platelet preparations did not hydrolyse B-glycerophosphate, p-nitrophenyl-phosphate, glucose-6-phosphate, cAMP, phosphate glass (a mixture of poliphosphates) or tripolyphosphate (all at 0.5 mM) when these instead of they (ATP or ADP) were used as substrates.

Action of inhibitors

Since many ATPases may interfere with platelet apyrase activity, to elucidate this possible relationship various specific ATPase inhibitors were tested in the presence of Ca^{2+} ion. Table 1 shows that the Na^+, K^+-ATPase inhibitors orthovanadate [35] or ouabain [36] did not inhibit ATP and ADP hydrolysis. The Ca^{2+}, Mg^{2+}-ATPase inhibitors orthovanadate, NEM and lanthanum were also ineffective in altering the hydrolysis of both substrates.

The mitochondrial ATPase activator (DNP) [37, 38] and the mitochondrial ATPase inhibitors oligomycin, efrapeptin [39] and NBD-Cl (7 chloro-4 nitro 2,1,3-benzoxodiazole) did not interfere with the hydrolysis of either enzyme substrate (Table 1). Low concentrations of sodium azide (1 mM) but far higher than that necessary to inhibit mitochondrial ATPase (100 μM) [40] did not inhibit the enzyme. When sodium azide was tested at high concentrations (5–10 mM) a significant and parallel ATP and ADP hydrolysis inhibition occurred. A similar inhibition of ATP diphosphohydrolase from various mammalian plasma membranes and cerebral cortex synaptosomes by 10 mM sodium azide has been reported by Knowles *et al.* [9] and Battastini *et al.* [11], respectively. The specific alkaline phosphatase inhibitor levamisole [41] also did not inhibit ATP or ADP hydrolysis by platelets, thus excluding this enzyme as a contaminant. Adenylate kinase activity (see above) was excluded by the lack of effect of Ap5A on ADP hydrolysis.

Table 2. Substrate specificity of Ca^{2+}-ATP diphosphohydrolase from rat blood platelets. Platelet preparations were obtained and enzyme assays were carried out as described in Materials and Methods. All substrates at 0.5 mM (5 mM Ca^{2+}). Data represent the mean ± SD of three different experiments

Substrate	Specific activity	Relative activity
ATP	6.23 ± 0.58	1.00
UTP	8.06 ± 0.50	1.29
ITP	7.43 ± 0.85	1.19
GTP	6.80 ± 0.60	1.09
CTP	5.09 ± 0.95	0.82
ADP	3.54 ± 0.42	0.57
UDP	4.08 ± 0.10	0.65
IDP	3.52 ± 0.17	0.57
GDP	3.23 ± 0.20	0.52
CDP	2.79 ± 0.54	0.45
ATETRAP	2.30 ± 0.11	0.37
AMP	1.01 ± 0.21	0.16

Substrate specificity

One of the characteristics of ATP diphosphohydrolase activity from various sources [7, 8, 11–14, 42, 43] is that the enzyme can promote the hydrolysis of different di- and triphosphate nucleotides. Platelet preparations showed little substrate specificity because at concentration of 0.5 mM all nucleotides tested were hydrolysed in the presence of Ca^{2+} ion (Table 2). The monophosphate nucleotide AMP was hydrolysed because our platelet preparations probably also contained a 5′-nucleotidase activity that catalyzed the formation of adenosine with AMP as substrate [44].

Effect of pH

The apparent pH optimum for the enzyme was determined in a medium with 50 mM histidine and 50 mM Tris buffer (pH range 6.0 to 9.0) and ATP or ADP as substrates (Fig. 1). The enzyme showed an apparent pH optimum in the range of 7.5–8.0 for both Ca^{2+}-ATP and Ca^{2+}-ADP hydrolysis, with a parallel profile for both substrates.

Kinetic parameters of the platelet enzyme

Ca^{2+}-ATP and Ca^{2+}-ADP hydrolysis was determined at Ca^{2+}-nucleotide complex concentrations from 20 to 600 μM for both substrates. The results (inset in Fig. 2) indicated that the enzyme activity increased with the increase in concentration of the complex (Ca^{2+} fixed at 5 mM and nucleotides at variable concentrations). The Lineweaver-Burk plot of the results obtained with complex in the range of 20 μM to 200 μM can be seen in Fig. 2. The apparent K_m (Michaelis constant) calculated from these results was 24 ± 0.56 μM (mean ± SD, n = 3)

and 31 ± 2.65 μM (mean ± SD, n = 3) for Ca^{2+}-ATP and Ca^{2+}-ADP, respectively. The calculated V_{max} (maximal velocity) was 5.73 ± 0.31 and 3.85 ± 0.78 nmol Pi/min/mg for Ca^{2+}-ATP and Ca^{2+}-ADP, respectively. The enzyme has a high affinity for Ca^{2+}-nucleotide complexes as substrates. It is important to note that similar K_m values for both substrates is a characteristic of apyrases. The enzyme presented a residual activity (without addition of Ca^{2+}) that was removed completely by the addition of 100 μM EGTA (results not shown), which preferentially chelates Ca^{2+} over Mg^{2+} ions. This result indicates the probable presence of divalent cations on the surface of platelets or leaking from the interior. To be certain that this inhibition by EGTA was caused by the specific depletion of calcium ions, as opposed to a direct inhibitory effect of EGTA on the enzyme, the reverse experiment was performed in which Ca^{2+} ions were replenished in the assay mixture with EGTA. ATP and ADP hydrolysis

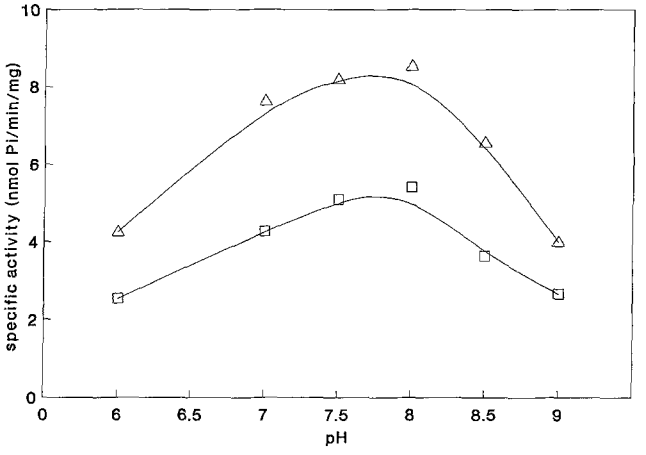

Fig. 1. Effect of pH on ATP diphosphohydrolase activity. Ca^{2+}-ATP (△) and Ca^{2+}-ADP (□) hydrolysis by intact platelets. Buffers used were 50 mM histidine and 50 mM Tris at all points, and pH was adjusted by the addition of HCl or NaOH. Date represent a typical experiment.

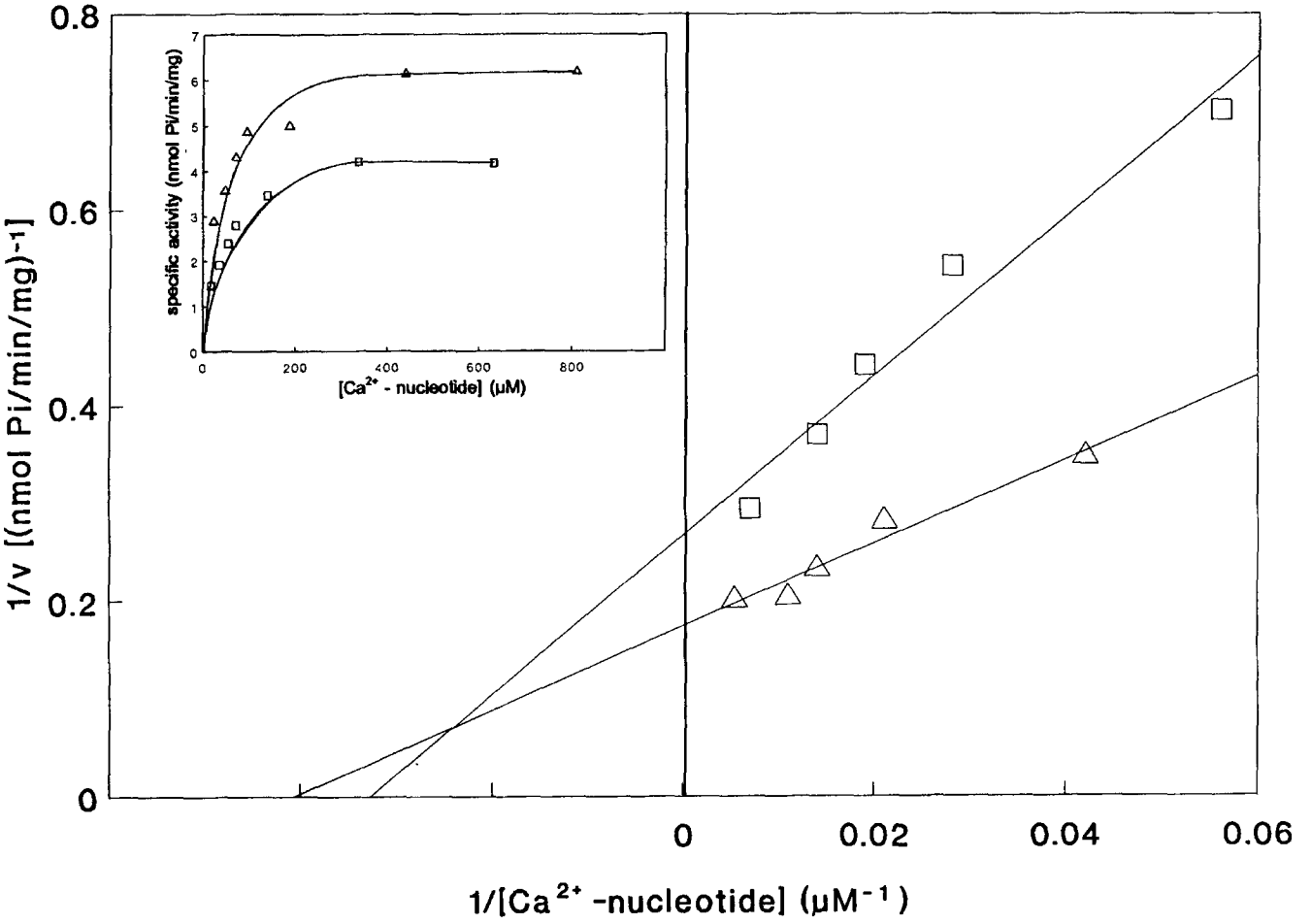

Fig. 2. Lineweaver-Burk plots of ATP diphosphohydrolase activity. All Ca^{2+}-ATP (△) or Ca^{2+} -ADP (□) hydrolysis was measured as described in Materials and Methods. Results were obtained with concentrations of Ca^{2+}-nucleotide complexes in the range of 20 to 200 μM. The K_m values and the maximal velocities (V_{max}) for the two substrates were calculated from the x and y intercepts, respectively. The inset shows the dependence of ATP diphosphohydrolase activity on Ca^{2+}-ATP and Ca^{2+}-ADP concentration (in the range of 20 to 600 μM).

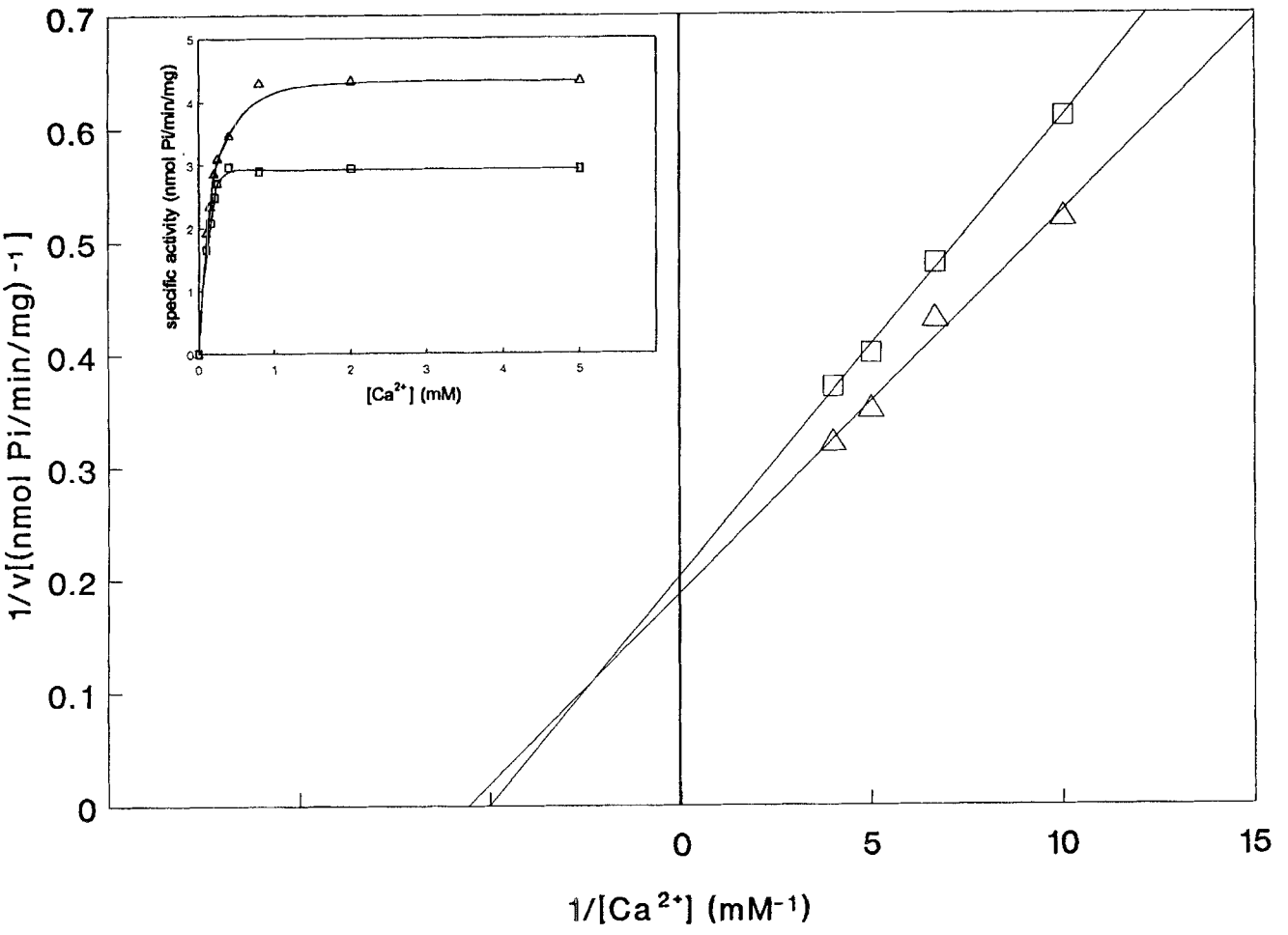

Fig. 3. Lineweaver-Burk plots of the dependence of ATP diphosphohydrolase activity on Ca^{2+}. Ca^{2+}-ATP (△) or Ca^{2+}-ADP (□) hydrolysis was measured as described in Materials and Methods. Residual activity was removed by the addition of 100 μM EGTA. Results were obtained with concentrations of Ca^{2+} in the range of 0.1 to 0.25 mM. The Km values for Ca^{2+} with ATP and ADP were calculated from the x intercept. The inset shows the activation of ATP diphosphohydrolase by Ca^{2+} at constant 0.2 mM ATP or ADP concentration.

as a function of calcium concentration was dose-dependent and saturable (inset in Fig. 3). The Michaelis-Menten constant (K_m) calculated for Ca^{2+} from the Lineweaver-Bruk plot (Fig. 3) was approximately 172 ± 30.3 μM (mean ± SD, n = 3) and 197 ± 41.8 μM (mean ± SD, n = 3) with ATP (0.2 mM) and ADP (0.2 mM), respectively. In a similar experiment (results not shown) but at high (0.5 mM) ATP or ADP concentration we observed an decrease in ATPase and ADPase activities with a consequent increase in K_m values for Ca^{2+} (when compared to the results obtained with 0.2 mM nucleotide), indicating that in this condition free ATP or ADP may bind to the enzyme and act as an inhibitor with respect to the calcium-nucleotide substrate. Similar hypothesis was presented to synaptosomal ATPases [45].

Integrity of platelets and localization of enzyme activity

The integrity of the platelet preparation was checked by measuring platelet LDH (lactate dehydrogenase) activity. The ratio of this enzyme activity measured in intact and disrupted platelets can be regarded as a measure of damaged particles [46]. Disruption of platelets by 0.1% Triton X-100 resulted in an 20-fold increase in LDH activity and also an increase in the rate of hydrolysis of either ATP and ADP (Table 3). Therefore, when we determined ADP hydrolysis in presence of 100 μM Ap5A the value was approximately the same as that determined in intact platelets. These results are a stronger evidence to suggest that the enzyme involved in ADP hydrolysis in intact platelets is an ecto-ATP diphosphohydrolase and the increase of the activity in lysed platelets is due to the liberation of citoplasmic adenylate kinase (Ap5A inhibited). The increase in ATPase activity

probably is due to the leakage of intraplatelet ATPases and exposition of inner membrane ATPases.

Discussion

The present results strongly suggest the presence of a true ATP diphosphohydrolase (apyrase) as an ecto-enzyme in intact platelets. The enzyme described here has the following general properties that characterize an apyrase:

(a) Low specificity for nucleoside tri- and diphosphates (Table 2).
(b) Activation by bivalent metal cation Ca^{2+} (inset in Fig. 3) or Mg^{2+} (results not shown).
(c) Optimum pH range from 7.5 to 8.0 (Fig. 1).
(d) Insensitivity to classical and well-known ATPase inhibitors (orthovanadate, ouabain, NEM, lanthanum, oligomycin, efrapeptin NBD-Cl, 1 mM azide) and to the alkaline phosphatase inhibitor levamisole (Table 1).
(e) Inhibition by azide at high concentrations (5 e 10 mM) (Table 1).
(f) Similar apparent Km values for both substrates (Ca^{2+}-ATP and Ca^{2+}-ADP) of the enzyme (Fig. 2).

Possible enzyme combinations that might lead to an apparent apyrase activity as an artifact were excluded. Adenylate kinase activity was ruled out on the basis of the absence of ATP production when platelets were incubated with ADP in the presence of Ca^{2+} (results not shown) and the insensitivity of ADP hydrolysis to Ap5A and NEM [47] (Table 1) which are inhibitors of the adenylate kinase enzyme. The combination of an ATPase and an ADPase was excluded by the mixed substrate experiment. The protocol of mixed substrate experiment do not exclude the possibility that a nucleotide might inhibit the hydrolysis of each other, for example, if we had two distinct enzymes but: a) this experiment is a classical procedure for characterizing enzymes acting on two substrates [7, 8, 11, 14, 48] and b) the parallelism observed for cation and pH dependence, substrate specificity and the lack of effect of different inhibitors suggest the presence of only one enzyme acting on the two substrates (ATP and ADP). The possible participation of pyrophosphatases or nonspecific phosphatases was also excluded because inorganic pyrophosphate and several phosphate esters were not substrates for the enzyme.

Since we used a well-described technique of gel filtration to separate intact blood platelets from plasma [27, 28, 49] and on basis in the results obtained from LDH activity and ATPase-ADPase activities (Table 3) determined in intact and disrupted platelets, we propose that the enzyme described here is an ecto-enzyme.

The importance of ATP and ADP hydrolysis in the regulation of hemostasis has been proposed by several authors [13, 18, 50–55]. In cardiac ischaemia and hypoxia the breakdown of circulating ATP and ADP to adenosine is a protective action to assure a sufficient supply of blood to vital regions of the body such as cardiac and cerebral tissues by prolonged vasodilatation. By converting ADP released from aggregated platelets and/or hemolysed red blood cells to AMP, the enzyme may play an important role in the prevention of microthrombus formation. In this paper we describe a platelet ecto-ATP diphosphohydrolase (ecto-apyrase) and we propose that this enzyme in association with a 5′-nucleotidase is able to form an 'enzyme chain' to regulate the concentrations of ATP (inducer of shock) [56] and ADP (inducer of platelet aggregation) in blood by increasing their hydrolysis, with a consequent increase in circulating adenosine which is a natural protective metabolite (inducer of vasodilatation).

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Table 3. Effects of platelet lysis on Ca^{2+}-ATPase, Ca^{2+}-ADPase and LDH activity. Data represent mean ± SD with the number of experiments given in parentheses a) Intact platelets were kept in an isoosmotic medium during the enzymatic assays. b) Platelets were lysed with 0.1% Triton X-100. c) The results are reported as percent of LDH activity in intact platelet preparations, compared to LDH activity in lysed platelets (100%)

Preparation	Ca^{2+}-ATPase	Ca^{2+}-ADPase	LDH
	(nmol Pi/min/mg)		% U.E. (c)
Intact platelets (a)	7.47 ± 0.85 (2)	4.32 ± 0.24 (2)	3.43 ± 0.72 (3)
Lysed platelets (b)	17.83 ± 1.34 (2)	12.14 ± 0.75 (2)	100 (3)
Lysed platelets + 100 μM Ap5A	18.03 ± 2.20 (2)	6.65 ± 0.39 (2)	100 (3)

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