Biochimica et Biophysica Acta 813 (1985) 277–281
Elsevier

BBA 72474

The effect of ethanol on the passive Ca permeability of human red cell ghosts measured by means of arsenazo III

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(Received September 4th, 1984)

Key words: Ca^{2+} transport; Ethanol; Passive transport; Arsenazo III; (Erythrocyte membrane)

Ethanol in the range of 0.76–2.40 M caused an immediate increase in the Ca permeability of the plasma membrane of resealed human red blood cell ghosts in which intracellular free Ca could be continuously monitored by means of the Ca chromophore arsenazo III. At a given concentration of ethanol, the Ca permeability increased markedly a few minutes following the mixing of the ghosts and the ethanol, and continued to increase over at least the next 30 min. Preincubating the ghosts in ethanol for 15, 60 and 120 min before measuring the rate of free Ca accumulation, progressively increased the effect of a given concentration of ethanol. These results indicate that the effect of a given concentration of ethanol is a complex function of concentration and exposure time. The effects of ethanol in this concentration range were completely reversible. The resealed ghosts used in these experiments were depleted of ATP to avoid interference from the Ca pump and all experiments were carried out with 150 mM KCl on both sides of the membrane to minimize changes in either the volume or membrane potential associated with activation of the Ca-dependent K channel.

Introduction

Ethanol is known to affect the transport mechanisms that control intracellular Ca [1,2] and has been reported to change the Ca-sensitivity of proteins that bind Ca [3]. Thus, the observed effects of ethanol on Ca-dependent mechanisms in cells could be due either to changes in the concentration of intracellular Ca or to changes in their sensitivity to Ca [4,5]. When studying the effects of ethanol in most cells, it is often difficult to evaluate the relative importance of these two factors due to the lack of an adequate method to measure intracellular free Ca.

Red blood cells contain a number of Ca-dependent transport mechanisms found in other cells, including a Ca-dependent K channel [6], a sodium pump [7,8], passive Ca transport mechanisms [9] and an ATP-driven Ca pump that regulates intracellular free Ca [10]. With these cells it is now possible to quantitatively monitor intracellular free Ca in resealed ghosts by means of incorporated metallochromic indicator dyes [11,12] and to study simultaneously the effects of Ca on cation transport [13–15]. For these reasons, we feel that red blood cells are a suitable experimental model to test whether ethanol affects Ca-dependent mechanisms more by altering intracellular Ca or by increasing the sensitivity of Ca-dependent mechanisms to Ca.

In this paper, we present data on the time course, reversibility and dose-response relationships of ethanol on passive Ca transport across the human red cell membrane. These experiments are the first step in evaluating the relative effects of

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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ethanol on the Ca-dependent transport mechanisms of red blood cells.

Materials and Methods

Preparation of resealed ghosts. Fresh blood was collected in heparin from healthy donors and the plasma and buffy coat were removed by aspiration after centrifugation at 12000 × *g* for 10 s at 4°C. The cells were then resuspended in 5 vol. 150 mM KCl/20 mM Hepes/0.1 mM EGTA (pH 7.4) (23°C) recentrifuged, and the residual plasma and buffy coat removed in three more repetitions of this procedure. The cells were then suspended at 20% hematocrit in the aforementioned wash solution containing a trace of chloramphenicol (1–2 mg/100 ml) and incubated with gentle shaking at 37°C for 20 h to deplete the cells of ATP. After the incubation, the cells were washed three more times following the above procedure, then centrifuged at 12000 × *g* for 5 min, and the supernatant was removed, leaving packed cells with a hematocrit exceeding 95%.

To make resealed ghosts, 1 vol. packed cells was hemolyzed at 0°C in 20 vol. of a solution which contained 0.088 mM arsenazo III/22 mM Hepes/0.055 mM EGTA titrated with Tris-hydroxide to pH 7.4 at 23°C. After 5 min, the tonicity of the hemolyzing solution was restored to 300 mosM by the addition of a small volume of 3 M KCl. The ghosts were then resealed by incubating them in the hemolyzing solution plus the KCl for 45 min at 37°C. After resealing, the ghosts were centrifuged at 49000 × *g* and the supernatant was removed by aspiration. The resealed ghosts were resuspended in 20 vol. of a solution comprising 150 mM KCl/20 mM Hepes/0.1 mM EGTA, centrifuged at 12000 × *g* for 5 min at 4°C, and the supernatant was removed. This procedure was repeated two more times after which the ghosts were layered on top of a 10% sucrose cushion comprising 20 mM Hepes/50 mM NaCl/0.1 mM EGTA (pH 7.4) and centrifuged at 4°C at 49000 × *g* for 60 min to reduce heterogeneity [16], thereby eliminating those ghosts which have a higher passive permeability to Ca [13]. After collection from the cushion, the ghosts were washed three more times as described above and then packed to a hematocrit exceeding 95%. The estimated concentrations of the constituents in the ghosts are: 150 mM KCl, 1 mM NaCl, 0.1 mM $MgCl_2$, 0.05 mM EGTA, 0.08 mM arsenazo III and 20 mM Hepes.

Calculation of intracellular free Ca. Intracellular free Ca was calculated from the change in absorbance of entrapped arsenazo III at 655 nm minus the absorbance at 700 nm by method A [13]. The change in absorbance at these two wavelengths were recorded in the dual wavelength mode of an Aminco DW-2a spectrophotometer (American Instrument Company, Urbana, IL) using methods that have previously been described [12]. In all cases, the ghosts were suspended at 1.67% hematocrit and 37°C in a solution which was mechanically stirred. The concentration of dye in each preparation of ghosts was determined by measuring the maximum Ca-sensitive change in the absorbance of dye after the membrane had been made freely permeable to Ca by the addition of ionophore A23187 [12]. The dye concentration was not determined after each measurement of Ca influx due to the danger of contaminating the cuvettes with the ionophore.

Chemicals. All chemicals were reagent grade, except where noted. EGTA was purchased from Sigma (St. Louis, MO), as was arsenazo III which was obtained as a practical grade and purified as previously described [11,12]. Ionophore A23187 was obtained from Calbiochem-Behring (La Jolla, CA). Calcium was purchased as a 1 M stock from British Drug House (distributed by Gallard-Schlesinger, Carle Place, NY).

Results

Ethanol increases the passive Ca permeability of human red cell ghosts in a dose-dependent manner as seen by the rate of change in the absorbance of arsenazo III (Fig. 1). The effect of ethanol on increasing the permeability appears to increase during the time course of the measurement of Ca influx, an effect that is especially noticeable at 1.71 M ethanol (Fig. 1). In this first experiment, the ghosts were exposed to ethanol for less than 5 min before Ca was added to the solution to begin the measurement of Ca influx. Note that increasing the ethanol from 1.71 M to 2.40 M had a much larger effect than raising the

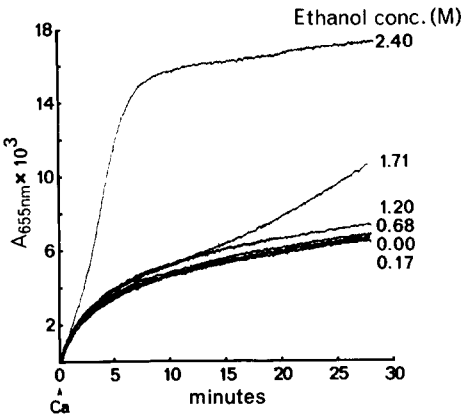

Fig. 1. The effect of ethanol on the rate of Ca accumulation without prior exposure of the resealed ghosts to ethanol. Resealed ghosts containing the Ca-sensitive dye, arsenazo III, were added to a solution consisting of 150 mM KCl/20 mM Hepes/0.1 mM EGTA, and the indicated concentrations of ethanol. 2–5 min later (time zero on the abscissa), a small volume of concentrated $CaCl_2$ was added to the extracellular solution to increase the extracellular free Ca concentration to 1 mM. The hematocrit was 1.67%; the temperature was 37°C; and the solutions were stirred. The concentration of intracellular free Ca at the end of the experiment is in the range of 15–16 μM for 0, 0.17 and 0.68 M ethanol, 18 μM for 1.20 M ethanol, and 50 μM for 1.71 M ethanol. In the case of the ghosts incubated in 2.40 M ethanol, the response began to level-off approx. 6 min after the addition of Ca, because the response of the arsenazo III to Ca was beginning to saturate [12].

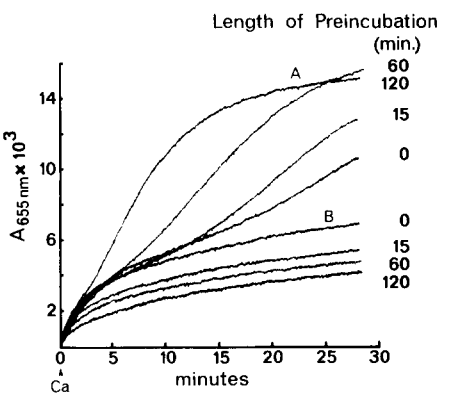

Fig. 2. The effect of preincubating the resealed ghosts in ethanol for various lengths of time prior to measuring the rate of Ca accumulation. The measurements were carried out as described in the legend to Fig. 1, except that the ghosts were preincubated with (A) and without (B) 1.72 M ethanol for the indicated times prior to the addition of Ca (time zero on the abscissa) to the extracellular solution. The ghosts were then washed in 150 mM KCl/20 mM Hepes/0.1 mM EGTA, either with (A) or without (B) ethanol and then resuspended in the original volume of the same solution. The concentration of

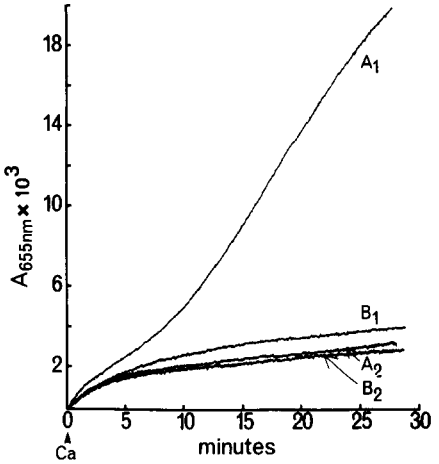

Fig. 3. The reversibility of ethanol-induced increases in Ca permeability. Resealed ghosts were preincubated for 15 min in 150 mM KCl/20 mM Hepes/0.1 mM EGTA, either with (A) or without (B) 1.72 M ethanol. At the end of the 15 min preincubation, a sample was taken from A and the rate of Ca accumulation was measured in the continued presence of 1.72 M ethanol (A_1). Likewise, a sample was removed from B after 15 min of preincubation and the rate of Ca accumulation was measured in the absence of ethanol (B_1). The remainder of the ghosts in both A and B were then washed three times in 500 vol. of the aforementioned buffer minus ethanol. After washing, the rate of Ca accumulation was measured in the absence of ethanol for the ghosts which had been exposed to ethanol in the preincubation period (A_2) and those that had been preincubated in the absence of ethanol (B_2). The concentration of intracellular free Ca at the end of the Ca accumulation period was in the range of 6–8 μM for B_1, B_2 and A_2, and over 100 μM for A_2. Note that the value for A_2 is an estimate, because the response of the dye at this high concentration of Ca is not linear [12].

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intracellular free Ca at the end of the Ca uptake period in the ghosts not preincubated in ethanol (group B) range from 16 (0 min of preincubation) to 9 μM (120 min of preincubation) assuming that all the ghosts contained the same concentration of arsenazo III. If any ghosts or arzenazo III were lost during the preincubation or subsequent washing, this would appear as a smaller change in absorbance and a corresponding smaller increase in itnracellular free Ca. The slopes of the curves for all the ghosts in group B are similar, indicating that they took up Ca at approximately the same rate. The concentration of free Ca at the end of the measurement in the ghosts preincubated in ethanol (group A) ranges from 50 (0 min of preincubation) to over 300 μM (120 min of preincubation). Again, values in this range are estimates, because the response of the dye to high concentrations of Ca is very nonlinear [12].

concentration from 1.20 M to 1.71 M (Fig. 1). The leveling-off of the signal after 5 min at the highest concentration of ethanol is due to saturation of the arsenazo III by Ca [12].

In the next type of experiment, the ghosts were preincubated for various lengths of time before Ca was added to the solution. In this case, it is apparent that the effect of a given concentration of ethanol increased with the length of time that the ghosts were exposed to alcohol prior to the addition of Ca (Fig. 2).

The large increases in passive Ca permeability caused by ethanol are reversible when the ethanol is removed from the solutions in which the ghosts are suspended and the ghosts are subsequently washed in solutions containing no ethanol (Fig. 3). The apparent small decrease in the rate of Ca influx in the control ghosts in Figs. 2 and 3 may be due to a minor loss of arsenazo III from the ghosts or loss of ghosts themselves during the washing procedure that followed the respective preincubation periods.

Discussion

This paper shows for the first time that ethanol alters the Ca permeability of the human red cell membrane. The changes in absorbance of arsenazo III as shown in this paper are indicative of net changes in intracellular Ca [12,13]. The addition of Ca to the extracellular solution at the beginning of each experiment resulted in an increase in intracellular Ca, because the electrochemical gradient after the addition of extracellular Ca favored a net influx of Ca, and the ghosts did not contain sufficient ATP to fuel the outwardly directed Ca pump. Initially, the ghosts probably contained less than 0.05 μM free Ca due to the incorporation of 50 μM EGTA inside the ghosts, but certainly less than 0.3 μM free Ca which is the minimum free intracellular Ca that can be detected with this concentration of arsenazo III under these conditions [12,13]. The addition of extracellular Ca at the beginning of each experiment increased the extracellular free Ca to 1 mM. Under these conditions, changes in the net intracellular Ca as detected by the arsenazo III are primarily a reflection of unidirectional Ca influx. Passive unidirectional efflux would be too small to affect the results, because the intracellular free Ca is so much lower than the extracellular concentration. Thus, the stimulation of Ca accumulation by ethanol was probably due to an increased rate of unidirectional Ca influx. The mechanisms of passive Ca transport across the human red cell membrane are not well understood, but could involve more than one mechanism [9]. If there is more than one mechanism, one may be affected differently by the ethanol than the other. These results show only that the overall effect of ethanol in this concentration range is to increase the Ca permeability. Since both the ghosts and the solutions in which the ghosts were suspended during the measurement of Ca influx contained 150 mM KCl, it is unlikely that there were major changes in the membrane potential due to the activation of the Ca-dependent K channel. Such changes in membrane potential have been previously shown to alter the rate of Ca influx when the intracellular K concentration is high and the extracellular concentration is low [13,17].

Alcohol and anesthetics are known to either stimulate or inhibit solute translocation across membranes, depending in part on the underlying transport mechanism [18]. Ethanol at a concentration of 2.2 M has been shown to increase the passive permeability of human red cells to Na and K [19]. In other experiments, 1 M ethanol did not affect the Na permeability of human red cells [20]. The effects of ethanol on the passive Ca permeability of red cells shown in the present paper are in the same range as that which inhibited Ca uptake and stimulated the Ca-ATPase in vesicles from sarcoplasmic reticulum [21] and inhibited Ca uptake and Ca binding in cardiac microsomes [22]. It is also in the same range that induced a selective increase in the K permeability of artificial bilayers composed of phospholipids from sheep red cells [23].

We interpret the effect of preincubation observed in Fig. 2 to mean that the threshold concentration of ethanol that could have an effect on passive Ca permeability may be a complex function of both ethanol concentration and time. Ethanol penetrates so rapidly [24] that increased permeability with time must be unrelated to the length of time it takes the intracellular concentration of ethanol to reach that on the outside of the

ghosts. Perhaps changes in Ca permeability with time depend on a relatively slow rearrangement of membrane components related to changes in membrane fluidity that are known to occur at these and lower concentrations of ethanol [25]. Whatever the mechanism by which the preincubation period affects the results, the effects are reversible, indicating that the basic structure of the membrane which controls Ca permeability has been preserved. Both the reversibility and the increase in potency with time are features also observed in measuring the effect of ethanol on the K permeability of bilayers [23] and on cardiac microsomes [22]. Thus, much lower concentrations of ethanol than those used in the present study may affect the Ca permeability after longer exposure periods.

Acknowledgments

This research was supported by a grant from the Neuroscience Program at Wayne State University, a Student Research Fellowship from the American Heart Association of Michigan to PMP and a Research Career Development Award from the National Institutes of Health (AM 01253-01) to D.R.Y.

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