Biochem. J. (1989) 259, 97–103 (Printed in Great Britain)

Identification and partial characterization of an adenosine(5′)tetraphospho(5′)adenosine hydrolase on intact bovine aortic endothelial cells

Adaling OGILVIE,*‡ Jürgen LÜTHJE,* Ulrich POHL† and Rudi BUSSE†

*Institut für Biochemie, Universität Erlangen, Erlangen, and †Institut für Angewandte Physiologie, Universität Freiburg, Freiburg, Federal Republic of Germany

The biologically active dinucleotides adenosine(5′)tetraphospho(5′)adenosine (Ap_4A) and adenosine(5′)-triphospho(5′)adenosine (Ap_3A), which are both releasable into the circulation from storage pools in thrombocytes, are catabolized by intact bovine aortic endothelial cells. 1. Compared with extracellular ATP and ADP, which are very rapidly hydrolysed, the degradation of Ap_4A and Ap_3A by endothelial ectohydrolases is relatively slow, resulting in a much longer half-life on the endothelial surface of the blood vessel. The products of hydrolysis are further degraded and finally taken up as adenosine. 2. Ap_4A hydrolase has high affinity for its substrate (K_m 10 μM). 3. ATP as well as AMP transiently accumulates in the extracellular fluid, suggesting an asymmetric split of Ap_4A by the ectoenzyme. 4. Mg^{2+} or Mn^{2+} at millimolar concentration are needed for maximal activity; Zn^{2+} and Ca^{2+} are inhibitory. 5. The hydrolysis of Ap_4A is retarded by other nucleotides, such as ATP and Ap_3A, which are released from platelets simultaneously with Ap_4A.

INTRODUCTION

Adenosine(5′)tetraphospho(5′)adenosine (Ap_4A) and adenosine(5′)triphospho(5′)adenosine (Ap_3A) are stored as metabolically stable pools in the dense granules of blood platelets [1–3]. Both dinucleotides are released into the blood during platelet aggregation. Experimental evidence suggests that Ap_4A and Ap_3A may be important in the regulation of platelet aggregation and therefore in the maintenance of haemostasis [4–7]. Ap_3A triggers platelet aggregation in platelet-rich plasma [4,5] and also in whole blood [6]. The mechanism of Ap_3A-induced aggregation has been elucidated [4,5] by demonstrating a continuous enzymic hydrolysis of Ap_3A in the plasma which produces ADP, a potent stimulus of platelet aggregation. Ap_4A, however, has antagonistic function as a competitive inhibitor of ADP-induced platelet aggregation and even disintegrates established platelet thrombi [4,6]. With an animal model of arterial thrombosis, Ap_4A has also been shown to have antithrombotic potency *in vivo* [8]. Recently, Ap_4A, as well as Ap_3A, at micromolar concentrations, have been found to influence the smooth-muscle tone of isolated rabbit arteries [9,10]. These potent vasomotor activities indicate that the unusual dinucleotides may affect target cells also beyond the bloodstream.

The plasma enzymes which degrade Ap_4A and Ap_3A have been identified and biochemically characterized [11,12]. On porcine aortic endothelial cells an ectoenzyme hydrolysing Ap_3A has been described [13]. Here we show that endothelial cells from bovine aortae harbour an enzyme on their luminal surface which catabolizes Ap_4A. Some biochemical characteristics of this activity have been explored.

MATERIALS AND METHODS

Reagents

[2,8-^{3}H]Ap_4A was purchased from Amersham (TRQ. 4405; 4.3 Ci/mmol). Analysis by t.l.c. revealed a purity of over 95%. [5′,8-^{3}H]ATP (46 Ci/mmol), [2-^{3}H]ADP (16 Ci/mmol), [2-^{3}H]AMP (19.3 Ci/mmol) were from Amersham International. Ap_4A, Ap_3A, ATP, ADP, AMP and adenosine were from Sigma (Munich, Germany). Thin-layer plastic sheets coated with PEI-cellulose were obtained from Merck (Darmstadt, Germany). Tissue-culture plastics were from Nunc, Wiesbaden, Germany. Media (RPMI 1640), fetal-calf serum, collagenase, phosphoenolpyruvate, pyruvate kinase and snake-venom phosphodiesterase were from Boehringer, Mannheim, Germany.

Ap_4A (Ap_3A) analyses by coupled luminescence assays

Luciferin–luciferase produces light with ATP that is generated from Ap_4A after hydrolysis with snake-venom phosphodiesterase [14]. The assay for Ap_3A additionally contained phosphoenolpyruvate and pyruvate kinase [15]. The assay contained, in a final volume of 0.3 ml, 30 μl of luciferin–luciferase (AMR; from LKB), 3.8 mM-$MgCl_2$, 25 mM-Hepes (pH 7.75) and 1–10 μl of diluted (with 1 mM-Na_2EDTA) cell-culture supernatant. The reaction was started at room temperature by the addition of snake-venom phosphodiesterase (0.1 μg/ml final concn.). The increase of luminescence was monitored with a photomultiplier (Lumacounter Model 2080; Lumac Systems AG, Basel, Switzerland). The light readings ('counts') were monitored every 30 s (integration mode) until reaching the maximum value after 3–5 min.

Abbreviations used: Ap_4A, adenosine(5′)tetraphospho(5′)adenosine; Ap_3A, adenosine(5′)triphospho(5′)adenosine; PEI, poly(ethyleneimine); PBS, phosphate-buffered saline (6.5 mM-Na_2HPO_4/1.5 mM-KH_2PO_4/2.7 mM-KCl/0.14 M-NaCl, pH 7.4).

‡ To whom correspondence and reprint requests should be sent at: Institut für Biochemie (Medizin Fakultät), Fahrstrasse 17, 8520 Erlangen, Federal Republic of Germany.

The assay for Ap_3A contained 1.9 mM-phosphoenolpyruvate and pyruvate kinase (0.1 mg/ml).

The high dilution of the samples in the assays (more than 100-fold) suppressed potential quench factors in the culture medium. This, however, was routinely controlled by adding standard solutions. The standard curves were linear, as published [14,15]. Release of ATP-consuming enzymes into the cell supernatant was also found to be negligible because of the high dilution.

T.l.c.

Cell-culture supernatants (3 μl) were directly spotted on to PEI-cellulose thin-layer sheets, a procedure that was found to stop all enzymic reactions within less than 30 s. The efficacy of the stopping method has been proven by determining the kinetics of Ap_4A hydrolysis in the presence of highly active snake-venom phosphodiesterase by comparing different techniques: (i) direct spotting on the thin-layer plate; (ii) stopping of the reaction by dilution with EDTA (or EDTA plus substrate in high excess) before spotting on PEI-cellulose. The kinetics were found to be indistinguishable (resolution limit about 30 s).The effective stop by EDTA has been controlled by a 30 min time interval between the dilution step and the spotting on the plate.

A mixture of marker nucleotides was added to each starting point of the layer. Separation was achieved with water and, after drying, with 0.75 M-LiCl. After drying the plates, the nucleotides were marked under u.v. light and cut off. The thin-layer pieces were counted in a scintillation mixture {toluene/0.5% PPO (2,5-diphenyloxazole)/0.03% dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene]}.

H.p.l.c.

Reverse-phase h.p.l.c. was used essentially as described [15]. Briefly, separation was achieved by two 30 cm-columns (Bondapak C18 from Waters) at 37 °C with a linear gradient of 0–6% methanol in 0.1 M-$NH_4H_2PO_4$ (pH 5.1) during the first 30 min, continued with a constant methanol concentration (6%) in the buffer.

Experiments on freshly extracted tissue

Fresh aortas from calves were kept on ice less than 2 h after slaughter. The aortae were cut once along their length. The luminal surface was then rinsed several times with cold phosphate-buffered saline (PBS). A bottomless plastic microtitre plate was gently pressed on to the luminal (endothelial) surface of the aorta. The bottom of the microtitre wells (i.e. the endothelial-cell layer) was then gently rinsed twice with prewarmed PBS before adding 100 μl of incubation mixture containing the nucleotides to be tested. At different times during the incubation at 37 °C in the CO_2 incubator, aliquots (3 μl) were withdrawn and spotted on thin-layer plates. Before any withdrawal, the supernatants were mixed by gentle sucking up and down with a micropipette.

Cell-culture methods

Primary cultures of bovine aortic endothelial cells were prepared by collagenase treatment of the luminal surface of calf thoracic aortae [16]. Briefly, aortae were cut lengthwise and rinsed several times with ice-cold sterile PBS. A sterile metallic frame (3 cm × 12 cm) was pressed on to the endothelial side of the aortae, which were kept horizontally in the sterile hood. After rinsing with prewarmed PBS, the cell surface was incubated with 0.2% collagenase in RPMI medium containing penicillin and streptomycin. After incubation (15 min, 37 °C) the endothelial cells were dissociated from the subcellular matrix as small sheets containing up to some dozens of polygonally shaped cells. The dissociation of the cells was forced by gentle pipetting of the collagenase solution on to the aortic surface. After low-speed centrifugation and washing, the resuspended cells were seeded into plastic dishes. The concentration of fetal-calf serum in the medium (RPMI 1640) was 20% (v/v). Secondary cultures were adapted to 15% serum. All experiments were done with dishes or multi-well plates, which were covered confluently with cells producing a typical 'cobblestone' appearance under the microscope. Kinetic studies of the ecoenzyme activities were performed as described above under Experiments on freshly extracted tissue.

RESULTS

Identification of Ap_4A hydrolase *in situ*

The inner (luminal) surface of cell aortae exhibits an enzymic activity which splits Ap_4A (Fig. 1*a*), yielding ATP plus AMP, which both only transiently accumulated because of further degradation by ecto-ATPase (Fig. 1*b*) and ecto-AMPase (5′-nucleotidase). The resulting adenosine was then taken up by the aortic tissue. Under identical conditions, at 1 μM initial concentration, ATP was hydrolysed about five times faster than Ap_4A, as reflected in the corresponding half-lives of 5.2 ± 1.2 min ($n = 5$) for ATP and of 24.8 ± 5.2 min ($n = 7$) for Ap_4A. The rate of degradation of ADP (not shown) was almost identical with that of ATP.

Because the inner surface of blood vessels is densely covered by a monolayer of endothelial cells, the experiments suggested that the Ap_4A hydrolase is an ectoenzyme of those cells, which are known to have hydrolytic ectoenzymes for nucleotides.

Identification of Ap_4A hydrolase and Ap_3A hydrolase on cultured endothelial cells

During cultivation *in vitro*, vascular endothelial cells from different sources retain ectoenzyme activities which degrade ATP, ADP and AMP [13,17]. Fig. 2 shows that cultured endothelial cells from bovine aortae expressed an Ap_4A hydrolase (half-life of 1 μM-Ap_4A, 75 ± 12 min; $n = 10$) as well as an Ap_3A hydrolase (half-life of 1 μM-Ap_3A, 245 ± 90 min; $n = 4$). Compared with the activities of ecto-ATPase and ecto-ADPase the activity of the Ap_4A hydrolase was about 10 times lower, the corresponding activity of the Ap_3A hydrolase being about 30 times lower. Long-term incubations of Ap_4A or Ap_3A, also at higher concentrations, revealed no accumulation of degradation products in the extracellular fluid. Also, adenosine could not be detected by h.p.l.c. analysis after long-term incubation with either Ap_3A or Ap_4A.

Systematic investigations of the relative rates of nucleotide degradation revealed a selective loss of ectoenzymes from the outer cell surface during serial cultivation of the cells *in vitro*. After more than 20 passages the hydrolytic capacities for Ap_4A and AMP were drastically diminished, whereas the rates of ATP or ADP degradation were almost unchanged and very similar to the condition *in situ*. Fig. 3 demonstrates the ADPase and Ap_4Aase after 26 passages. The 5′-nucleotidase (AMPase) was almost completely lost, as

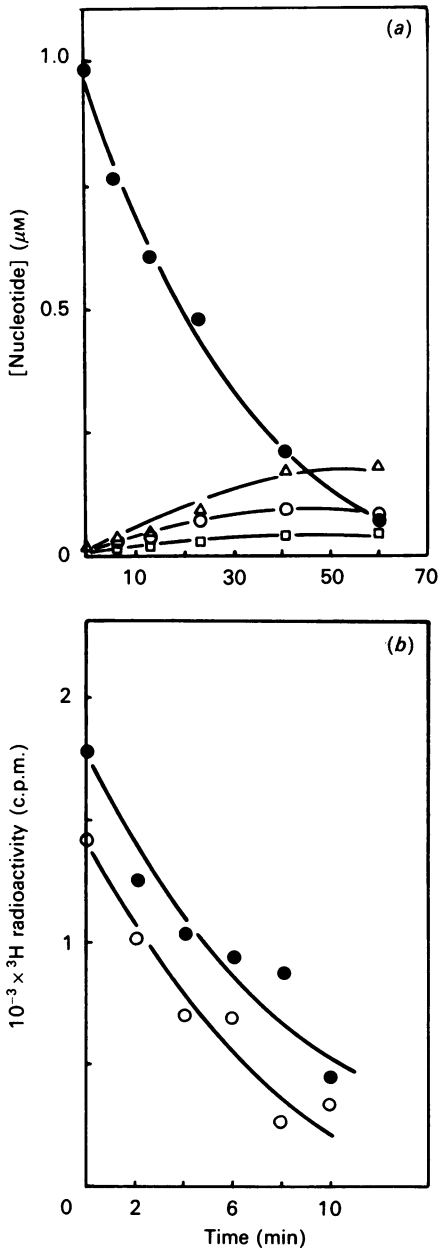

Fig. 1. Degradation of Ap_4A and ATP by bovine aortic endothelial cells *in situ*

The luminal surface of an aorta from a freshly slaughtered calf was incubated with nucleotides as described under in the Materials and methods section. (*a*) Tritiated Ap_4A (●; 1 μM initial concn.) was incubated for 1 h at 37 °C. Aliquots of the cell supernatant were separated by thin-layer chromatography. The identified radioactive products were: △, AMP; ○, ATP; □, adenosine. (*b*) [^{3}H]ATP (○, 1 μM; ●, 10 μM) was incubated for 10 min under identical conditions; 1.4×10^3 c.p.m. of ^{3}H corresponds to 1 μM-ATP (○), and 1.8 ± 10^3 c.p.m. corresponds to 10 μM (●).

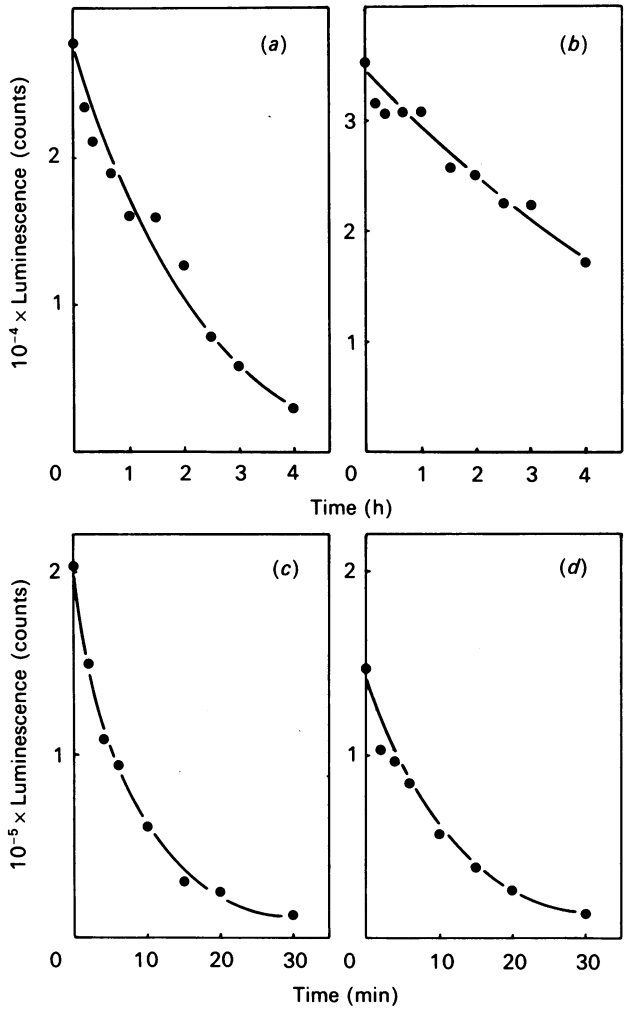

Fig. 2. Catabolic capacities of nucleotide ectoenzymes of intact endothelial cells grown in culture dishes

Ap_4A (*a*; 1 μM initial concn., corresponding to 2.7 ± 10^4 luminescence counts) and Ap_3A (*b*; 1 μM, corresponding to 3.6×10^4 counts) were incubated for 4 h. Samples (5 μl) were withdrawn at various times and diluted 10-fold with ice-cold EDTA. The samples were kept frozen until measurements were made. ATP (*c*; 3 μM, corresponding to 2×10^5 counts) and ADP (*d*; 3 μM, corresponding to 1.4 ± 10^5 counts) were incubated for 30 min. The amounts of nucleotides in the extracellular milieu were determined by bioluminescence techniques.

can be concluded from the quantitative accumulation of AMP in both assays. For further characterization of Ap_4A hydrolase, cells from passages between 4 and 12 were used.

In order to confirm that Ap_4A hydrolysis was due to an ectoenzyme and not due to leakage of cytoplasmic enzymes, the following experiments were carried out. Firstly, 'conditioned media', i.e. media which had been incubated with the cultured cells for up to 90 min, were tested for Ap_4A hydrolase activity. Compared with the activity of the 'complete' assay mixture, the activity of the extracellular fluid was less than 10% (7.3 ± 3.7; $n = 4$). Alternatively, the incubation with the cells was interrupted by transferring the medium conditioned over the cells into cell-free vessels. The degradation of Ap_4A decreased to less than 10% (Fig. 4). This experiment was performed several times at different times of medium transfer. Secondly, the release of lactate dehydrogenase was measured as a cytoplasmic marker. The activity of lactate dehydrogenase was less than 15% (7.8 ± 6.1; $n = 4$) after 90 min of incubation (as compared with total cellular lysates). The release of ATP-degrading activity

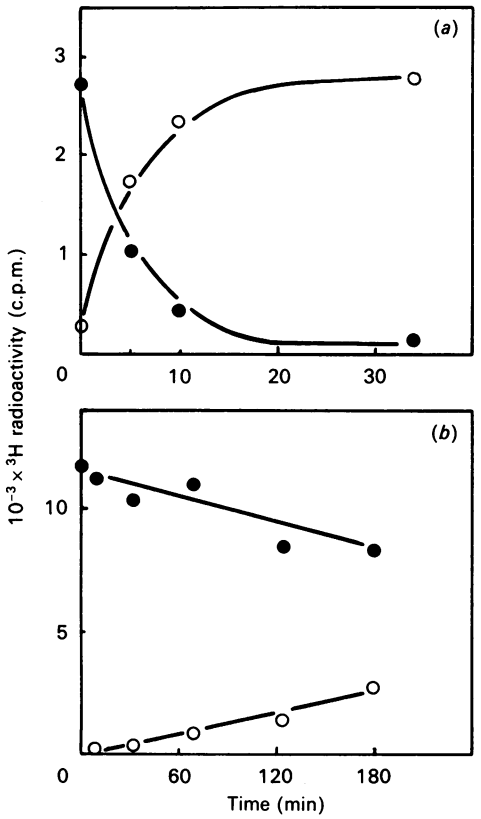

Fig. 3. Degradation of Ap_4A and ADP by endothelial cells after long-term culturing (passage 26)

(*a*) [^{3}H]ADP (●; 2.8×10^3 c.p.m. correspond to 1 μM-nucleotide) was incubated for 30 min. AMP (○) is shown as a product of hydrolysis. (*b*) [^{3}H]Ap_4A (●; 12×10^3 c.p.m. correspond to 1 μM-nucleotide) was incubated for 3 h. AMP (○) is shown as product.

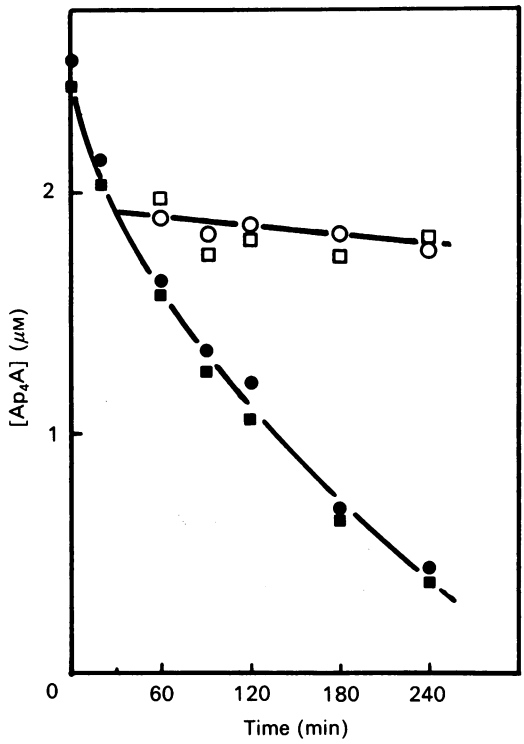

Fig. 4. Ap_4A hydrolase is cell-associated

Two wells (squares, circles) with endothelial cells grown to confluency were incubated with [^{3}H]Ap_4A (2.5 μM initial concn.). At the times indicated (closed symbols), aliquots were withdrawn and analysed for nucleotide concentration by thin-layer technique. After 30 min of incubation, part of the extracellular medium was transferred to cell-free vessels and further incubated. Aliquots were analysed analogously (open symbols).

after 90 min of incubation was 11% (mean of two determinations). Thirdly, total cellular lysates (prepared with 0.2% Triton X-100), as well as mechanically prepared homogenates of the scraped cells, revealed almost identical activities of Ap_4A hydrolysis when compared with the media containing the cells. This suggests that a major part of the Ap_4A-hydrolysing capacity is membrane-bound and also that partial release of cytoplasm would hardly alter the time course of Ap_4A hydrolysis.

Kinetic analysis of Ap_4A and ATP hydrolysis

The concentration-dependence of initial velocity of Ap_4A degradation revealed saturation between 10 μM- and 20 μM-AP_4A, suggesting a Michaelis constant in the micromolar range. Up to 50 μM there was no indication for a second constant of lower affinity. To determine the apparent K_m value, two techniques have been applied for initial-rate analysis, which gave fairly consistent results (Fig. 5). The K_m value for Ap_4A was 9.9 ± 7.8 μM ($n = 6$).

The affinity of ecto-ATPase for its substrate ATP was much lower (Fig. 6), with a K_m of 780 μM (mean of three determinations). This is in good accordance with published data on the ecto-ATPase of endothelial cells from other species [17,18].

Effects of metal ions on Ap_4A hydrolase

Fig. 7 demonstrates that ecto-Ap_4A hydrolase needs bivalent-metal ions for optimal activity. Millimolar concentrations of Mg^{2+} were necessary for maximal stimulation, whereas at 10 μM and also at 200 μM the hydrolysis of Ap_4A was drastically inhibited. The Figure shows the results of one experimental series, which was repeated with another passage of the cells and gave identical results. Analyses in the absence of Mg^{2+} or in the presence of EDTA were hampered by the sensitivity of the endothelial cells, which lost intercellular contact and changed their surface morphology within 1 h of treatment. Ecto-ATPase remained fully active at 10 μM-Mg^{2+} (result not shown).

With Mn^{2+} (2 mM), maximal velocity could be reached after washing the cells in the presence of 10 μM-Mg^{2+}. Ca^{2+} and Zn^{2+}, however, were not able to substitute for Mg^{2+} but were found to be inhibitory (results not shown).

Effect of other nucleotides on the hydrolysis of Ap_4A

The hydrolysis of Ap_4A was not inhibited by the presence of equimolar amounts of Ap_3A. Higher concentrations of Ap_3A reduced the rate of Ap_4A degradation. Initial-rate analysis with variable substrate concentrations suggested a competitive mode of inhibition of Ap_3A. ATP at 25-fold excess over Ap_4A

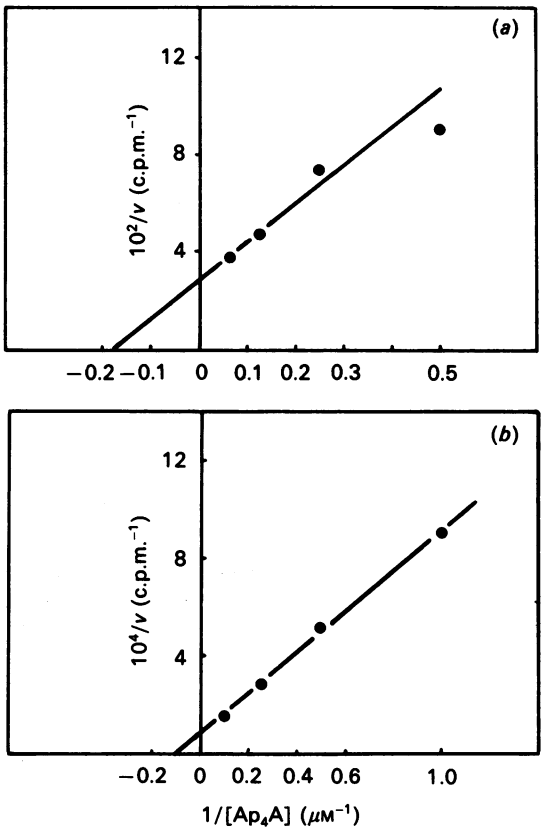

Fig. 5. Lineweaver–Burk analysis of Ap_4A hydrolysis by endothelial cells

(*a*) Measurement by thin-layer technique with [^{3}H]Ap_4A at variable concentration (2–16 μM). The double-reciprocal plot gives a Michaelis constant of 6 μM (10^3 c.p.m. correspond to 2 pmol of product formed/min). (*b*) Measurement by bioluminescence technique with unlabelled Ap_4A at 1–10 μM. The double-reciprocal plot gives a Michaelis constant of 10 μM (4×10^3 luminescence counts/min correspond to 1 picomol of substrate degraded/min).

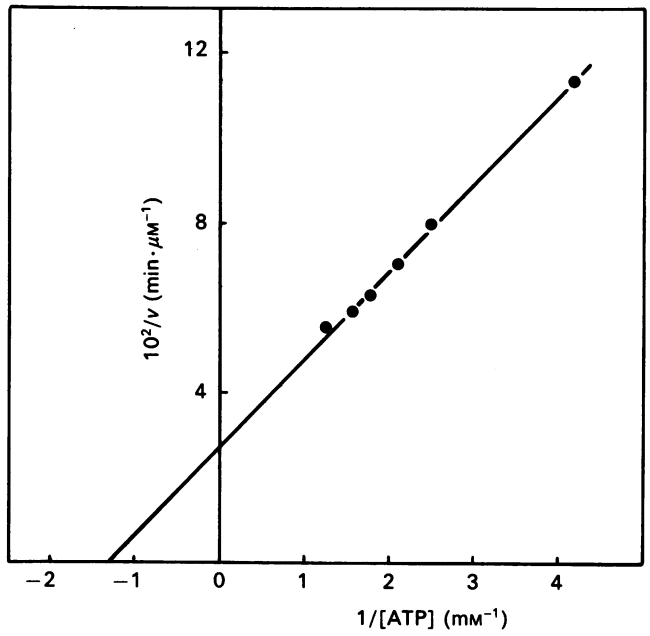

Fig. 6. Lineweaver–Burk analysis of ATP hydrolysis by endothelial cells

[^{3}H]ATP at variable concentrations (0.24–0.8 mM) was added and analysed by the thin-layer technique.

(1 μM initial concn.) did not retard the hydrolysis of Ap_4A. ATP at 125 μM and 500 μM, however, significantly inhibited the hydrolysis of Ap_4A. Nevertheless, there was no extracellular accumulation of radioactive ATP and AMP as products.

DISCUSSION

Ap_4A and Ap_3A are stored in dense granules of blood platelets and can be quantitatively released after platelet activation. Their extracellular concentration is then in the micromolar range [2,3], which was found to be sufficient for the modulation of platelet aggregation [4,5,7] as well as of the tone of vascular smooth muscle [9,10]. Intact endothelial cells derived from bovine calf aortae degrade extracellular Ap_4A and Ap_3A. Thus, besides the soluble enzymes of blood plasma [11,12,20], the endothelial lining of blood vessels is involved in limiting the biological effects of the dinucleotides. The ectoenzymes of endothelial cells might become predominantly important in very small vessels or, especially, at the platelet/endothelial-cell interface during thrombus formation.

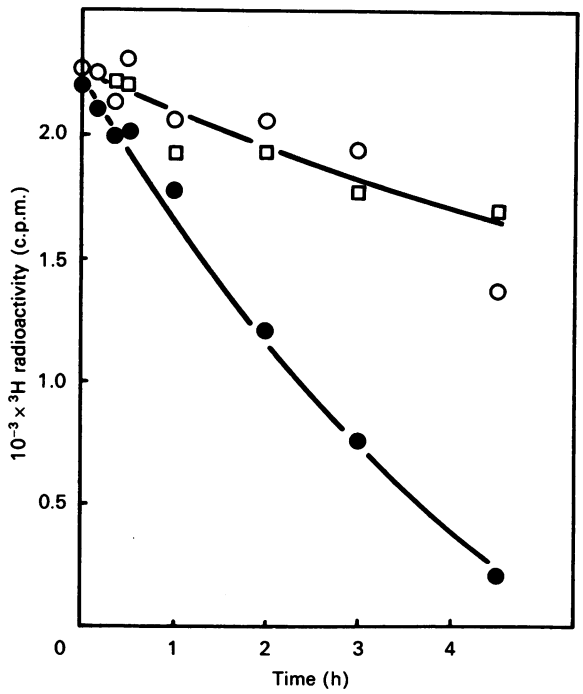

Fig. 7. Mg^{2+}-dependence of Ap_4A hydrolase

Confluently grown endothelial cells were washed with phosphate-buffered saline containing 10 μM-$MgCl_2$. [^{3}H]Ap_4A in buffer containing variable amounts of $MgCl_2$ was added to measure Ap_4A hydrolysis (□, 10 μM-$MgCl_2$; ○, 200 μM; ●, 5 mM); 2.4×10^3c.p.m. of ^{3}H correspond to 1 μM-Ap_4A (initial concn.).

Tissue-culture techniques provide a pure cell type which in many aspects resembles the corresponding cell *in vivo*. Cultured endothelial cells of porcine aortae have been successfully investigated for their role in extracellular nucleotide metabolism [13,17,18,21,22]. Quite uniquely for enzymological studies, ectoenzymes can easily be investigated in their physiological surroundings, which might be crucial for activity and function. During cultivation *in vitro*, however, we have observed a gradual loss of ectoenzymic activities towards Ap_4A, Ap_3A and also AMP. Ecto-ATPase and ecto-ADPase were constantly expressed at the cell surface. Whether this reflected a controlled and selective down-regulation of the genes coding for ectoenzymes during cultivation *in vitro* is not known. It would also be important to know whether factors in the blood or in the subendothelial tissue are required for continuous ectoenzyme expression in the endothelial cells. The selective loss of Ap_4A hydrolase supports the conclusion that this ectoenzyme is a protein moiety not identical with ATPase, ADPase or AMPase. The differential behaviour upon withdrawal of Mg^{2+} gives additional support. Ap_4A hydrolase is possibly identical with Ap_3A hydrolase, since competitive inhibition has been observed. The hydrolase purified from blood plasma [11], which also degrades Ap_4A and Ap_3A, is not identical with, or derived from, the ectoenzyme described here, since the dependence on metal ions is different. Intracellularly, however, highly specific hydrolases exist for either Ap_4A [23] or Ap_3A [24].

For physiological aspects the relative rates of nucleotide degradation as well as the affinities of the enzymes for their corresponding substrates seem to be relevant. Compared with ATP and ADP, the rate of hydrolysis of Ap_4A is slower by one order of magnitude (when measured at micromolar concentrations). Because of the high affinity of Ap_4A hydrolase, which also means early saturation by substrate, the difference between ATP or ADP is even more pronounced at higher nucleotide concentrations. The Michaelis constant for ATP has been found to be more than 50 times higher, in good accordance with reports on porcine endothelial cells [17,18].

Thus, on the endothelial lining, the dinucleotides live significantly longer than the normal adenine nucleotides. A longer half-life of Ap_4A and Ap_3A has also been found in blood plasma, serum or in whole blood [6,25].

The manner by which the dinucleotide is cleared by Ap_4A hydrolase seems to be asymmetrical, yielding ATP plus AMP, which could at least transiently be detected in the extracellular fluid. A higher accumulation of ATP would be a prerequisite for detecting ADP as well as to substantiate a stepwise further pathway of degradation which has been suggested for porcine endothelial cells [17,18]. We failed to accumulate radioactive ATP by adding high amounts of unlabelled ATP to the assay containing labelled Ap_4A. We were also not able to block the degradation of AMP by the ecto-AMPase inhibitor adenosine 5′-[$\alpha\beta$-methylene]diphosphate [36] without also inhibiting Ap_4A hydrolysis.

We did not measure the activity of probably co-existing ecto-ATP pyrophosphatase or nucleoside diphosphate kinase, which have been found on porcine endothelial cells [17,37] and could take part in the catabolism of extracellular nucleotides. ATP pyrophosphatase could even be identical with Ap_4A hydrolase, since highly purified Ap_4A hydrolase from human plasma also cleaves ATP, yielding AMP [11]. A detailed analysis of the branched degradation route of all products resulting from Ap_4A degradation was beyond the scope of this investigation.

The recovery of radioactivity in the extracellular milieu could be improved by adding dipyridamole, which inhibits adenosine uptake by endothelial cells [17]. When analysing trichloroacetic acid-extracts of cells, it could be shown that radioactivity stemming from Ap_4A degradation ended up as intracellular nucleotides and macromolecular incorporation.

The recovery of radioactivity in the extracellular fluid, however, was complete when aged cultures, which had lost ecto-AMPase (Fig. 3), were analysed. With those cells the final product of nucleotide catabolism was AMP.

In common with other ectoenzymes which hydrolyse nucleotides [17], the Ap_4A hydrolase needs bivalent-metal ions for maximal catalytic activity. Mg^{2+} can be replaced by Mn^{2+}. The concentration-dependence of Mg^{2+} revealed that maximal activity is reached only at millimolar concentrations, which is much higher than the dissociation constant of the Mg–Ap_4A complex (28 μM; ref. [19]). This suggests that the enzyme needs more Mg^{2+} for its optimal conformation in the membrane.

Ap_4A hydrolase activity is affected by other nucleotides. ATP and ADP, which are co-released from activated platelets in high excess [2], may retard the catabolism of the dinucleotides at the endothelial surface, giving them the chance to reach other target cells. A retarding effect of ATP has also been found for Ap_3A hydrolysis on porcine endothelial cells [13]. The inhibition of Ap_4A degradation by ATP is even more pronounced with the catabolic enzymes found in blood plasma [25].

The dinucleotides Ap_3A and Ap_4A, together with their hydrolytic enzymes, probably exhibit dual functions in the blood and in the vessel wall tissues.

1. The dinucleotides are biologically active in themselves in interfering with purinergic receptors, which have been found on platelets, endothelial cells, smooth-muscle cells and other cell types (for a review, see [26,27]). The catabolic enzymes seem to regulate the concentration of the extracellular nucleotides and thus modulate receptor-mediated cell responses [28,29].

2. The dinucleotides are relatively 'long-lived' sources for ATP and ADP, which are both enzymically releasable. The aggregating effect of Ap_3A on blood platelets, for instance, strictly depends on the activity of hydrolases in the plasma which produce ADP, a highly potent agonist of platelet activation [4,5]. It is tempting to speculate that the Ap_4A hydrolase described here plays a functional role in generating short-lived ATP near the ATP receptor, which has been functionally identified on endothelial cells from various species [30–35]. It is also conceivable that Ap_4A hydrolase is part of such receptor systems. The hydrolytic enzymes for ATP and ADP on endothelial cells, however, seem not to be identical with the corresponding purine receptor, as has been concluded from indirect evidence [22].

This work was supported by the Deutsche Forschungsgemeinschaft (grants to A.O. and R.B.) and we gratefully acknowledge the continuous support of Professor W. Kersten and the excellent technical assistance of Mrs. R. Zitzmann, Mrs. I. Zenger and Mr. O. Wiesinger.

REFERENCES

1. Flodgaard, H. & Klenow, H. (1982) Biochem. J. **208**, 737–742
2. Luthje, J. & Ogilvie, A. (1983) Biochem. Biophys. Res. Commun. **115**, 253–260
3. Luthje, J., Miller, D. & Ogilvie, A. (1987) Blut **54**, 193–200
4. Lüthje, J. & Ogilvie, A. (1984) Biochem. Biophys. Res. Commun. **118**, 704–709
5. Lüthje, J., Baringer, J. & Ogilvie, A. (1985) Thromb. Haemostasis **54**, 469–471
6. Lüthje, J., Baringer, J. & Ogilvie, A. (1985) Blut **51**, 405–413
7. Harrison, M. J., Brossmer, R. & Goody, R. (1975) FEBS Lett. **54**, 57–60
8. Louie, S., Kim, B. K. & Zamecnik, P. (1988) Thromb. Res. **49**, 557–566
9. Pohl, U., Busse, R., Herrmann, I. & Ogilvie, A. (1987) Biol. Chem. Hoppe-Seyler **368**, 1094
10. Busse, R., Ogilvie, A. & Pohl, U. (1988) Am. J. Physiol. **254**, H828–H832
11. Lüthje, J. & Ogilvie, A. (1985) Eur. J. Biochem. **149**, 119–127
12. Lüthje, J. & Ogilvie, A. (1987) Eur. J. Biochem. **169**, 385–388
13. Goldman, S. J., Gordon, E. L. & Slakey, L. L. (1986) Circ. Res. **59**, 362–366
14. Ogilvie, A. (1981) Anal. Biochem. **115**, 302–307
15. Ogilvie, A. & Jakob, P. (1983) Anal. Biochem. **134**, 382–392
16. Jaffe, E. A., Nachman, R. L., Becker, C. G. & Minick, C. R. (1973) J. Clin. Invest. **52**, 2745–2754
17. Pearson, J. D., Carleton, J. S. & Gordon, J. L. (1980) Biochem. J. **190**, 421–429
18. Gordon, E. L., Pearson, J. D. & Slakey, L. L. (1986) J. Biol. Chem. **261**, 15496–15504
19. Holler, E., Holmquist, B., Vallee, B. L., Taneja, K. & Zamecnik, P. (1983) Biochemistry **22**, 4924–4933
20. Lüthje, J. & Ogilvie, A. (1987) Clin. Chim. Acta **169**, 183–188
21. Cusack, N. J., Pearson, J. D. & Gordon, J. L. (1983) Biochem. J. **214**, 975–981
22. Pearson, J. D. & Cusack, N. J. (1985) Eur. J. Biochem. **151**, 373–375
23. Ogilvie, A. & Antl, W. (1983) J. Biol. Chem. **258**, 4105–4109
24. Lobaton, D., Vallejo, C. G., Sillero, A. & Sillero, M. A. G. (1975) Eur. J. Biochem. **50**, 495–501
25. Lüthje, J. & Ogilvie, A. (1988) Eur. J. Biochem. **173**, 241–245
26. Burnstock, G. (ed.) (1981) Purinergic receptors, Chapman and Hall, London
27. Gordon, J. L. (1986) Biochem. J. **233**, 302–319
28. Manery, J. F. & Drydon, E. E. (1979) in Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides (Baer, H. P. & Drummond, G. I., eds.), pp. 323–339, Raven Press, New York
29. Pearson, J. D. & Gordon, J. L. (1985) Annu. Rev. Physiol. **47**, 617–627
30. Furchgott, R. F. (1984) Annu. Rev. Pharmacol. Toxicol. **24**, 175–195
31. Pearson, J. D., Slakey, L. L. & Gordon, J. L. (1983) Biochem. J. **214**, 273–276
32. Kennedy, C., Delbro, D. & Burnstock, G. (1985) Eur. J. Pharmacol. **107**, 161–168
33. Lückhoff, A. & Busse, R. (1986) J. Cell. Physiol. **126**, 414–420
34. Needham, L., Cusack, N. J., Pearson, J. D. & Gordon, J. L. (1987) Eur. J. Pharmacol. **134**, 199–209
35. Forsberg, E. J., Feuerstein, G., Shohami, U. & Pollard, H. B. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 5630–5634
36. Pearson, J. D. (1985) Methods Pharmacol. **6**, 83–107

Received 20 May 1988/13 October 1988; accepted 21 October 1988