Analogues of diadenosine 5',5''-P1,P4-tetrathosphate (Ap4A) as potential anti-platelet-aggregation agents

PAUL C. ZAMECNIK†, BYUNG KIM‡, MAO JUN GAO§, GRAHAM TAYLOR§, AND G. MICHAEL BLACKBURN§

*Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545; †Platelet Research Products, Inc., Watertown, MA 02171; and §Department of Chemistry, The University, Sheffield, S3 7HF United Kingdom

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ABSTRACT Dense granules of platelets contain a high content of diadenosine 5',5''-P1,P4-tetrathosphate (Ap4A). We have previously demonstrated an antithrombotic effect of this compound in a live rabbit model. In the present study we find that certain synthetic Ap4A analogues are superior to Ap4A in inhibiting ADP-induced aggregation of human platelets. Analogues having a P—C—P bridge located in the P4,P3 position of Ap4A are the most potent inhibitors. These analogues are also resistant to hydrolytic enzymes. Analogues having the above characteristics exhibit competitive inhibition with ADP in the ADP-induced platelet aggregation reaction. These compounds, such as AppCHFPpSpA, may be useful as antithrombotic agents. The analogues ApSpSpSpA and ApSpCHFSpSpA also showed good inhibitory effects on ADP-induced platelet aggregation. In addition, this action of Ap4A and its analogues provides an example of a dinucleotide inducing an antagonistic effect by occupying an extracellular mononucleotide binding site on platelets. It calls attention to the possibility that Ap4A and its analogues may act in a similar way in whole organisms, triggering effector or inhibitory responses in any one of a variety of cells.

Diadenosine 5',5''-P1,P4-tetrathosphate (Ap4A) was first observed as a minor byproduct in the chemical synthesis of ATP (1). It was then found to be present as a biological compound formed in the back reaction of amino acid activation (2, 3) and to be a ubiquitous component of living cells (4, 5). Various metabolic roles for intracellular Ap4A have been reported (6, 7). It is formed principally but not exclusively by the following reactions:

\[
\text{amino acid} + \text{ATP} \rightarrow \text{cognate aminoacyl-tRNA synthetase} \Rightarrow \text{aminoacyl adenylate} + \text{synthetase} + \text{pyrophosphate} \quad (1)
\]

\[
\text{aminoacyl adenylate} + \text{synthetase} + \text{ATP} \Rightarrow \text{Ap4A} + \text{amino acid} + \text{synthetase}. \quad (2)
\]

Blood platelets were observed unexpectedly to contain a relatively high amount of Ap4A, almost exclusively in their dense granules (8). Platelet Ap4A has been regarded as being metabolically inert while in situ, and the physiologic role of Ap4A located in blood platelets has not yet been completely defined. It is, however, known to be a competitive inhibitor for adenosine 5'-diphosphate (ADP) in the platelet aggregation response (9-11). We have previously demonstrated an antithrombotic effect of this compound in an animal model (12). Ap4A has a short half-life in the blood, due to rapid enzymatic hydrolysis (12), a possible limitation to its use as an intravascular therapeutic agent.

A number of Ap4A analogues containing phosphonate and phosphorothioate moieties have been synthesized and found to be resistant to hydrolytic enzymes (13-16). If such analogues were to inhibit platelet aggregation in a manner similar to Ap4A, and to retain a low toxicity, they would be of special value as anti-platelet-aggregation drugs. In the present study, we have found the β-chloromethyl and β-fluoromethyl phosphonates and the α,α'-thiophosphate analogues of Ap4A to be effective anti-platelet-aggregation agents.

The intravascular extracellular presence of Ap4A and its unanticipated high concentration in platelets (8) have stimulated interest in its possible role in the regulation of vascular events. The term “concentration” is used with the caveat that the Ap4A is located inside one tiny dense granule within a platelet. Highly active diadenosyloligophosphate hydrolases are present in serum (17, 18). Vasodilatory and vasoconstrictive effects of Ap4A and Ap3A have been reported, depending on the intactness of the vascular endothelium (19). These ectoenzymatic activities of Ap4A are related to membrane receptors, particularly those in which ADP participates (20-22).

MATERIALS AND METHODS

Venous blood was collected, in 0.1 vol of 3.8% sodium citrate, from healthy human volunteers who had abstained from anti-platelet-aggregation drugs for at least 10 days. Platelet-rich plasma (PRP) was separated from the blood by centrifugation at 150 x g for 10 min at 22°C. The remaining blood was further centrifuged at 150 x g for 10 min at 22°C. Platelet counts were performed with an electronic particle counter (Coulter model S-plus).

Platelet aggregation was measured with PRP (3.5 x 10⁵ platelets per ml) by the turbidimetric method of Born (23) in an aggregometer (Chromo-Log model 530VS, Chromo-Log, Haverstown, PA). The in vitro [¹⁴C]serotonin (5-hydroxy-[side-chain-2-¹⁴C]tryptamine creatinine sulfate, Amersham) release was measured according to a method described by Holmsen and Dangelmaier (24). Platelet factor 3 (PF3) was measured by Stypven clotting time (25). PRP (4 x 10⁵ platelets per ml) was preincubated with 10 μM ADP (Sigma) in the absence or presence of an inhibitor (50 μM Ap4A or 10 μM AppCHICpSpA) at 37°C for 5 min. One-tenth milliliter of preincubated PRP sample and 0.1 ml of saline were admixed with 0.1 ml of Russell viper venom (1.2 μg) (Wellcome) in a 37°C water bath to activate coagulation factor X. The clotting end point was then determined by the addition of 0.1 ml of CaCl₂ (25 mM in imidazole-buffered saline, pH 7.4) at 30 sec from addition of Russell viper venom.

Twelve synthetic phosphonate and three phosphorothioate analogues of Ap4A were synthesized (13-16) and employed (26, 27) essentially as previously presented. Abbreviations for the compounds are listed in Table 1; for further details on similar abbreviations, see ref. 16. The chemical moieties —CH₂,—CH₂CH₂,—CHF,—CF₂,—CHCl—, and

Abbreviations: PRP, platelet-rich plasma; Ap4A, diadenosine 5',5''-P1,P4-tetrathosphate; PF₃, platelet factor 3.

†To whom reprint requests should be addressed.
The concentration—aggregate of time. In three cases sulfur was substituted for bridge oxygens at α and α’ positions or at the β position. In three cases sulfur was substituted for bridge oxygens at the α and α’ positions. The analogues were grouped into three categories, including the S-analogues or phosphorothioates, on the basis of the positions of the substitutions in the Ap4A molecule. Some of these data have been presented in abstract form (26, 27).

RESULTS

Time-Dependent Inhibition. PRP containing 3.5 × 10⁹ platelets per ml prewarmed to 37°C was incubated with 100 μM Ap4A or 10 μM AppCHClppA for 1, 5, 15, and 30 min, followed by addition of 10 μM ADP to induce platelet aggregation. Aggregation was suppressed by more than 90% at 1 min of preincubation and completely at 5 min of preincubation (data not shown). The results indicate that the inhibitory activity of these agents on the ADP-induced platelet aggregation is a rapid reaction. The amount of Ap4A required to inhibit platelet aggregation is in general 5- to 10-fold higher than that of AppCHClppA.

Dispersal Activity of Platelets Previously Aggregated. PRP preincubated for 1 min with 10 μM AppCHClppA showed an almost complete inhibition of the platelet aggregation induced by ADP in control experiments. When aggregation was induced by ADP, and AppCHClppA was added 30 sec later, the aggregated platelets were completely dispersed. When such aggregation had, however, already progressed for 3-4 min, addition of AppCHClppA resulted in only slight dispersion (Fig. 1). This observation suggested that a secondary phase of platelet aggregation, induced by ADP, could not be reversed by Ap4A or its analogues. Immediate dispersive effects of AppCHClppA were at least 10-fold greater than those of Ap4A, as observed previously (12).

Dose-Dependent Inhibition and the IC₅₀ Values. Immediate inhibitory effects of Ap4A analogues on ADP-induced platelet aggregation were dose dependent, and an apparent IC₅₀ value (the concentration required for 50% inhibition) could be estimated by plotting the percent inhibition against the log of inhibitor concentration (Fig. 2). The IC₅₀ values for different analogues of Ap4A determined thus are illustrated in Table 1. The IC₅₀ values indicate that β-analogues containing a methylene bridge between two molecules of ADP in the Ap4A molecule are more potent inhibitors of ADP-induced platelet aggregation than other methylene bridge analogues tested. Dichloromethylene-substituted analogues (AppCCl₂ppA) were exceptions from these general trends, since they showed similar inhibitory potency between α,α’- and β-analogues. Thus the presence of a pyrophosphate group in the α-β and α’-β’ positions of Ap4A is desirable for effective inhibition of ADP-induced platelet aggregation. Phosphorothioate analogues were also promising, particularly ApSpCHFpSpA and ApSpSpSpA, as shown in Table 1.

Kinetic Analysis of Inhibition. Platelet aggregation was induced by four different concentrations (3, 5, 10, and 20 μM) of ADP in the absence or presence of 5 μM AppCHClppA, as shown in Fig. 3. Maximal percent aggregation in 4 min from the time of addition of ADP was taken as the velocity for kinetic analysis of inhibition. The aggregability was dose dependent and was suppressed by a dose of inhibiting agent (Fig. 3). When the double-reciprocal plot, a typical enzyme kinetic model, was adapted to the analysis of the data, the inhibition mechanism appeared to be competitive (Fig. 4).

![Fig. 1. Dispersive effects of AppCHClppA on ADP-induced platelet aggregation. Visible light transmission is plotted as a function of time. Normal fresh PRP, 3.5 × 10⁹ platelets per ml, was induced to aggregate by 10 μM ADP. PRP preincubated with 10 μM AppCHClppA for 1 min showed a potent inhibition of aggregation. An advanced aggregation could be dispersed by addition of the inhibitor, within 30 sec, to the aggregated platelets. However, by the end of 4 min the aggregation was irreversible; by this time secondary interactions of the aggregated platelets had occurred. Dispersive potency of this analogue is greater than that observed with Ap4A, for which the concentration required is 10- to 20-fold higher. This Ap4A effect was described previously (12).](https://example.com/Fig1.png)

![Fig. 2. Dose-dependent inhibition of ADP-induced platelet aggregation. Aggregation was induced in an aggregometer by 5 μM ADP in the presence of the inhibitors Ap4A and AppCHClppA at various concentrations. Suppression of the aggregability is expressed as percent inhibition and is plotted against the logarithm of the inhibitor concentration. Arrows indicate the IC₅₀ values with 5 μM ADP.](https://example.com/Fig2.png)
The same mechanism was found also for other inhibitors, including AppCHFppA and AppCF$_2$ppA. The apparent $K_i$ values calculated from the plots were 6.1 $\mu$M for Ap$_4$A, 1.1 $\mu$M for AppCHClppA, 2.1 $\mu$M for AppCHFppA, and 2.8 $\mu$M for AppCF$_2$ppA.

**Effects on Other Platelet Responses to ADP.** PRP preincubated with [1$^4$C]serotonin was stimulated by 10 $\mu$M ADP in the absence or presence of either 50 $\mu$M Ap$_4$A or 10 $\mu$M AppCHClppA. The reaction mixture contained 2 $\mu$M imipramine (10,11-dihydro-5H-N,N-dimethyl-5H-dibenz(b,f)azepine-5-propanamine hydrochloride) to block the re-uptake of the serotonin previously released. Both Ap$_4$A and AppCHClppA inhibited the ADP-induced platelet release reaction (Table 2). The activity of PF$_3$ in PRP can be determined by the Stypven clotting time (25). The activity is enhanced by preincubation of platelets with ADP, as shown by a shortened clotting time (Table 3). When the platelets were preincubated with ADP, in the presence of Ap$_4$A or AppCHClppA, ADP stimulation of PF$_3$ activity was completely prevented, as shown by the column headed "AP$_4$A" vs. the column headed "Saline." The results indicate that the inhibitory effects of these agents are specific for this ADP reaction on platelets.

**DISCUSSION**

We have evaluated the inhibitory effects of 12 phosphonate-substituted analogues and 3 phosphorothioates of Ap$_4$A on ADP-induced platelet aggregation. The analogues having a P–C–P bridge in place of P–O–P links are known to be resistant to hydrolytic enzymes (13, 14). These analogues may therefore have a longer lifespan in the blood and be more useful anti-platelet-aggregation drugs than the natural compound, Ap$_4$A. The latter is hydrolyzed rapidly in the blood and has a $t_{1/2}$ value of only a few minutes (12). Determination of the IC$_{50}$ was utilized as a screening tool to evaluate the

<table>
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<th>Inhibitor</th>
<th>Total activity in platelets, cpm</th>
<th>Plasma activity after ADP stimulation (release reaction), cpm</th>
<th>With inhibitor</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ap$_4$A</td>
<td>12.200</td>
<td>2010</td>
<td>147</td>
<td>93</td>
</tr>
<tr>
<td>AppCHClppA</td>
<td>11.600</td>
<td>2100</td>
<td>109</td>
<td>95</td>
</tr>
</tbody>
</table>
Table 3. Stypven clotting time: Inhibitory effects of Ap4A and AppCHClppA on PF3 activation by ADP

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Normal PRP</th>
<th>Saline</th>
<th>With Ap4A</th>
<th>With AppCHClppA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>30</td>
<td>36</td>
<td>38</td>
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<tr>
<td>2</td>
<td>39</td>
<td>25</td>
<td>39</td>
<td>40</td>
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<tr>
<td>3</td>
<td>49</td>
<td>34</td>
<td>48</td>
<td>51</td>
</tr>
</tbody>
</table>

Ap4A and AppCHClppA both inhibit ADP stimulation of PF3 activity, as described in the text. Compare times in saline columns with those in Ap4A and AppCHClppA columns.

relative potency of the agents. Our findings indicate that analogues having a P—C—P bridge located in the P2, P3 position are more potent inhibitors than those in other positions—e.g., P1, P2 and P3, P4. The phosphorothioate analogues also have good inhibitory potency and deserve further scrutiny.

It is reasonable to consider that these agents compete with ADP at the receptor sites on the surface of platelets, since the agents are composed of two ADP moieties linked symmetrically by a carbon bridge. It is interesting that the analogue linked by an ethylene bridge (AppCH2CH2ppA), thus lengthening the intermolecular distance, showed an IC50 value lower than that with a single-carbon bridge.

The kinetic studies of inhibition performed with two of the most potent inhibitors, AppCHClppA and AppCHFppA, in fact showed the same inhibition mechanism, that is, competitive with ADP. The same was true for Ap4A itself. The Ki values were of comparable magnitude for both of these above-mentioned analogues, indicating their higher potency as inhibitors of ADP-induced platelet aggregation as compared with the native compound Ap4A. Similar studies have yet to be carried out with the phosphorothioate analogues.

Other platelet responses to ADP, such as the release reaction and PF3 activation, were also suppressed by either Ap4A or AppCHClppA, suggesting strongly that these agents are specific inhibitors for the interaction of ADP with platelets, by occupying the same receptor sites on the platelet surface.

Thus, the potential anti-platelet-aggregation activity and the resistance to hydrolysis of the phosphonate and phosphorothioate analogues of Ap4A make them potentially useful antithrombotic agents, poised at a primary stage in the cascade of reactions leading to thrombosis.

An early review (6) illuminated some of the intracellular functions of Ap4A. More recently it has been recognized that Ap4A and its analogues play an important role in extracellular signaling events as well as in specific enzyme inhibition (7, 28–37). These extracellular functions draw attention to the possible role of Ap4A and its analogues as pharmacologically active compounds in cardiovascular, neurotransmitter, stress-related, and diverse purinergic fields.

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