Mode of hemolytic action of the antifungal polyene antibiotic filipin

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Filipin at low concentrations rapidly hemolyzes bovine erythrocytes. Of the compounds tested, which were known as constituents of cell membranes, only cholesterol and phosphatidyl-choline prevented hemolysis. A specific complex was formed only with cholesterol. Filipin treatment caused a release of cholesterol from erythrocytes. Since all cell sterol is located in the cell membrane, the hemolytic action of filipin may be due to a displacement of cholesterol from the cell membrane thus weakening its stability. The internal pressure of the cell then could force the hemoglobin through the defective membrane.

It has been shown 1, 2 that the antifungal polyene, filipin, inhibited growth and respiration of Saccharomyces cerevisiae. The selective permeability of the cell membrane was altered and leakage of cell constituents occurred. This condition probably leads to a deficiency of certain metabolites and finally results in cell death. Such alteration of the selective permeability of cell membranes seems to be a general phenomenon in the action of antifungal polyenes 3—11. These results form the basis of the hypothesis that the principal site of action for antifungal polyenes is in the cell membrane. Nystatin is mainly bound by lipid-rich particles of Saccharomyces cerevisiae 12, Neurospora crassa 13 and Leishmania Donovani 14. These particles were considered to be of membrane origin and thus further support the polynene-membrane hypothesis. Erythrocytes from mammalian blood seem to follow the same pattern of reaction, for they are immediately hemolyzed by a number of polyenes 15, 16. Since the leakage of hemoglobin is an indicator of changes in cell membrane integrity, intact bovine erythrocytes were used in this study to get more detailed information about the mode of action of filipin. The primary focus of attention was the interaction between sterols and filipin. Prevention of filipin activity by addition of exogeneous sterols had been established earlier 1, 17—19, and was confirmed 20. Though some relationship between sterols and filipin has been shown, a filipin sterol complex from the cell has not yet been identified. Nor has it been shown that the antibiotic causes a release of sterol from the cell. This investigation presents data indicating that filipin causes both complex formation with cell constituents and the release of cell sterols.

Materials and Methods

The chemicals used were of the highest purity available and were obtained from the following sources: 1) Sigma Chemical Company, St. Louis, Missouri: Phosphatidyl-choline, -serine, -ethanolamine, -glycerophosphate, glycerophosphorylcholine, glyceroltripalmitate, bovine albumin; 2) East-Ton D. D. S.}

HEMOLYTIC ACTION OF THE ANTIFUNGAL POLYENE ANTIBIOTIC FILIPIN

Preparation of packed red blood cells: Cells for each experiment were obtained from 200 ml citrated bovine blood. The blood was centrifuged for 30 minutes at 3500 x g and 2°C, the plasma was removed by aspiration. The red blood cells (RBC) were then washed with 150 ml isotonic (0.9%) sodium chloride and centrifuged for 30 minutes at 3500 x g. The supernatant was discarded and the cells were again washed with 170 ml isotonic sodium chloride. Resulting RBC were stored at 3°C if not used immediately.

Hemolysis test: Suspensions of 0.005 ml packed RBC in 10 ml isotonic sodium chloride were usually used. Compounds which were added to prevent hemolysis were first dissolved in methanol, propanol or acetone at final concentrations which per se did not cause hemolysis (0.1 – 3.0%). Filipin dissolved in methanol was added to the suspensions. After 10 minutes at room temperature (24°C), the suspension was centrifuged at 1500 x g and 3°C. The degree of hemolysis was determined by measuring the OD of the supernatant at 550 m,u in a “Coleman Junior Spectrophotometer”, using “Lumetron” matched tubes.

Spectrophotometric studies on the formation of filipin complexes were carried out with a “Beckman DU” in 1 cm cuvettes at 355 m,u. This is the filipin peak that decreases most in complex formation 1. For assays involving lipids all values were corrected for turbidity.

To determine the release of cholesterol from the cell membranes, 15 ml packed RBC were suspended in 100 ml isotonic sodium chloride in a separatory funnel and 13.14 mg filipin were added to each sample; the controls contained only 1 ml methanol. After 15 minutes the samples were extracted with hexane. The insoluble cholesterol filipin complex that appeared at the interphase between the aqueous and hexane layer was collected with the hexane phase. The interfacial layer was freed from erythrocytes by washing it twice with 100 ml isotonic sodium chloride. Twenty ml methanol were added to break the sterol antibiotic complex in the interphase. Since filipin is insoluble in hexane this phase contained no filipin, which would have interfered with the Liebermann-Burchard test for sterols. The hexane phase was dried in a “Rinco-evaporator”, the residue was dissolved in chloroform and the sterols were determined by the Liebermann-Burchard test 21. The remainder of the chloroform solution was used for a qualitative determination of phospholipids. When 1 M sucrose was used as osmotic stabilizer in the isotonic salt solution, the procedure was slightly altered; only 5 ml packed RBC, 1.5 mg filipin and 75 ml hexane were used. One molar sucrose was then employed in washing the interfacial layer.

Phospholipids were estimated qualitatively by paper chromatography on silica gel paper (Schleicher and Schüll No.966) 22. A mixture of chloroform: methanol:2,6-dimethyl-4-heptanone:water (40:9:5:1) was used as solvent and 0.01% Rhodamine B in 25% aqueous methanol plus 0.5% ammonia as the developing reagent. Pure phospholipids served as standards.

The molarity of filipin solutions was based on the brutto formula C35H58O11 for filipin 23.

Results

Bovine erythrocytes were immediately hemolyzed by filipin (Fig. 1) the reaction being complete within 10 – 20 seconds. Hemolysis began at 0.001 and was complete at 0.002 µM/ml. The fact that there is no gradual release of hemoglobin with increasing filipin concentration indicates a all-or-nothing pattern of reaction.

To determine which constituents of the cell membrane could possibly interact with filipin, we investigated a number of compounds for their ability to antagonize filipin caused hemolysis, when applied prior to the addition of the antibiotic (Table 1). Only cholesterol, phosphatidyl-choline and bovine plasma were effective. Complex formation occurred with those materials that prevented hemolysis. Cholesterol which gave the greatest protection against

22 U. Beiss, J. Chromatogr. 13, 104 [1964].
Table 1. Prevention of filipin induced hemolysis and complex formation of filipin. Ineffective were: bovine albumin (6 mg); palmitic acid, glyceromonostearate, glycerol-1-lauryl-2,3-palmitate, phosphatidyl-serine and -ethanolamine, α-glycerophosphate, glycerophosphoryl-choline, glycerotripalmitate, all 0.6 μM; cholesteryl-palmitate, -acetate, -proprionate, all 0.2 μM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>μM</th>
<th>OD 550</th>
<th>% Hemolysis compared to filipin</th>
<th>μM</th>
<th>OD 355</th>
<th>% Reduction of OD 355</th>
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<tbody>
<tr>
<td>filipin</td>
<td>0.025</td>
<td>0.136</td>
<td>—</td>
<td>0.05</td>
<td>0.197</td>
<td>—</td>
</tr>
<tr>
<td>bovine plasma</td>
<td>(0.3 ml)</td>
<td>0.016</td>
<td>11.8</td>
<td>(0.1 ml)</td>
<td>0.128</td>
<td>35.0</td>
</tr>
<tr>
<td>cholesterol</td>
<td>0.2</td>
<td>0.003</td>
<td>2.2</td>
<td>0.2</td>
<td>0.104</td>
<td>47.2</td>
</tr>
<tr>
<td>phosphatidyl-choline</td>
<td>0.6</td>
<td>0.014</td>
<td>10.3</td>
<td>0.6</td>
<td>0.170</td>
<td>13.7</td>
</tr>
</tbody>
</table>

Table 2. Release of cholesterol from bovine erythrocytes after filipin treatment (values are averages from triplicates and refer to 15 ml packed red blood cells).

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Cholesterol released (μg)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>735</td>
<td>1735</td>
</tr>
<tr>
<td>2</td>
<td>1035</td>
<td>2175</td>
</tr>
<tr>
<td>3</td>
<td>475</td>
<td>1365</td>
</tr>
<tr>
<td>4</td>
<td>442</td>
<td>1335</td>
</tr>
</tbody>
</table>

Fig. 1. Hemolytic effect of filipin on bovine erythrocytes. Hemolysis also caused the greatest reduction of the 355 mJ filipin peak. Phosphatidyl-choline prevented hemolysis but decreased the U.V.-absorption only 13% compared to 47% by cholesterol.

Apparently the β-3-hydroxyl group of cholesterol must be free in the reaction with filipin since acylation of cholesterol caused a complete loss of the filipin binding capacity. The entire molecule of phosphatidyl-choline is needed to prevent hemolysis and none of the different parts of the molecule reacted with the antibiotic (Table 1).

When erythrocytes were treated with filipin there was a 2 – 3 fold increase of cholesterol in the suspension medium compared to the untreated control (Table 2). The sterols from the untreated cells could have been derived from aged red blood cells which could not withstand the general treatment. Aged erythrocytes are generally more sensitive to adverse conditions.

No release of phospholipids was observed, as shown in paper chromatographic studies.

It was reported that when sucrose was used as an osmotic stabilizer, it minimized the lytic action of nystatin and amphotericin B. Our experiments confirmed these observations for filipin (Fig. 2). In isotonic sodium chloride plus filipin hemolysis was complete within 1 minute; in 1 M sucrose the release of hemoglobin was considerably suppressed. Even prolonged incubation in osmotic stabilizer revealed only a slow and slight increase in hemoglobin release with time. Hypertonic sucrose alone causes hemolysis to a certain degree.

Fig. 2. Filipin induced hemolysis of bovine erythrocytes suspended in isotonic sodium chloride (○—○) and isotonic sodium chloride containing sucrose (1 M) as osmotic stabilizer (○○○). 0.0025 μM filipin were added per ml.

Although no appreciable hemolysis occurred with filipin in 1 M sucrose, there was a considerable release of cholesterol from both, filipin treated and untreated erythrocytes (Table 3). Thus sucrose treatment alone causes the release of cholesterol but it does not allow the movement of hemoglobin from the cells.

<table>
<thead>
<tr>
<th>Suspension medium</th>
<th>µg cholesterol released</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>+ filipin</td>
</tr>
<tr>
<td>isotonic NaCl</td>
<td>248</td>
<td>495</td>
</tr>
<tr>
<td>1 M sucrose</td>
<td>248</td>
<td>595</td>
</tr>
<tr>
<td>isotonic NaCl</td>
<td>710</td>
<td>927</td>
</tr>
<tr>
<td>+ 1 M sucrose</td>
<td>710</td>
<td>974</td>
</tr>
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</table>

Table 3. Effect of osmotic stabilizer on cholesterol release from filipin treated bovine erythrocytes (values are averages from triplicates and refer to 5 ml packed red blood cells).

Discussion

Of all the compounds tested, which are found in cell membranes, only cholesterol, phosphatidylcholine and bovine plasma could prevent hemolysis. This points to the importance of the sterol-polyene interaction. The effect of the plasma was most probably due to its cholesterol bound to lipoproteins. Prevention of filipin induced hemolysis by phosphatidylcholine is probably of a less specific type since it caused no change in the U.V-absorption of filipin. It might coat a specific site on the membrane and thus prevent filipin from entering.

It was observed\(^1\) that incubation with filipin caused a sharp decrease in bound sterols of *Saccharomyces cerevisiae*. This result could be interpreted in two ways. Either the sterols were degraded by an enzyme or enzyme system induced by the antibiotic or that the sterols were released from the cells into the medium. The fact that filipin causes a release of cholesterol from bovine erythrocytes favors the latter concept. This observation also militates against the assumption that sterols bind filipin in the membranes. It rather points out that the sterols in the membranes are displaced by the antibiotic.

The lipid part of the red blood cell membrane is in general composed of cholesterol and phospholipids in approximately a 1:1 molar ratio\(^25\). The cholesterol appears to be exclusively located in the membrane\(^26\). Splitting off up to 1/2 of its lipid constituents (the theoretical value for cholesterol) should result in a change of membrane stability. The short time needed for completion of hemolysis and the all-or-nothing pattern of reaction indicates that the final effect of filipin on erythrocytes could be bursting due to osmotic shock, which can be prevented by hypertonic sucrose.

For sheep erythrocytes in isotonic solution an internal hydrostatic pressure of 2 mm water has been reported which is absent in hypertonic solution\(^26,28\). Thus the hemolytic action of filipin appears to be due to the displacement of cholesterol from the erythrocyte membrane which weakens its stability. The internal pressure of the cell could then force the hemoglobin through the defective membrane.

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