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CHARACTERIZATION OF ECTO-ATPASE ON HUMAN BLOOD CELLS

A PHYSIOLOGICAL ROLE IN PLATELET AGGREGATION?

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Abstract—Ecto-ATPase (EC 3.6.1.15) is a plasma membrane-bound enzyme which degrades extracellular triphosphate nucleotides. Although its physiological function is still unclear, the enzyme obscures the study of P_2 purinoceptors (i.e. receptors for ATP and other di- and triphosphate nucleotides), since it is capable of metabolizing the pharmacological ligands, such as ATP, for these receptors. We characterized the ecto-ATPase activity on human blood cells with a [γ^{32}P]ATP assay and HPLC measurements. We also determined whether ecto-ATPase activity could affect the anti-aggregatory role of ATP in whole human blood. The K_m for ATP of the ecto-ATPase on human blood cells was 8.5 ± 2.3 μM and the maximum degradation rate, at 37°, was 2.7 ± 1.1 nmol ATP/(min × mL whole blood). In whole blood the major part of ATP was broken down by the blood cells, predominantly by the leukocytes. ATP and UTP were broken down equally well, mainly yielding the corresponding di- and monophosphates. In search of inhibitors for the ecto-ATPase, we studied several analogs of ATP. 8-Bromo-ATP as well as 2′- and 3′-deoxy-ATP were substrates for the enzyme. In contrast, modification of the phosphate side chain yielded inhibitors. Subsequently, a possible role of the ecto-ATPase in platelet aggregation was verified. To assess the role of the plasma membrane-bound enzyme, platelet aggregation was determined in whole blood instead of platelet-rich plasma. In the presence of ATP alone, an antagonist of ADP-induced platelet aggregation, some aggregation was still observed. As breakdown of ATP by the ecto-ATPase leads to gradual formation of ADP, as mentioned above, we compared the effects of a stepwise versus bolus addition of ADP. Subsequent dosing of ADP (1.5, 2.5, 5 and 10 μM) resulted in platelet aggregation but to a much smaller extent, at most approximately 60%, compared tc the amount of platelet aggregation obtained with a bolus addition of ADP (10 μM). In conclusion, human blood cells possess a high affinity ecto-ATPase which degrades ATP as well as ATP analogs with modified base and ribose moieties. ATP analogs with a modified phosphate chain are inhibitors of the ecto-ATPase. A direct role of the ecto-ATPase activity on platelet aggregation is probably small, as degradation of ATP to ADP proceeds slowly and cumulative addition of ADP to platelets in whole blood results in a modest amount of aggregation. In view of the need for ecto-ATPase inhibitors to characterize and classify the P_2 purinoceptors, the combination of the [γ^{32}P]ATP assay and the HPLC system appears to be a powerful tool for screening ligands for this inhibitory effect. Moreover, the blood cells provide an easily obtainable human source for ecto-ATPase.

The presence of ATP in cells and its role as an energy transducing agent have been known for a very long time. However, the existence of adenine nucleotides in plasma, under physiological conditions, was not demonstrated until 1969 [1]. These extracellular nucleotides are able to interact with purinergic receptors, which are present on various blood cells and on the endothelium of the vascular wall. These receptors may be subdivided in two classes: the P_1 receptors interacting with adenosine and AMP and the P_2 receptors which interact with ADP and ATP [2]. The biological importance of these purinergic receptors in the vasculature lies in their effect on the vascular tone. The platelets contain a special kind of P_2 purinergic receptor, the P_{2T} receptor, for which ADP is an agonist mediating aggregation of platelets whereas ATP is a competitive antagonist.

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In addition to their interaction with receptors extracellular nucleotides also interact with ecto-nucleotidases. The first characteristics of ecto-ATPase activity were described on avian erythrocytes [3]. A more detailed analysis of the metabolism of extracellular nucleotides in human blood revealed a stepwise hydrolysis of ATP via ADP to AMP and adenosine [4–6]. The resulting concentration of nucleosides and nucleotides determines whether and which purinergic receptors will be activated. Both plasma and various blood cells degrade extracellular nucleotides [5, 7, 8].

Current research on the effects and potency of nucleotides, mediated via the purinergic receptors, is biased by the presence of this ecto-nucleotidase activity. The availability of selective inhibitors for these ecto-enzymes, which do not interact with the P_2 purinoceptors, would provide a powerful research tool for investigating the contribution of these enzymes in experiments designed to study the purinergic receptors. In this study we have focused our attention on the breakdown of extracellular ATP

by the ecto-ATPase of human blood cells. This preparation provides the unique possibility of studying the ecto-ATPase on freshly obtained human cells, and may potentially serve as a simple test system for novel enzyme inhibitors.

First this ecto-ATPase activity was characterized. Next, the affinity and the maximum hydrolysis rate of ATP for this ecto-ATPase on whole blood cells were determined using a [γ^{32}P]ATP assay. These enzyme kinetic parameters were compared to those of lymphocytes, plasma and whole blood. Also the structural requirements for interaction of the nucleotides with the enzyme have been determined. With the [γ^{32}P]ATP assay an effect of the nucleotides on ATP breakdown could be verified. A HPLC system was used to determine whether these nucleotides themselves were substrates for the ecto-ATPase.

Furthermore, we studied a possible role of the ecto-ATPase in platelet aggregation in whole human blood. As this enzyme degrades ATP, an antagonist for the P_{2T} receptor, to ADP, which is an agonist, a role for this enzyme in platelet aggregation might be expected.

MATERIALS AND METHODS

Chemicals. Active charcoal, particle size 4–7 μm, was purchased from Serva (Heidelberg, Germany). Adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS*), AMP, α,β-methylene-ATP (AMPCPP), β,γ-methylene-ATP (AMPPCP), β,γ-imido-ATP (AMPPNP), adenine 9-β-D-arabinofuranoside 5′-triphosphate (araATP), adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), 8-bromo-ATP (BrATP), 2′-deoxy-ATP (2′dATP), 3′-deoxy-ATP (3′dATP) and (4-nitrobenzyl)-6-thioinosine (NBI) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). ADP, ATP and tetrabutylammoniumphosphate (TBAP) were from Aldrich Chemie (Steinheim, Germany). (−) Bromotetramisole was from Janssen (Beerse, Belgium), 8-*para*-sulfophenyltheophylline (pSPT) from RBI (Natick, MA, U.S.A.) and UTP from Boehringer (Mannheim, Germany). [γ^{32}P]ATP was provided by Amersham (U.K.).

All chemicals were of analytical grade. Nucleotides were dissolved in deionized water. Stock solutions of ouabain and NBI were made in dimethyl sulfoxide. The final dimethyl sulfoxide concentration in the incubation mixture did not exceed 2.5%, a concentration which had no effect on enzyme activity.

Human blood cells. Fresh blood from healthy volunteers (Bloodbank, Academic Hospital, Leiden) was collected in heparinized glass tubes and was centrifuged for 10 min at 2000 *g*. Plasma was carefully removed to prevent disturbance of the buffy coat. Subsequently, the blood cells were washed twice to remove remaining plasma, again being careful not to disturb the buffy coat. Washing steps were carried out with assay buffer containing 20 mM Tris, 5 mM glucose, 140 mM NaCl, 5 mM KCl, pH 7.4. With a Coulter counter the optimal centrifugation speed was determined. At 2000 *g* for 10 min all the blood cells were present in the pellet. At a lower speed recovery of leukocytes and platelets was not complete whereas at a speed ≥ 3000 *g* hemolysis occurred. Blood cells were diluted seven times to determine K_m and V_{max} values and to determine the effect of ATP analogs on ATP degradation. Time-course HPLC experiments were carried out with 2.5-times diluted blood cells. A 3.5-times diluted blood cell preparation was used to assess the stability of ATP analogs against breakdown.

Lymphocytes. Blood was drawn from healthy volunteers and B- and T-lymphocytes, obtained via elutriation, were provided by the Department of Experimental Infectious Diseases of the Academic Hospital in Leiden. Cells were kept on ice during the whole isolation procedure and were washed three times with phosphate-buffered saline via centrifugation at 600 *g* for 10 min. [γ^{32}P]ATP experiments were performed with freshly obtained cells at a concentration of 2.7×10^6 cells/mL. The viability of the cells was determined with eosine (5% in phosphate-buffered saline) and was >95%.

Plasma. Blood obtained from healthy volunteers was centrifuged at 700 *g* for 15 min to remove erythrocytes and the buffy coat. Next the plasma was centrifuged at 16,000 *g* for 20 min to obtain platelet-free plasma. Plasma was diluted three times prior to [γ^{32}P]ATP analysis.

[γ^{32}P]ATP assay. The total incubation volume was 200 μL assay buffer containing in addition 5 mM NaN_3, 0.5 mM $CaCl_2$, ATP as indicated and approximately 100,000 dpm [γ^{32}P]ATP. ATP analogs were tested at a concentration of 100 μM. Cells, plasma or whole blood were added in a volume of 75 μL. After a 30 min incubation at 37°, 400 μL of an active charcoal suspension (20 *g* in 500 mL 0.1 N HCl) were added. The suspension was centrifuged at 6500 *g* upon which 350 μL of supernatant was dissolved in 4 mL scintillation cocktail (Emulsifier Safe, Packard) and counted in an LKB-Wallac 1214 Rackbeta Excel Spectrometer. Controls, without cells, plasma or whole blood, were processed identically.

Inhibitors, concentrations as indicated, were added in a volume of 25 μL. (−) Bromotetramisole and NBI were preincubated with blood cells for 10 min at 37° and 15 min at room temperature, respectively. In controls blood cells were preincubated with assay buffer. Association of (−) bromotetramisole and NBI was very rapid as shown before [9, 10].

HPLC assay. HPLC experiments were carried out similarly to the [γ^{32}P]ATP experiments. Blood cells were incubated for 30 min at 37° in a total volume of 200 μL, containing 5 mM NaN_3, 0.5 mM $CaCl_2$, and 100 μM ATP or other nucleotide(analog)s. After this period 400 μL assay buffer were added. Following a 2 min centrifugation at 6500 *g*, the supernatant was

* Abbreviations: ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); AMPCPP, α,β-methylene-ATP; AMPPCP, β,γ-methylene-ATP; AMPPNP, β-γ-imido-ATP; araATP, adenine 9-β-D-arabinofuranoside 5′-triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); BrATP, 8-bromo-ATP; 2′dATP, 2′-deoxy-ATP; 3′dATP, 3′-deoxy-ATP; NBI, (4-nitrobenzyl)-6-thioinosine; pSPT; 8-*para*-sulfophenyltheophylline; TBAP, tetrabutylammoniumphosphate.

stored at −80°, or analysed directly. The supernatant was diluted 1:1 with assay buffer before injection.

Nucleotides were separated on a nucleotide–nucleoside column (Alltech, 250 mm × 4.6 mm, 7 μm) at a flow rate of 1 mL/min. Analyses were performed by a linear gradient elution which ran from 0–35% buffer B in buffer A in 16 min. Buffer B was HPLC-grade methanol with 5 mM TBAP. Buffer A contained 20% buffer B in 60 mM $NH_4H_2PO_4$, 5 mM TBAP adjusted to pH 5.0 with 1 N NaOH. Buffers were filtered over a Millipore LS 5 μm filter prior to use. A total of 100 μL of supernatant was injected and analysed at 268 nm, with a Spectra 100 variable wavelength detector from Spectra Physics. The HPLC system consisted of an LKB Bromma 2152 controller, two LKB Bromma 2150 pumps and a Jasco 880-30 solvent mixing module.

Whole blood aggregation. Heparinized blood from healthy volunteers was diluted 1:1 with phosphate-buffered saline. Blood was used between 30 min and 3 hr after venipuncture. Diluted blood (2 mL) was put in a siliconized glass vial. The sample was stirred at 1200 rpm using a siliconized rod and was kept at 37° throughout the experiment. An electrode was inserted to perform impedance measurements [11]. Once a stable baseline signal was obtained, that is the electrode was coated with a monolayer of platelets, pSPT (25 μM) and nucleotide analogs were added. To prevent disturbance of the temperature or of the platelet monolayer, all additions were made in a volume of 20 μL. Aggregation of platelets to the platelet monolayer on the electrode led to an increase in resistance. This increase of the impedance was recorded with a Chrono-Log aggregometer model 540. Calibration was carried out using a constant impedance increase of 5 Ω.

Calculations. The K_m and V_{max} values were calculated using a non-linear curve fitting program based on the Michaelis–Menten equation. ATP concentrations were used after subtraction of degraded ATP. The effect of nucleotide analogs on the degradation of ATP is expressed as per cent relative to control, to which no nucleotide analogs were added. HPLC data were corrected for the purity/breakdown of added nucleotide using controls without blood cells. For HPLC experiments the amount of nucleotide and nucleoside present in the supernatant was expressed as percentage of the total amount of nucleotide added.

RESULTS

Characterization of the ecto-ATPase

The ecto-ATPase on human blood cells requires divalent cations for its activity. Without addition of divalent cations some ecto-ATPase activity was still present. This activity was probably due to endogenously present divalent cations as addition of EDTA (3 mM) abolished all activity (data not shown).

ATP degradation was not sensitive to ouabain, a Na^+/K^+ ATPase inhibitor, at a concentration of 2 mM. All the experiments were performed with 5 mM NaN_3, a general inhibitor of intracellular ATPases. An equal amount of ATP was degraded when the NaN_3 concentration was increased to 25 mM. (−) Bromotetramisole, an inhibitor of alkaline phosphatase at a concentration of 1 or 2 mM, inhibited ATPase activity 15% or 30%, respectively [9]. Also NaF at 10 mM reduced ATP degradation by approximately 15%. To determine whether the inhibitory effect of (−) bromotetramisole and NaF indeed indicated a contribution of alkaline phosphatase activity, *p*-nitrophenylphosphate was used. *p*-Nitrophenylphosphate is a substrate of alkaline phosphatase with a 2-fold higher affinity than ATP [9]. At a concentration of 100 μM, *p*-nitrophenylphosphate had no effect on ATP degradation. In contrast, 100 μM ATP reduced the breakdown of [γ^{32}P]ATP by 75%. These data rule out that the ecto-ATPase activity is contaminated with alkaline phosphatase activity.

Kinetic parameters of ecto-ATPase

The enzyme kinetic parameters, namely the

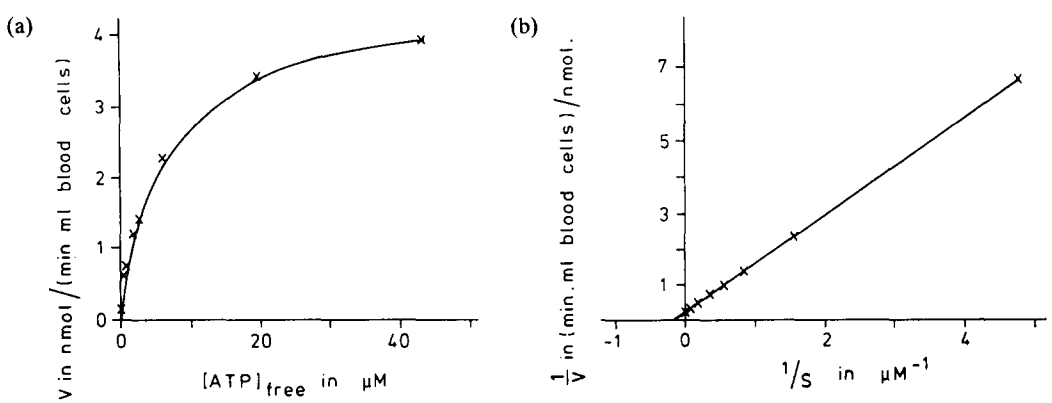

Fig. 1. Michaelis–Menten (a) and Lineweaver–Burk (b) plots of ecto-ATPase activity of human blood cells. Blood cells were incubated with ATP in a concentration range of 0.5–50 μM, spiked with [γ^{32}P]-ATP. The plots are a representative of 14 independent experiments with all data points determined in duplicate.

Table 1. K_m and V_{max} values of ecto-ATPase activity on blood cells and lymphocytes, and in plasma and whole blood

	K_m (μM)	V_{max} (nmol/min × mL blood)*	N
Blood cells	8.5 ± 2.3	2.7 ± 1.1	14
Lymphocytes	7.1 ± 2.9	0.3 ± 0.1	4
Plasma	7.5 ± 1.4	0.4 ± 0.1	3
Whole blood	5.8 ± 1.5	1.8 ± 0.6	5

* The maximum degradation rate is expressed per mL whole blood. The assumption has been made that plasma makes up half of the blood volume and that the other half is accounted for by blood cells. In the case of the lymphocytes, the maximum degradation rate has been calculated assuming that 2.2×10^6 lymphocytes are present per mL of blood.

Values are means ± SEM.

affinity of the enzyme for ATP (K_m) and the maximum degradation rate (V_{max}) of the ecto-ATPase on intact human blood cells, were determined with the [γ^{32}P]ATP assay. Blood cells were incubated with 0.5–50 μM ATP for 30 min at 37°. Figure 1 shows a curvi-linear Michaelis–Menten plot (a) and a linear representation of the data according to Lineweaver–Burk (b). The K_m and the V_{max} values were determined with a non-linear curve fitting program based on the Michaelis–Menten equation. Blood cells from 14 different donors revealed a mean K_m of 8.5 ± 2.3 μM and a mean V_{max} of 5.5 ± 2.2 nmol ATP/(min × mL blood cells). Similarly, we determined the ATPase activity of plasma and isolated lymphocytes. In Table 1 the maximum degradation rates have been expressed per milliliter of blood. In consequence, the V_{max} of blood cells is 2.7 ± 1.1 nmol ATP/(min × mL blood) assuming that blood cells make up half of the blood volume. Plasma, accounting for the other half of the blood volume, degraded ATP as well, but the V_{max} was much lower, 0.4 ± 0.1 nmol/(min × mL blood). Lymphocytes degraded ATP at a maximum rate of 0.3 ± 0.1 nmol/(min × mL blood) assuming that 2.2×10^6 lymphocytes are present per milliliter of blood. The K_m values in whole blood and plasma, and on lymphocytes and blood cells were similar, approximately 6–8 μM. These data have been summarized in Table 1.

Time-dependent degradation of ATP and UTP, and degradation of nucleotide analogs

Incubation of ATP or UTP with human blood cells resulted in step-wise degradation via diphosphate and monophosphate formation (Fig. 2a and b, respectively). Purine (ATP) and pyrimidine (UTP) nucleotides were degraded to the same extent. After 30 min incubation approximately 50% of the triphosphate nucleotides were degraded, mainly to the diphosphate and monophosphate nucleotides. The degradation of UDP to UMP seemed to be slightly less efficient compared to ADP degradation. Low amounts of nucleosides were formed. Also the addition of an inhibitor of the nucleoside transport protein, 1 μM NBI, did not lead to increased concentrations of extracellular nucleosides.

Besides the effects of different base moieties, the effects of deoxy sugars and phosphate chain alterations were investigated. With the [γ^{32}P]ATP assay the effect of these nucleotide analogs on ATP degradation was examined (Table 2). In the control situation, 10 μM of ATP was present of which 3.4 ± 0.7 μM was degraded after 30 min at 37°. Thus 34% of the added ATP was broken down and was defined as 100% activity. BrATP and ATP itself reduced the degradation of [γ^{32}P]ATP by approximately 75%. Also, upon addition of the ribose-modified ATP analogs, 2'dATP, 3'dATP and araATP, the remaining ATPase activity was

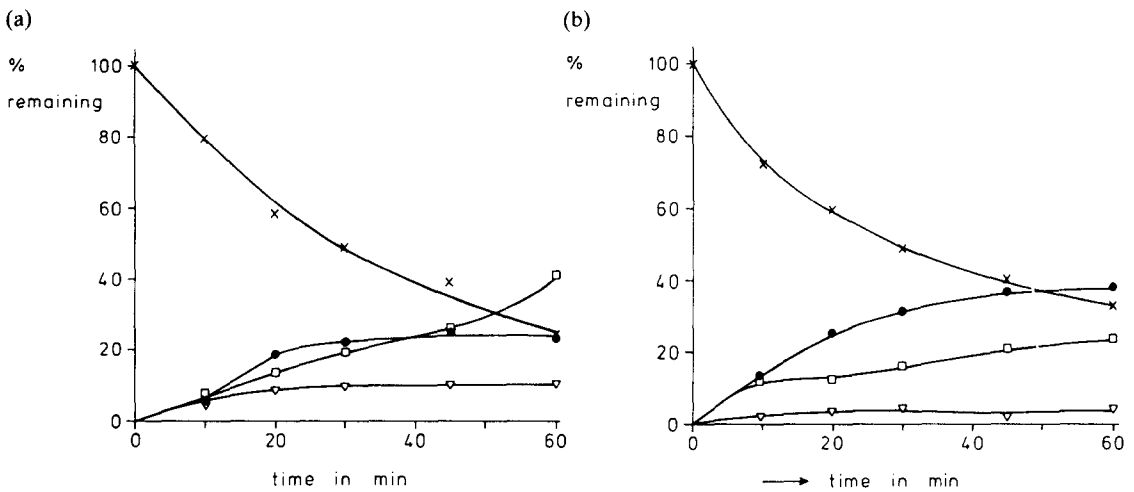

Fig. 2. Time-dependent degradation of ATP and UTP by human blood cells. Blood cells were incubated with 100 μM of ATP (a) or UTP (b) for the time indicated. Triphosphate (×), diphosphate (●) and monophosphate nucleotides (□) as well as nucleoside (▽) concentrations were determined with HPLC. Means of three independent experiments are shown. The SEM did not exceed 12%.

Table 2. Effect of nucleotide analogs on ecto-ATPase activity on human blood cells using the [γ^{32}P]ATP assay

Nucleotide analog*	Modification	Activity remaining (% of control)
BrATP	Purine	25.4 ± 5.9
2'dATP	Ribose	38.5 ± 4.0
3'dATP	Ribose	60.0 ± 8.2
araATP	Ribose	52.6 ± 8.8
AMPPCP	Phosphate	75.9 ± 2.1
AMPPNP	Phosphate	32.0 ± 4.9
AMPCPP	Phosphate	38.6 ± 2.5
ADPβS	Phosphate	54.2 ± 3.3
ATPγS	Phosphate	19.3 ± 3.7
ADP	Phosphate	38.9 ± 4.3
ATP (control)		100
ATP		23.9 ± 5.3

* For the control 10 μM of ATP was added of which 3.4 ± 0.7 μM was degraded. This 34% breakdown was defined as 100% activity. In all other cases 100 μM of analog was also present.

Values are means ± SEM.

decreased. Of these, 2'dATP reduced the breakdown of ATP the most.

Phosphate chain-modified ATP analogs decreased the amount of ATP broken down as well. With AMPPNP, only 32.0 ± 4.9% of the ATPase activity remained, a reduction of approximately 70%. In contrast, AMPPCP was less effective and ATP degradation was diminished by 25%. Replacement of the oxygen linking the α and β phosphates by methylene (AMPCPP) reduced the activity much more, by approximately 60%. The addition of ATPγS had the most prominent effect of all analogs tested, with only 20% of the ATPase activity remaining. The diphosphate nucleotides, ADP and ADPβS, reduced the breakdown of ATP by 60% and 45%, respectively.

To determine whether these analogs were themselves substrates for the ecto-ATPase, HPLC experiments were performed. From these experiments, it appeared that the nucleotides with modified base moieties, UTP and BrATP, were still substrates for the ecto-ATPase enzyme. Both 2'dATP and 3'dATP were degraded also, whereas araATP was relatively stable to degradation; that is 67%, 59% and 82%, respectively, of the triphosphate nucleotides remained after 30 min of incubation with the human blood cells. All analogs with modified phosphate side chains were resistant to degradation; over 90% of these triphosphate nucleotides remained after incubation with the human blood cells. ADP, the product of ecto-ATPase activity, was subject to degradation itself, a finding in agreement with the time-course experiment of ATP breakdown.

Effect of adenine nucleotides on platelet aggregation in whole blood

The use of impedance instead of optical density measurements allowed the determination of platelet aggregation in whole blood. Thus, the influence of ecto-ATPase activity, which is present on blood cells, on platelet aggregation could be determined. In Fig. 3 a typical effect of ADP and ATP on platelet aggregation is shown. Addition of ADP at a concentration of 10 μM elicits a rapid and maximal response. After approximately 15 min maximum aggregation is achieved. In the presence of 5 and 25 μM ADP a similar, rapid aggregatory response was seen. A concentration of 2.5 μM ADP resulted again in a rapid response leading to approximately 50% of maximum aggregation. Simultaneous addition of 10 μM ATP and 10 μM ADP resulted in maximum aggregation, similar to the aggregation obtained with ADP alone. At 50 μM, ATP counteracted the aggregatory response of 10 μM ADP partially. ATP alone (10 μM) caused only modest aggregation.

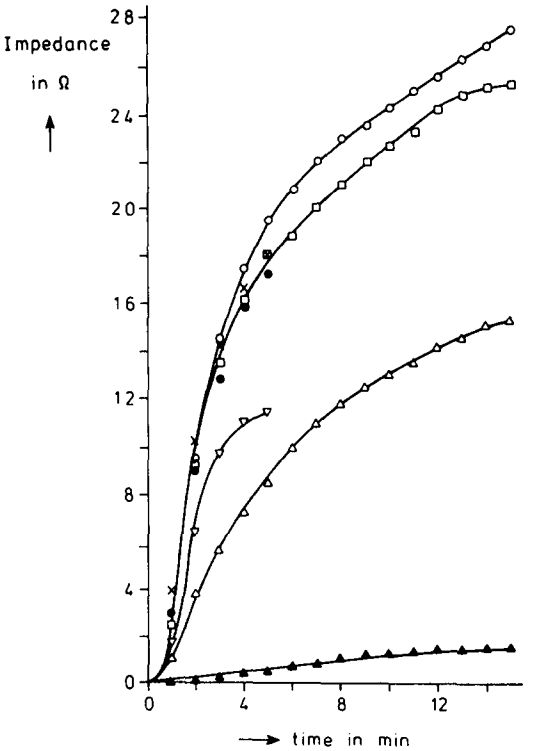

Fig. 3. Platelet aggregation in whole blood in the presence of ATP and different ADP concentrations. Aggregation of platelets in human blood in response to ADP 2.5, 5, 10 and 25 μM (▽, ●, □ and ×, respectively), ATP (▲), ATP and ADP both 10 μM (○), and ATP 50 μM and ADP 10 μM (△) was determined with impedance measurements.

The maximum response upon addition of ADP varied somewhat between blood donors as can be seen when comparing the curves of 10 μM ADP in Figs 3 and 4. The data presented in each of these figures were obtained with blood from one donor. In Fig. 4, the effect of cumulative dosing of ADP on platelet aggregation is presented. Cumulative dosing as opposed to a bolus addition was used here to simulate the breakdown of ATP by the ecto-ATPase. Although the amount of aggregation resulting from cumulative dosing of ADP varied between blood donors, aggregation was decreased

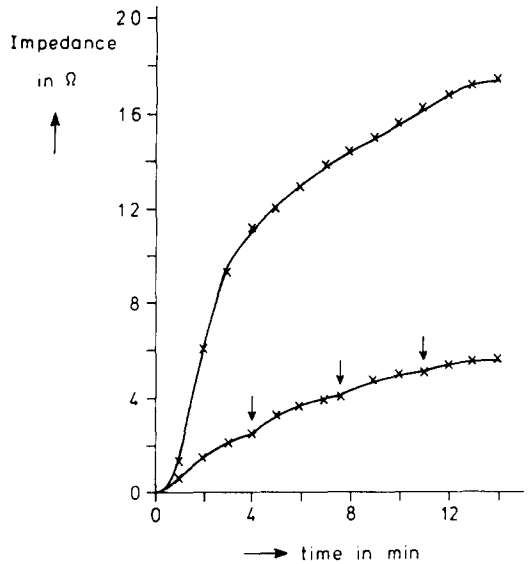

Fig. 4. Effect of cumulative dosing of ADP on platelet aggregation was determined in whole blood. At $t = 0$, 1.5 μM ADP was added. The arrows indicate successive additions of ADP 2.5, 5 and 10 μM. For comparison the response to a bolus of 10 μM ADP alone is presented.

by at least 60%. With platelet-rich plasma (10 min centrifugation of heparinized blood at 150 g) similar results were obtained. Again, cumulative administration of ADP resulted in a reduction of the aggregatory response of 75–90%.

DISCUSSION

Characterization of the ecto-ATPase

The extracellular location of ATP breakdown by intact human blood cells was verified in the following ways. First of all, the concentration of ATP used here was insufficient to penetrate the cell membrane or even lyse the cells, as has been reported for millimolar concentrations of ATP [12]. Second, the supernatant remained colorless during the course of the experiment, showing that there was no hemolysis. Third, no inhibitory effects of ouabain, an inhibitor of the Na^+/K^+ ATPase, which degrades intracellular ATP, were found [13]. Finally, damage of the blood cells with divalent cations, like Cu^{2+} or Hg^{2+}, at a concentration of 0.5 mM, resulted in an increase in ATP breakdown, presumably due to the contribution of intracellular ATPases (data not shown).

Kinetic parameters of ecto-ATPase

The affinity of ATP for the ecto-ATPase on human blood cells was relatively high, namely 7–8 μM. With human plasma a similarly high affinity, again approximately 7–8 μM, was found. The maximum hydrolysis rate in whole blood was approximately 2 nmol of ATP/(min × mL blood). A similar hydrolysis rate was obtained by Coade and Pearson [5]. The physiological concentration of ATP in human blood is at most 20 nM; however, upon exercise a 50-fold increase was found [1]. Moreover, disruption of microvessels might result in a concentration of 20 μM in the blood [14]. These amounts of ATP will be subject to degradation by the ecto-ATPase.

The maximum degradation rate on the blood cells was relatively high compared to whole blood (Table 1). An explanation might be that the concentration of Ca^{2+} ions was optimized for the human blood cells (ATP degradation was hampered by a concentration of $Ca^{2+} > 0.5$ mM), whereas human blood contains higher concentrations of Ca^{2+} ions as well as Mg^{2+} ions.

Plasma was responsible for approximately one quarter of the total ATP breakdown in whole blood, assuming that plasma makes up half of the blood volume. Thus, the cells are the major factor in ecto-ATPase activity. The lymphocytes we tested represent the physiological ratio of B- and T-cells. Previously, Gutmann *et al.* [7] and Barankiewicz *et al.* [15] have shown that the ecto-ATPase activity resides on the B-lymphocytes, especially the mature ones. Several investigators have shown in parallel experiments that the enzymatic activity on lymphocytes and granulocytes is comparable in terms of V_{max} values expressed per 10^6 cells [16, 17]. Thus, the contribution of granulocytes will be substantial, especially since these cells represent the majority of leukocytes.

Time-dependent degradation of ATP and UTP, and degradation of nucleotide analogs

ATP and UTP were degraded to a similar extent as was monitored with time in the HPLC assay (Fig. 2). The ability to degrade purine as well as pyrimidine nucleotides is characteristic of ecto-ATPase and might be of physiological relevance not just for ATP extracellular receptors but also for UTP as previously described [18, 19].

ADP and UDP were subject to degradation also, with the degradation of ADP being somewhat faster. Whether the degradation of these diphosphate nucleotides is mediated by the same enzyme (e.g. ecto-ATPase) or whether these activities reside on separate enzymes is still a matter of debate [20]. The resulting monophosphate nucleotides were degraded only to a moderate extent to the respective nucleosides. Addition of NBI (1 μM), which completely blocks the nucleoside transport protein, had no effect [21]. Thus, the low extracellular levels of nucleosides were not a result of uptake by the blood cells. This breakdown pattern resembles the pattern reported for endothelial cells. Both show the formation of low amounts of nucleosides, in contrast to smooth muscle cells where accumulation of nucleosides occurs [22].

In the [γ^{32}P]ATP assay we determined whether ATP analogs affected the breakdown of ATP. Degradation of ATP can be reduced either by the presence of another substrate or by the presence of an inhibitor. The combination of [γ^{32}P]ATP and HPLC experiments enabled us to discriminate between substrates and inhibitors.

The HPLC experiments indicated that nucleosides with modified base moieties, like UTP and BrATP, were broken down by the ecto-ATPase. Similarly,

the ribose-modified analogs, 2'- and 3'dATP, were also substrates for the ecto-ATPase. In contrast, araATP, also a ribose-modified analog (the 2' OH has the opposite configuration), was resistant to breakdown. The decrease in ATP degradation in the presence of araATP is therefore due to inhibition, as araATP is not a substrate. Modification of the phosphate chain, as in AMPCPP, AMPPCP, AMPPNP or ATPγS, yields ecto-ATPase inhibitors, since these analogs were not broken down by the ecto-ATPase. These data are in agreement with results obtained on guinea-pig taenia coli and urinary bladder [23, 24]. Interestingly, AMPPNP and AMPCPP were much more potent inhibitors than AMPPCP. ATPγS was the most potent inhibitor of the ecto-ATPase activity of the analogs tested.

ADP itself decreased the breakdown of ATP, which might be regarded as product inhibition of the enzyme. ADPβS, a stable analog of ADP, also inhibited the ecto-ATPase activity.

Effect of adenine nucleotides on platelet aggregation in whole blood

Platelet aggregation is mediated by various stimuli, e.g. ADP. ADP mediates this aggregatory response via interaction with the P_{2T} receptor, for which ATP is a competitive antagonist [25]. With the ADP concentrations used here (>1 μM) irreversible platelet aggregation occurs [26]. To assess the role of the blood cell ecto-ATPase we used impedance measurements to study platelet aggregation in whole blood. In contrast to light transmission, impedance measurements are not disturbed by the presence of other blood cells. These experiments were carried out with heparinized instead of citrated blood, as reproducibility was much better with heparinized blood, a finding in agreement with a report by Lüthje and Ogilvie [27].

Upon addition of 10 μM of ADP, a rapid aggregatory response was seen, which was inhibited by 50 μM of ATP. In the presence of ATP alone, a slight amount of platelet aggregation was found. To simulate the breakdown of ATP by the ecto-ATPase, we added ADP in a cumulative way as opposed to a bolus. From the [γ^{32}P]ATP assay we knew that the maximum degradation rate of ATP was approximately 2 nmol/(min × mL blood), or 2 μM/min. The cumulative addition of ADP began with a concentration of 1.5 μM, which caused a slight amount of aggregation. Subsequent additions of ADP, up to 10 μM after 12 min, did not elicit maximum platelet aggregation. Apparently, repeated additions of ADP are much less effective than a bolus addition.

A plausible explanation for this reduced aggregatory response upon cumulative dosing of ADP could be the gradual formation of AMP and adenosine, which are inhibitors of platelet aggregation. However, we performed these experiments in the presence of 25 μM pSPT, an adenosine receptor antagonist, to prevent inhibitory effects of those breakdown products. Similar results were obtained when 50 μM of pSPT was used (data not shown). Thus, the reduced aggregatory response with cumulative dosing of ADP is presumably not a result of the formation of breakdown products, but might instead be a receptor phenomenon, such as desensitization.

In conclusion, human blood cells possess high affinity for ecto-ATPase which degrades ATP as well as ATP analogs with modified base and ribose moieties. Interestingly, modification of the phosphate side chain yielded inhibitors of the ecto-ATPase enzyme. However, these inhibitors are non-selective as they are also agonists for the P_2 purinoceptors. Future experiments are directed towards the identification of ecto-ATPase inhibitors which do not interact with the P_2 purinoceptors.

A direct role of the ecto-ATPase activity on human blood cells in platelet aggregation is presumably small, as degradation of ATP to ADP proceeds slowly, and cumulative addition of ADP to platelets in whole blood results in a modest amount of aggregation.

As mentioned in the introduction, there is a need for selective ecto-ATPase inhibitors to characterize and classify the P_2 purinoceptors. The combination of the [γ^{32}P]ATP and HPLC experiments is very well suited for the search for potential inhibitors of the ecto-ATPase. Moreover, the blood cells provide a convenient human preparation to screen for such inhibitors.

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