Hydrolysis of Diadenosine 5',5''-P',P''-Triphosphate (Ap₃A) by Porcine Aortic Endothelial Cells

SAMUEL J. GOLDMAN, ELLEN L. GORDON, AND LINDA L. SLAKEY

Diadenosine triphosphate is present in platelet-dense granules and released quantitatively on platelet aggregation. We have found that intact porcine aortic endothelial cells can efficiently hydrolyze extracellular diadenosine triphosphate. The products of diadenosine triphosphate hydrolysis are adenosine monophosphate and adenosine diphosphate. Adenosine diphosphate is a potent stimulus of platelet aggregation. Since platelet-dense granules contain high concentrations of adenosine triphosphate and adenosine diphosphate, we examined endothelial cell hydrolysis of a mixture of diadenosine triphosphate and adenosine triphosphate. We find that the presence of adenosine triphosphate severely inhibits the hydrolysis of diadenosine triphosphate. Thus, although endothelial cells can rapidly clear extracellular diadenosine triphosphate, during platelet aggregation the hydrolysis of diadenosine triphosphate may be slow due to the presence of high concentrations of other adenine nucleotides. This phenomenon may be important physiologically if, as current evidence implies, diadenosine triphosphate is involved in the maintenance of hemostasis. (Circulation Research 1986; 59:362-366)

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Materials and Methods

Pig aortic endothelial cells were grown as described in Dickinson and Slakey. Pig skin fibroblasts were obtained by trypsin digestion of subcutaneous connective tissue from a newborn pig. Tissue from different areas was pooled, minced, and incubated for 30 minutes before plating out in a small Falcon flask. Nonadherent debris was rinsed away 1 day later. Serial subcultures were prepared from the confluent primary cultures. Cells were grown in DME + 10% fetal calf serum.

Experiments to determine the products of Ap₃A hydrolysis and the kinetic constants of hydrolysis were done with endothelial cells grown to confluence on 35-mm petri dishes as described previously. Cells were incubated in 0.5 ml of DME buffered with 20 mM HEPES, pH 7.4 at 37°C with gentle shaking. At appropriate times a small aliquot (20–50 μl) was removed and analyzed by reverse-phase HPLC (Shandon ODS-Hypersil) on a Varian Vista 5500 Liquid Chromatograph. HPLC was performed by a modifica-
tion of the procedures of Simmonds et al.\textsuperscript{11} The method runs isocratically for 10 minutes using 0.05 M \(NH_4H_2PO_4\) (Solvent A) followed by a linear gradient from 100\% Solvent A to 70\% 0.05 M \(NH_4H_2PO_4\)-30\% methanol (Solvent B). Solvent B then runs isocratically from 25 to 30 minutes. Absorbance of peaks was monitored with an ultraviolet detector set at 254 nanometers, and peak areas were quantitated using a Varian 402 Chromatography Data System. For kinetic experiments aliquots from a single dish were taken at two successive time points (e.g., 2 minutes and 4 minutes) and the rate of hydrolysis was determined. Only samples where the reaction rate was linear over the tested time range were designated initial velocities for kinetic calculations.

Experiments designed to assess the effect of ATP on Ap\(_3\)A hydrolysis were done using a minicolumn of endothelial cells grown on Biosilon beads (Nunc) as described by Pearson et al.\textsuperscript{12} One milliliter of HEPES-buffered DME containing an appropriate concentration of substrate(s) was recirculated through a minicolumn containing approximately 3 \(\times\) 10\(^6\) endothelial cells. At appropriate times small aliquots (20 \(\mu\)l) were taken and analyzed for hydrolysis products by HPLC. Control experiments show that there is no detectable release (by HPLC analysis) of adenine nucleotides from endothelial cells during 15 minutes incubation at 37\° C. Ap\(_3\)A is stable in medium alone at 37\° C for 30 minutes.

Tissue culture reagents were obtained from Flow Laboratories, Rickmansworth, Herts, U.K. HPLC grade solvents were from Rathburn Chemical Co., Ltd., Poole, Dorset, U.K. Shandon ODS-Hypersil 5 \(\mu\)m Hyperspheres column packing was obtained from Shanda Southern Products Ltd., Cheshire, U.K. Ap,A and ATP were from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

**Results**

**Hydrolysis of Ap,A Produces ADP and AMP**

Figure 1A is an HPLC trace of the degradation products generated by incubating 15 \(\mu\)M Ap,A with intact endothelial cells. The major products are AMP and adenosine, with smaller amounts of ADP and inosine. Since vascular cells are known to possess ectoenzymes that efficiently hydrolyze ATP, ADP, and AMP,\textsuperscript{13} these products could be generated if Ap,A were directly hydrolyzed to ADP and AMP (open arrows, Figure 1 inset) or alternatively if Ap,A were initially hydrolyzed to ATP and adenosine (closed arrows, Figure 1 inset). To distinguish between these possibilities we incubated Ap,A with intact endothelial cells in the presence of \(\alpha,\beta\)-methylene ADP (AOPCP), a potent inhibitor of 5' nucleotidase, the enzyme that converts 5' AMP to adenosine. If the hydrolysis of Ap,A is generating ADP and AMP, then the addition of AOPCP should block the production of adenosine. If ATP and adenosine are the initial products of hydrolysis, AOPCP will not affect the generation of adenosine. Figure 1B shows that in the presence of 25 \(\mu\)M AOPCP there is no adenosine produced by hydrolysis of 15 \(\mu\)M Ap,A. The concentrations of nucleotides observed are summarized in Table 1 and show that ADP and AMP are the initial products of Ap,A hy-

![Figure 1](http://circres.ahajournals.org/)

**Figure 1. Products of hydrolysis of Ap,A.** High-pressure liquid chromatograph tracing showing the products of endothelial cell hydrolysis of 15 \(\mu\)M Ap,A in the absence (A) and presence (B) of 25 \(\mu\)M AOPCP. Peaks are labelled to indicate products. Unlabelled peaks are components of DME. Incubation was for 15 minutes at 37\° C.
TABLE 1. Products of Hydrolysis of Ap₃A

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>− AOPCP concentration (µM)</th>
<th>+ AOPCP concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>ADP</td>
<td>1.3</td>
<td>1.7</td>
</tr>
<tr>
<td>AMP</td>
<td>11.3</td>
<td>18.3</td>
</tr>
<tr>
<td>ADO</td>
<td>7.5</td>
<td>0.1</td>
</tr>
<tr>
<td>INO</td>
<td>2.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Ap₃A</td>
<td>4.2</td>
<td>7.0</td>
</tr>
<tr>
<td>AOPCP</td>
<td>0</td>
<td>25</td>
</tr>
</tbody>
</table>

N.D. = Not Detected.

Conditions of the experiment are identical to those described in Figure 1. The concentrations of the products were calculated from the integrated absorbance of each peak at 254 nm.

hydrolysis. Since ADP is further metabolized to AMP, the final concentration of AMP after 15 minutes incubation is substantially greater than the concentration of ADP.

In order to determine whether this activity is present in comparable levels in cultured pig cells from other tissues, we have also measured hydrolysis of Ap₃A by pig skin fibroblasts. The extent of hydrolysis of Ap₃A by intact skin fibroblasts is very much less than by intact aortic endothelial cells. The initial velocity for hydrolysis of 100 µM Ap₃A was 0.3 nmol/minute per 10⁶ cells for skin fibroblasts compared to 3.9 nmol/minute per 10⁶ cells for aortic endothelial cells.

Kinetic Analysis of Ap₃A Hydrolysis

The dependence of initial velocity of Ap₃A hydrolysis on substrate concentration is shown in Figure 2. The enzymatic activity appears to obey simple Michaelis-Menton kinetics. The Eadie-Hofstee plot yields a Km of 20 µM. The Vₘₐₓ is 4.6 nmol/minute per 10⁶ cells.

Effect of ATP on the Hydrolysis of Ap₃A

The above data show that intact endothelial cells have a high-affinity activity that can hydrolyze extracellular Ap₃A. Since Ap₃A is present in platelet-dense granules and released on platelet aggregation with high concentrations of other adenine nucleotides (predominantly ATP and ADP), it was of interest to determine whether the hydrolysis of Ap₃A was affected by ATP. Lütjhe and Ogilvie have shown that the ratio of ATP:Ap₃A in a platelet releasate is about 25:1. Thus we have examined hydrolysis of a mixture containing 500 µM ATP and 20 µM Ap₃A by endothelial cells. These concentrations were chosen to reflect the high concentrations of nucleotides that are likely to be present at the platelet-endothelial cell interface during thrombus formation.

Figure 3 shows the pattern of hydrolysis of 500 µM ATP in the presence and absence of 20 µM Ap₃A. As mentioned previously, the endothelial cell surface has ectoenzymes capable of hydrolyzing ATP, ADP, and AMP. It is apparent that the presence of 20 µM Ap₃A has no effect on these activities. However, when comparing the rates of hydrolysis of 20 µM Ap₃A in the absence and presence of 500 µM ATP, the difference is profound (Figure 3B). In the absence of ATP 20 µM Ap₃A is rapidly and quantitatively hydrolyzed. When 500 µM ATP is present in the incubation mixture the rate of hydrolysis of Ap₃A is severely inhibited. The rate of Ap₃A hydrolysis in the presence of ATP remains constant even though the relative concentrations of ATP, ADP, AMP, and adenosine are continually changing throughout the time course of the experiment (see Figure 3A).

Discussion

The presence of Ap₃A and Ap₃A in a metabolically stable, releasable pool in platelets has led us to investigate the pathways of extracellular metabolism and the possible functions of these unusual nucleotides. The data presented here show that intact endothelial cells can hydrolyze extracellular Ap₃A. Current evidence suggests that the cellular location of both Ap₃A and Ap₃A is the platelet-dense granule and that both compounds are quantitatively released on platelet aggregation. Thus in vivo Ap₃A will be released at the endothelial cell surface in the presence of high concentrations of ATP and ADP. Since enzymatic activities are present on the endothelial cell surface that can hydrolyze ATP, ADP, AMP, and Ap₃A, the effective concentrations of these nucleotides and adenosine as well, will be continually changing during the time following their release from platelets. In this context, we have shown that the rate of hydrolysis of 20 µM Ap₃A by endothelial cells is profoundly inhibited by co-incubation with 500 µM ATP. Even after 45 minutes of
incubation, when there is no detectable ATP and the ADP concentration has been reduced to <40 μM, the bulk of the initial Ap₃A remains intact. The ultimate effect of a mixture of adenine nucleotides and Ap₃A on hemostasis would necessarily reflect the relative concentration of each compound at a particular point in time. Thus, it is possible that after all the ATP and ADP released from aggregating platelets are cleared and the inhibition of Ap₃A hydrolysis is relieved, Ap₃A may serve as an additional source of ADP. Our data are consistent with the suggestion by Lüthje and Ogilvie that Ap₃A, via its conversion to ADP, may function as a "long lived" stimulant of platelet aggregation. These same authors have purified a large glycoprotein complex from human plasma which hydrolyzes both Ap₃A and Ap₄A with very high affinity (Kₘ = 1.0 μM and 0.6 μM, respectively). The relation between this activity and the endothelial cell activity is unclear. We have detected Ap₃A hydrolytic activity in a sample of serum-free conditioned medium that has been incubated for 24 hours on a confluent monolayer of cultured endothelial cells (S. Goldman, unpublished observation). Cultured endothelial cells have been shown to synthesize and release in soluble form a number of plasma proteins. Further studies characterizing the substrate specificity and cellular turnover of the endothelial cell nucleotidase described here will be necessary to determine if these activities are related.

Intracellular effects on DNA synthesis and protein kinase activity have been reported for Ap₃A. The data on the intracellular effects of Ap₃A in eucaryotes is sparse. In procaryotes, Ap₃A is one of several adenylated nucleotides proposed to act as "alarmones" signalling cellular crisis events such as oxidation stress and heat shock. A similar role for adenylated nucleotides as alarmones has been proposed for eucaryotes. Ap₃A and Ap₄A presumably do not penetrate normal, intact cell membranes, although in vitro uptake of intact (H, β, γ,32P)-labelled Ap₃A has been reported in virally transformed cells. The question of whether some component(s) released during platelet aggregation can render normal cells permeable to Ap₃A or Ap₄A has not been directly addressed.

If, as current data imply, Ap₃A plays a role in hemostasis, an understanding of its turnover is crucial. Since platelet aggregation and the concomitant release of the platelet's dense granule contents occur in close proximity to the endothelial cell surface, it seems likely that the endothelial cell activity we describe will be an important factor in modulating extracellular Ap₃A levels in vivo.

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References

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FIGURE 3. Hydrolysis of Ap₃A in the presence of ATP. A. The patterns of hydrolysis of 500 μM ATP, in the presence (○△○) or absence (▼▼▼) of 20 μM Ap₃A. □-ATP, △-ADP, ▲-AMP, ▼-adenosine. B. The hydrolysis of 20 μM Ap₃A in the presence (○) or absence (▼) of 500 μM ATP. All incubations were at 37° C.


**KEY WORDS** • diadenosine triphosphate • platelets • endothelial cells • hemostasis • nucleotidase
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