EFFECT OF BENZYL ALCOHOL ON ADENOSINE TRIPHOSPHATASE, p-NITROPHENYLPHOSPHATASE AND ACETYLCHOLINESTERASE IN RAT ERYTHROCYTE MEMBRANE

Reiko TANAKA
Department of Pharmacology, Akita University School of Medicine,
Akita 010, Japan
Accepted January 11, 1984

Abstract—The irreversible effect of benzyl alcohol on ATPase, p-nitrophenylphosphatase and acetylcholinesterase in the erythrocyte membrane of rats was demonstrated. The ATPase activity in the membranes was stimulated with 10-70 mM benzyl alcohol and inhibited by concentrations greater than 80 mM. p-Nitrophenylphosphatase was gradually inhibited by concentrations of benzyl alcohol greater than 30 mM. The acetylcholinesterase activity was not affected by concentrations below 100 mM and strongly inhibited by concentrations of benzyl alcohol greater than 150 mM. With the uptake studies of 14C-labeled benzyl alcohol by membranes, the highest uptake was obtained in the presence of 200 mM of benzyl alcohol. And SDS-polyacrylamide gel electrophoresis showed a binding of benzyl alcohol to the major protein bands of the erythrocyte membrane. Therefore, stimulation of the ATPase activity appeared to be the result of an increase in ion uptake due to an increase in the fluidity of the membrane lipid by benzyl alcohol, and the inhibition of the enzymes may be the result of benzyl alcohol-induced denaturation of the membrane components. The difference in the observed inhibition patterns among ATPase, p-nitrophenylphosphatase and acetylcholinesterase may be related to the sensitivity of benzyl alcohol on those enzymes.

Key words: Benzyl alcohol, ATPase, AChE, p-Nitrophenylphosphatase, Erythrocyte membrane

INTRODUCTION

Benzyl alcohol (BA) has been used in many drug preparations of injectable form as a local anesthetic, antiseptic and solubilizing agent. Our previous investigations...
Reiko TANAKA

(Tanaka, et al., 1977; Ohmiya et al., 1978) have demonstrated the lysis and morphological changes of the erythrocytes of rats exposed to generally used concentrations for BA parenteral therapy. And in concentrations more than 2% BA, precipitation of the erythrocytes and membrane preparations was observed.

Recently, Schleifer et al., (1982) warned about the toxic effect of BA, especially in premature animals, including respiratory failure, vasodilation, hypotension, convulsions, paralysis and death.

In the present paper, we studied the effect of BA on enzymes in the erythrocyte membrane, adenosine triphosphatase (ATPase), p-nitrophenolphosphatase (PNPase) and acetylcholinesterase (AChE) to explicate the effect of BA on cell membranes.

**METHODS**

**Membrane preparation**

Erythrocytes were freshly obtained from adult male Sprague-Dawley rats using heparin as the anticoagulant, and completely lysed with hypotonic phosphate buffer (10 mM, pH 7.4), after being washed three times with a phosphate buffered saline (PBS, pH 7.4). For some experiments, erythrocytes (2% in PBS) were pretreated with BA for 60 min at 37°C, and washed three times with PBS following the hypotonic hemolysis.

The erythrocyte membranes collected by centrifugation (10,000 g, for 15 min) were washed four times with a hypotonic buffer. For the determination of the enzyme activity, the membranes were washed with a 5 mM tris buffer (pH 7.7). The membrane protein was determined by the Lowry et al. method (1951) after solubilization in 1 N NaOH. Bovine serum albumin (albumin stock standard, Daiichi Pure Chemicals) was used as the protein standard. BA was purchased from Nakarai Chem., Co.

**Determination of ATPase activity**

The ATPase activity was measured under two different conditions, i.e. in the presence of Na+, K+ and Mg++ (total ATPase) and in the presence of Mg++ only (Mg++-ATPase). The (Na+ + K+)-ATPase activity was obtained by subtracting Mg++-ATPase from the total ATPase activity.

The standard reaction medium for total ATPase assay consisted of 140 mM NaCl, 14 mM KCl, 5 mM MgCl₂, 40 mM Tris and 3 mM ATP-Tris (Sigma Chemical Co.) in a final volume of 3.0 ml adjusted to pH 7.7 with 1 N HCl. For Mg++-ATPase assay, the standard reaction medium (pH 7.7) contained 5 mM MgCl₂, 40 mM Tris and 3 mM ATP-Tris.

Erythrocyte membrane preparations (0.5 mg protein) were incubated for 60 min at 37°C in a different reaction media (3.0 ml). The reaction was stopped by the addition of 15% trichloroacetic acid (3.0 ml).

After the 15 min, supernatant was separated by the centrifugation for 15 min at 1,500 g, and the concentration of the inorganic phosphate, liberated into the supernatant, was determined by the method of Fiske and Subbarow (1925).
Effect of benzyl alcohol on enzymes in the membrane

Determination of AChE activity

The AChE activity was determined by the method of Ellman et al. (1961). Three milliliters of the membrane suspension (0.1 mg protein) in a 0.1 M phosphate buffer (pH 8.0) and 100 μl of the dithiobisnitrobenzoic acid reagent were mixed in a spectrophotometer cuvette. Starting immediately after the addition of 10 μl of the 0.075 M acetylthiocholine solution, a change in the optical absorbance at 412 nm was recorded for 6 min.

Determination of PNPase activity

The PNPase activity was assayed by the same method as total ATPase, except that NaCl was omitted and 3 mM ATP-Tris was replaced by 3 mM p-nitrophenylphosphate. Liberated p-nitrophenol in the supernatant was measured in a spectrophotometer at 420 nm after the addition of 2 M Tris (Fujita et al., 1966).

BA uptake by membrane

Membrane preparations (0.5 mg protein) were incubated for 60 min at 37°C with different concentrations of BA and ¹⁴C-labeled benzyl alcohol (¹⁴C-BA, the Radiochemical Centre, Amersham) in PBS.

Radioactivity in the supernatant, separated by 15 min centrifugation at 10,000 g was determined with a liquid scintillation spectrometer (Mark II, Nuclear Chicago) in a Bray (1960) scintillation liquid.

SDS-polyacrylamide gel electrophoresis of the membranes, labeled by 60 min incubation with ¹⁴C-BA in PBS at 37°C, and staining of the proteins in the gels were performed as described previously (Tanaka, 1978).

¹⁴C-radioactivity in 2 mm gel slices was determined with a Mark II liquid scintillation spectrometer in Bray's scintillation liquid.

RESULTS

Effect of BA on ATPase activity

Taking the mean ± S.E. for the four experiments, the activity of total ATPase in the erythrocyte membranes of rats was 2.59 ± 0.05 μ moles Pi/mg protein/hr, that of Mg⁺⁺-ATPase was 1.19 ± 0.07, and that of (Na⁺ + K⁺)-ATPase was 1.49 ± 0.10. The values of Km and Vmax for total ATPase were 0.56 mM and 3.33 μ moles Pi/mg protein/hr, respectively, by the Lineweaver-Burk plots; for Mg⁺⁺-ATPase, these values were 0.40 mM and 1.47 μ moles Pi/mg protein/hr, respectively.

Figure 1 shows that the ATPase activities were stimulated by the lower BA concentrations (10-70 mM) and inhibited by higher (above 80 mM) BA concentrations. Maximum of total ATPase and (Na⁺ + K⁺)-ATPase occured at a concentrations of 30 mM and that of Mg⁺⁺-ATPase at 50 mM.

In the Lineweaver-Burk plots, BA was a noncompetitive inhibitor for ATP-ase. In the presence of 100 mM BA, the value of Vmax was 2.00 μ moles Pi/mg protein/hr for total ATPase and 1.14 μ moles Pi/mg protein/hr for Mg⁺⁺-ATPase.

When the erythrocytes were pretreated with various concentrations of BA, and the
Fig. 1. ATPase activities in the erythrocyte membrane of rats in the presence of different concentrations of benzyl alcohol.
A: Total ATPase (●●)  B: Mg\textsuperscript{2+}-ATPase (▲▲)
C: (Na\textsuperscript{+}+K\textsuperscript{+})-ATPase (○○)

Table 1. Effect of benzyl alcohol on ATPase activity in the erythrocyte membrane

<table>
<thead>
<tr>
<th>Concentration of benzyl alcohol</th>
<th>Total ATPase (% of control)</th>
<th>Mg\textsuperscript{2+}-ATPase (% of control)</th>
<th>(Na\textsuperscript{+}+K\textsuperscript{+})-ATPase (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>96.7</td>
<td>106.7</td>
<td>83.5</td>
</tr>
<tr>
<td>30 mM</td>
<td>101.7</td>
<td>101.5</td>
<td>85.0</td>
</tr>
<tr>
<td>50 mM</td>
<td>100.3</td>
<td>103.5</td>
<td>91.3</td>
</tr>
<tr>
<td>70 mM</td>
<td>103.3</td>
<td>116.4</td>
<td>94.4</td>
</tr>
<tr>
<td>80 mM</td>
<td>142.8</td>
<td>258.9</td>
<td>65.5</td>
</tr>
<tr>
<td>90 mM</td>
<td>73.9</td>
<td>89.0</td>
<td>63.6</td>
</tr>
<tr>
<td>100 mM</td>
<td>63.8</td>
<td>80.8</td>
<td>59.8</td>
</tr>
<tr>
<td>150 mM</td>
<td>2.8</td>
<td>3.4</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Membranes were prepared from erythrocytes pretreated with various concentrations of benzyl alcohol.
membranes were prepared by hypotonic hemolysis, stimulation of ATPase was greatest at 80 mM. At BA concentrations above 90 mM, the activity of all three ATPases was clearly inhibited, and the inhibitions were concentration-dependent (Table 1).

**Effect of BA on AChE activity**

As showed in Fig. 2, AChE in the membranes was clearly inhibited in the presence of BA concentrations greater than 150 mM but not in concentrations below 100 mM.

At 300 mM BA, the activity of AChE was not detected in the membranes.

**Effect of BA on PNP activity**

Figure 3 shows the PNPase activity in the membranes performed in the presence of various concentrations of BA. Inhibition of the activity occurred at BA concentrations greater than 30 mM, and stimulation of the activity was not observed. When the erythrocytes were pretreated with BA and the membranes were prepared by hypotonic hemolysis, inhibition of PNPase was observed at BA concentrations greater than 80 mM in the erythrocyte suspensions.

**BA uptake by membrane**

Table 2 indicates that the uptake of $^{14}$C-BA by membranes was dose-dependent in the presence of between 50–200 mM concentrations of BA. Analysis of the membrane protein by SDS-polyacrylamide gel electrophoresis (Fig. 4) revealed the binding of $^{14}$C-BA to the major protein bands, especially band I (200,000 dalton), and band III (108,000 dalton) (Tanaka, 1978).

![Graph](attachment:image.png)

**Fig. 2.** Effect of different benzyl alcohol concentrations on the AChE activity in the erythrocyte membrane of rats.
Reiko TANAKA

Fig. 3. Effect of different benzyl alcohol concentrations on the PNPase activity in the erythrocyte membrane of rats.

Fig. 4. SDS-polyacrylamide gel electrophoresis of the erythrocyte membranes labelled with $^1^4$C-benzyl alcohol.
I, II, III, IV: protein band

Table 2. Uptake of benzyl alcohol by the erythrocyte membrane

<table>
<thead>
<tr>
<th>Concentration of benzyl alcohol (mM)</th>
<th>Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>50</td>
<td>10.2</td>
</tr>
<tr>
<td>70</td>
<td>8.6</td>
</tr>
<tr>
<td>80</td>
<td>9.0</td>
</tr>
<tr>
<td>90</td>
<td>10.3</td>
</tr>
<tr>
<td>100</td>
<td>11.7</td>
</tr>
<tr>
<td>150</td>
<td>16.7</td>
</tr>
<tr>
<td>200</td>
<td>21.9</td>
</tr>
<tr>
<td>300</td>
<td>13.7</td>
</tr>
</tbody>
</table>

Membranes (0.5 mg protein) were incubated with various concentrations of benzyl alcohol and $^1^4$C-labelled benzyl alcohol in 1.0 ml PBS for 60 min at 37°C.

* Uptaked radioactivity in membranes of total $^1^4$C-radioactivity

— 114 —
Effect of benzyl alcohol on enzymes in the membrane

DISCUSSION

The effect of BA on the enzymes in the erythrocyte membranes of rats was studied. Our results indicated that the patterns of effect of BA on the membrane-bound enzymes, ATPase, AChE and PNPase were different. While the ATPase activity was stimulated by low concentrations and inhibited by high concentrations of BA (Fig. 1), the AChE activity was not affected by concentrations below 100 mM and strongly inhibited by concentrations greater than 150 mM (Fig. 2). At the concentrations examined (10-200 mM), BA did not stimulate PNPase, and inhibitions was noted when concentrations exceeded 30 mM (Fig. 3).

In the hemolysis and BA uptake experiments, precipitations of the erythrocytes and membranes was frequently noted. Furthermore, in the Lineweaver–Burk plots, BA was a noncompetitive inhibitor of ATPase. These findings suggest that the BA-induced inhibition of ATPase, AChE and PNPase would be the result of denaturation, brought about by the strong binding of BA to the membrane components, especially protein. The observed different inhibition patterns may reflect the difference of the enzyme sensitivity to BA.

At the BA concentrations examined, only the ATPase activity was stimulated; similar results have been obtained by others.

Mitjavila et al., (1975) who examined the effects of deoxycholate, Triton X-100, Tween 20, 60 and 80 on the enzymes in the isolated brush border of the intestinal epithelium of rats, found no effect on the alkaline phosphatase activity, however, the ATPase activity, especially (Na⁺+K⁺)-ATPase activity was stimulated at low concentrations of those surface active agents, and progressively inhibited as the concentrations increased. Chan (1967) reported that the ATPase activity in the erythrocyte membranes of humans was stimulated by low concentrations of sodium dodecyl sulfate and inhibited by concentrations greater than 0.4 mM.

Others have reported a lipid–protein interaction to be an important factor in the ATPase activity (Kimelberg et al., 1974; Warren et al., 1974; Warren et al., 1975). And BA has been reported to bind to lipid and protein (Colley et al., 1971) and to possess the ability to increase fluidity of the lipid bilayers (Hubbell et al., 1970). As showed in Fig. 1 and Table 1, stimulation and inhibition of ATPase in BA-treated membranes was observed at lower concentrations of BA than when membranes were prepared from BA-treated erythrocytes suggesting the uptake and binding of BA to components in erythrocytes inspite of different concentrations of proteins and lipids in those two experiments.

Based on these reports, results and findings that the stimulation and inhibitions of the enzymes were considered to be irreversible, because those effects did not decreased by washings the preparation with PBS (data not showed), the toxic effect of BA would be an important problem for animals including humans.

— 115 —
REFERENCES


