Studies on the Mechanism of Ristocetin-induced Platelet Aggregation

By Herman E. Kattlove and M. Hugo Gomez

Adenine nucleotide metabolism and the release reaction were studied during ristocetin-induced platelet aggregation. Decreasing platelet ATP by incubation with metabolic poisons did not decrease ristocetin-induced aggregation. ADP and ATP were released from platelets during ristocetin-induced aggregation, and ATP was converted to hypoxanthine. However, these occurred after aggregation was almost complete. Aggregation was inhibited by p-chloromercuribenzoic acid. By studying platelet suspensions, we were able to determine that this effect was on platelets and not on the plasma cofactor needed for aggregation. We postulate that ristocetin and its cofactor aggregate platelets by binding platelet membranes and that the platelet plays a passive role in this reaction.

RISTOCETIN, AN ANTIBIOTIC derived from Nocardia lurida, aggregates human platelets. This aggregation requires a plasma cofactor which is reduced in the plasma of patients with von Willebrand's disease. The purpose of this study was to examine the platelet response to ristocetin with particular regard to adenine nucleotide metabolism in order to understand the mechanism of this aggregation.

MATERIALS AND METHODS

Platelet-rich plasma (PRP) was prepared by differential centrifugation of blood obtained from normal volunteers and anticoagulated with one-tenth volume of 3.1% sodium citrate. Platelet suspensions were prepared by gel filtration from this PRP or by the method described by Weiss et al., which utilizes EDTA anticoagulated blood (0.1% final concentration) and repeated centrifugation and resuspension of platelets in Tris-buffered saline containing EDTA, pH 7.3.

Radioactive adenine 8-3H (specific activity, 17-19 Ci/m mole, 0.5 mCi/mL) dissolved in water, was obtained from Schwartz/Mann, Van Nuys, Calif. Ristocetin was obtained as a white powder from Abbott Laboratories, North Chicago, Ill., Lot No. 757-2493. Prostaglandin E2 was obtained from Upjohn Company, Kalamazoo, Mich. All other chemicals were obtained from Sigma Co., St. Louis, Mo.

Ristocetin-induced platelet aggregation was studied in the Chronolog aggregometer attached to a Kontron recorder. PRP was placed in the aggregometer which kept the plasma at 37°C, and 1/11 volume of ristocetin dissolved in saline was added (final concentration, 1.8 mg/mL). When platelet suspensions were studied, 0.3 mL of the suspension was mixed with 0.3 mL of platelet-poor plasma (PPP) which had been collected in 1/10 volume 3.1% sodium citrate. This mixture was also aggregated with 1.8 mg/mL ristocetin.

The role of energy metabolism in ristocetin-induced aggregation was studied as follows: Citrated PRP was incubated with 1/40 volume of 3H adenine at 37°C for 75 min, at which time the metabolic pool of adenine nucleotides was completely labeled. The PRP was incubated at 37°C with 16 mM 2-deoxy-D-glucose (2 DG) and oligomycin, 50 μg/mL. (These large doses were...
Ristocetin (1.8 mg/ml) added to PRP in aggregometer and aliquots removed into EIA at stated intervals. Figure in parentheses represents platelet count of PRP. Results expressed aszmol/liter PRP.

... used to ensure rapid decrease in ATP.) At intervals, aliquots were removed to the aggregometer to test for ristocetin-induced aggregation. Simultaneously, a sample was extracted with an equal volume of a mixture of 9 parts ethanol and 1 part 0.1 M EDTA to measure radioactive ATP content. The adenine metabolites were separated on 1-inch-wide strips of Whatman 3MM paper by high-voltage electrophoresis in the presence of known standards which facilitated their identification. Their radioactivity was measured in a liquid scintillation counter, and the ATP was expressed as a percent of the total radioactivity. With this technique approximately 95% of the total radioactivity of the extract is recovered.

Adenine nucleotide release was studied in the aggregometer. PRP was aggregated with 1.8 mg/ml ristocetin. At 30, 60, and 180 sec, aliquots were removed and pipetted into 1/10 volume of 0.1 M EDTA and chilled in melting ice. These mixtures were centrifuged 10 min at 10,000 g in a Sorval RC 2B centrifuge at 4°C. The supernatant PPP was extracted with an equal volume of ethanol and its ATP and ADP content measured by the firefly-luciferase technique.

ATP utilization was also studied in the aggregometer. 3H adenine-labeled PRP was aggregated with 2 mg/ml ristocetin. At time intervals varying from 10 to 180 sec, the PRP was extracted by rapidly injecting an equal volume of the ethanol-EDTA mixture into the aggregometer cuvette. The supernatant of these extracts were studied by high-voltage electrophoresis for changes in distribution of radioactive adenine metabolites.

The effect of p-choromercuribenzoic acid (PCMB) was studied by adding this sulfhydryl reactive agent to PRP at 37°C at a concentration of 2 mM. At intervals the PRP was tested for ristocetin-induced aggregation. To determine whether the PCMB reacted with the platelets or with the plasma cofactor, platelet suspensions were prepared. PCMB (2 mM) was added to one-half the suspension and to a specimen of citrated PPP. Saline was added to the rest of the suspension and PPP. After appropriate incubation, ristocetin-induced aggregation was studied in equivolume mixtures of (1) PCMB-treated platelet suspension and control plasma, (2) untreated platelet suspension and PCMB-treated plasma, and (3) untreated platelet suspension and control plasma.

RESULTS

Release Reaction Induced by Ristocetin

During the first 60 sec of ristocetin-induced aggregation, only small amounts of adenine nucleotides were released (Table 1). As can be seen from Fig. 1, most of the aggregation was complete by 60 sec. Significant amounts of adenine nucleotides were released, however, by 3 min. This experiment was repeated with 3H adenine-labeled PRP. No radioactive ATP or ADP was released into the supernatant PPP.

During ristocetin-induced aggregation, 3H ATP declined with a corresponding increase in 3H AMP, 3H IMP, and 3H hypoxanthine (Fig. 2). There was also a small drop in 3H ADP during the first 10 sec and a subsequent rise above baseline levels.

Table 1. Release of Adenine Nucleotides by Ristocetin

<table>
<thead>
<tr>
<th>Time After Ristocetin Addition (sec)</th>
<th>Exp. 1 (P.C. 327.000/Ml)</th>
<th>Exp. 2 (P.C. 606.000/Ml)</th>
<th>Exp. 3 (P.C. 400.000/Ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>ADP</td>
<td>ATP</td>
</tr>
<tr>
<td>30</td>
<td>0.2</td>
<td>0.2</td>
<td>0.9</td>
</tr>
<tr>
<td>60</td>
<td>0.24</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>180</td>
<td>1.5</td>
<td>3.0</td>
<td>3.5</td>
</tr>
</tbody>
</table>

For pH and temperature control, plus volume-time recording, we give you the works:
Effect of Metabolic Poisons on Ristocetin-induced Platelet Aggregation

Ristocetin-induced platelet aggregation was unaffected by decreasing $^3$H ATP content (Table 2). In the presence of the metabolic inhibitors 2 DG and oligomycin, platelet $^3$H ATP rapidly decreased. In spite of this, normal aggregation persisted even when $^3$H ATP had dropped to 20% of its original concentration.

Table 2. Effect of Decreasing Radioactive Platelet ATP on Ristocetin-induced Platelet Aggregation

<table>
<thead>
<tr>
<th></th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Δ O.D.</td>
<td>ATP</td>
<td>Δ O.D.</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>15 min</td>
<td>43</td>
<td>98</td>
<td>62</td>
</tr>
<tr>
<td>30 min</td>
<td>29</td>
<td>90</td>
<td>42</td>
</tr>
<tr>
<td>60 min</td>
<td>16</td>
<td>98</td>
<td>24</td>
</tr>
</tbody>
</table>

Citrated $^3$H adenine-labeled PRP was incubated with 16 mM 2 DG and 50 μg/ml oligomycin. At intervals radioactive ATP content and ristocetin-induced aggregation were measured. ATP content and aggregation of control PRP which was studied at 0 time and after 60 min did not change. ATP is expressed as percent of control. Aggregation (Δ O.D.) is expressed in arbitrary units (1 U equals 0.1 inch of recorder paper).
Fig. 3. (A) Effect of PCMB on ristocetin-induced aggregation. PRP was incubated with 2 mM PCMB 15 and 30 min and tested with 1.8 mg/ml ristocetin. Representative experiment. (B) Effect of PCMB on platelet suspensions. (A) represents platelet suspension incubated 15 min with 2 mM PCMB and then mixed with control PPP. (B) represents control platelet suspension mixed with PPP which had been incubated with 2 mM PCMB. Representative experiment.

However, these inhibitors completely prevented the release of adenine nucleotides (results not shown).

**Effect of PCMB on Platelet Aggregation**

PCMB inhibited ristocetin-induced aggregation in platelet-rich plasma (Fig. 3A). After 15 min of incubation, inhibition was partial; inhibition was complete by 30 min. To determine whether PCMB affected platelets or the plasma cofactor, platelet suspensions were prepared. Gel-filtered platelet suspensions proved unsuitable for this study because they aggregated normally in response to ristocetin without plasma addition. This confirms previous work which indicated that the cofactor filters through the gel with the platelets. Therefore, we prepared platelet suspensions in EDTA. These would aggregate in response to ristocetin only when mixed with plasma. Varying amounts of plasma were not tested.

PCMB appears to primarily affect the platelet, not the plasma cofactor (Fig. 3B). When PCMB was incubated with the platelet suspension and tested with control plasma, aggregation was inhibited. A mixture of PCMB-treated plasma and untreated platelet suspension aggregated normally.

**DISCUSSION**

Ristocetin, which was introduced into clinical medicine in the late 1950s, was subsequently withdrawn, in part, because it caused thrombocytopenia. It was later demonstrated that this drug would aggregate platelets in vitro. Howard and Firkin further clarified the action of this drug when they demonstrated that the PRP of two patients with von Willebrand’s disease would not aggregate in response to this agent. Recently, Howard et al. and Weiss et al. have demonstrated that a plasma factor, which is reduced in the plasma of most patients with von Willebrand’s disease, is necessary for ristocetin-induced aggregation.

Platelet aggregation induced by physiologic stimuli can be related to platelet energy metabolism. During in vitro aggregation induced by thrombin, collagen, and epinephrine, the storage pool of ADP is released from the platelet. This is associated with the catabolism of the metabolic pool of platelet ATP to IMP.
and hypoxanthine, which presumably supplies energy for this release reaction. Metabolic poisons inhibit the release caused by collagen and epinephrine. During primary aggregation induced by ADP, vasopressin, and serotonin, ATP is also catabolized. This usually occurs within 5 sec after the agent is added and may relate to the shape change induced by these agents. Primary aggregation induced by ADP and epinephrine is inhibited by metabolic poisons.

The main thrust of this study was to determine if energy is required for ristocetin-induced aggregation. Our results indicate that at the dose of ristocetin we used (1.8–2 mg/ml), energy is not required. This is supported by the following data. (1) Ristocetin-induced aggregation was intact even when radioactive platelet ATP levels were 20% of normal. At this level of ATP, ADP- and collagen-induced aggregation are completely inhibited. (2) Although some ADP was released during ristocetin-induced aggregation, most of it was released when aggregation was almost complete. This was also demonstrated by Weiss et al. (3) Similarly, the ATP conversion to hypoxanthine appeared late in aggregation.

The results with metabolic inhibitors differ from those of Howard and Firkin as well as Weiss et al., who found that metabolic poisons inhibited platelet aggregation. The difference in our observations is probably best explained by the amount of ristocetin used. The other studies used 1-1.2 mg/ml of ristocetin. This dose causes slow suboptimal aggregation; 50% aggregation requires 90 or more seconds. At this time ADP is released and probably contributes to aggregation. Since both release and ADP-induced aggregation are inhibited by metabolic poisons, this could account for the inhibition these investigators found. Our study differs in that we used 1.8 mg ristocetin per ml, and aggregation was almost complete by 60 sec, which is before significant amounts of ADP were released.

Howard and Firkin and Weiss et al. also noted decreased ristocetin-induced aggregation in a patient with Glanzmann’s thrombasthenia. However, in a patient with this disorder whom we have studied, ristocetin-induced aggregation in response to 1.8 mg/ml was normal. This difference is probably due to the partial dependence on released ADP with the lower doses of ristocetin used by those investigators.

Thus it does not appear that platelets need be metabolically intact to respond to optimal aggregating concentrations of ristocetin. Indeed, this agent will aggregate platelets which have been fixed in paraformaldehyde. The inhibition of ristocetin-induced aggregation by PCMB, which binds to membrane thiol groups, suggests that ristocetin and the plasma cofactor act directly on platelet membranes. Since the antibiotic activity of ristocetin is probably due to its specific binding to C-terminal tripeptides of bacterial cell wall mureopeptide precursors, it is probable the ristocetin that binds to the platelet membrane. Perhaps the plasma factor causes aggregation by forming a bridge between ristocetin molecules. Although this is extremely speculative, it may explain the electron-microscopic studies of Tsao et al., who found electron-dense material between membranes of ristocetin-aggregated platelets.

In summary, ristocetin-induced aggregation may represent intercellular bind-
ing of sulfhydryl-dependent peptides of platelet membranes, caused by the drug along with its cofactor, which is not dependent on any energy-requiring reaction of the intact platelet.

REFERENCES


19. Kattlove HE: unpublished observations


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