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HEMOLYSATE INCREASES CALCIUM-INHIBITION OF THE Na^+,K^+ PUMP OF RESEALED HUMAN RED CELL GHOSTS

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The Na^+,K^+ pump of resealed human red cell ghosts is more sensitive to inhibition by intracellular Ca (Ca_i) when they contain diluted hemolysate compared to ghosts without hemolysate. The activity of the Na^+,K^+ pump was assessed by measuring ouabain-sensitive ^{22}Na efflux in ghosts that, in addition to the presence or absence of hemolysate, also contained arsenazo III to measure free Ca_i and a regenerating system to maintain a constant concentration of ATP. Incorporating hemolysate diluted 20-fold compared to in situ conditions doubled the inhibitory effects of 1–50 μM free Ca_i on the Na^+,K^+ pump and caused 50% inhibition to occur between 5 and 10 μM free Ca_i. Increased inhibition in the presence of the hemolysate was not due to a cytoplasm-induced decrease in the ATP content of the ghosts. These findings are consistent with the suggestion that the cytoplasm of human red cells contains a factor which increases the sensitivity of the Na^+,K^+ pump to inhibition by Ca_i.

The $(Na^+ + K^+)$-ATPase in human red cell membranes washed free of cytoplasm is inhibited 50% by 50–100 μM Ca [1–4]. Adding diluted red cell hemolysate to these membranes increases Ca inhibition 10–20-fold so that the $(Na^+ + K^+)$-ATPase is inhibited 50% by 5–10 μM free Ca [4]. Boiling the hemolysate or digesting it with trypsin before adding it to the membranes eliminates the increased inhibition, indicating that an active agent is present in the hemolysate, and that it might be proteinaceous [4]. Adding purified calmodulin has no effect on Ca-inhibition of the $(Na^+ + K^+)$-ATPase [4]. These results suggest that the cytoplasm of human red cells contains a factor distinct from calmodulin that alters the inhibitory effect of Ca on the $(Na^+ + K^+)$-ATPase [4], the biochemical activity underlying the $(Na^+ + K^+)$-ATPase [4], the biochemical activity underlying the Na^+,K^+ pump. This study was designed to test whether hemolysate also modifies the inhibitory effect of Ca on ion translocation through the Na^+,K^+ pump.

Human blood was drawn by venipuncture from healthy young adults, heparin was added, and the buffy coat removed by aspiration after centrifuging the cells for 1 min at 9000 × *g* (Sorvall RC-5B, Newton, CT). The blood was then washed three times in 10 vol. of 146 mM NaCl/20 mM Pipes-$Tris_2$ (pH 7.6) (23°C), again centrifuging at 9000 × *g* for 1 min and aspirating off the supernatant to leave packed cells with greater than 90% hematocrit. A portion of the cells was then used to make resealed ghosts by the agarose column method of Wood and Passow [5]. The remainder were kept at 0°C to make a 'suspending' solution

Abbreviation: Pipes, 1,4-piperzindieethanesulfonic acid.

containing hemolysate. The agarose (Bio-Gel A-50m, 50–100 mesh; Bio-Rad, Richmond, CA) column (Pharmacia K50/60, Piscataway, NJ) had a bed volume of 700 ml and was preequilibrated at 0°C with 15 mM K_2Pipes, 0.1 mM K_2EDTA (pH 6.0) (23°C). 20 ml of 146 mM NaCl/20 mM Pipes-$Tris_2$ (pH 7.6), was applied to the top of the column. This volume was immediately followed by the cells suspended at 12% hematocrit in 40 ml of 146 mM NaCl/20 mM Pipes-$Tris_2$ (pH 7.6). White unsealed membranes were eluted from the column at 0°C in 15 mM K_2Pipes/0.1 mM K_2EDTA (pH 6.0) (23°C) well separated from hemoglobin and accompanying cytoplasmic constituents. Membranes were then centrifuged 5 min at $21000 \times g$, the solution aspirated off, and the packed membranes mixed with 5 vol. of 'suspending' solution containing 22 mM Pipes-$Tris_2$/1.8 Na_2ATP/0.12 mM arsenazo III/0.12 mM EDTA/3.0 mM $MgCl_2$/288 μg/ml creatine phosphokinase (pH 7.6) prepared with or without the addition of membrane-free hemolysate (see below). After stirring the unsealed column membranes and the appropriate suspending solutions for 5 min at 0°C, a trace of ^{22}Na was added, followed by an appropriate volume of concentrated reversing solution so that the suspensions contained 20 mM NaCl, 10 mM KCl, 10 mM choline chloride, 60 mM $Tris_2$phosphocreatine, 240 μg/ml creatine phosphokinase, 0.1 mM arsenazo III, 1.5 mM ATP and 2.5 mM $MgCl_2$ (pH 7.3) (0°C) and, where appropriate, hemolysate diluted 20-fold compared to in vivo concentrations. The ghosts were then resealed by incubating them for 30 min at 37°C and subsequently washed three times in 20 vol. 20 mM NaCl/10 mM KCl/89 mM choline chloride/20 mM Pipes-$Tris_2$/5.5 mM $MgCl_2$/5 mM EDTA-$Tris_2$ (pH 7.3) (23°C).

The hemolysate to be added to the suspending solution was prepared by adding 1 vol. of packed red cells to 6 vol. of distilled water at 0°C followed by 5 min of stirring. This solution was then centrifuged at $40000 \times g$ for 15 min at 4°C. 6 ml of the membrane-free supernatant were removed and added to 7.3 ml of a solution comprising 46 mM Pipes-$Tris_2$/3.72 mM Na_2ATP/0.248 mM arsenazo III/0.248 mM EDTA/6.2 mM $MgCl_2$/595 μg/ml creatine phosphokinase. The solution was adjusted to pH 7.6 and brought to a final volume of 15 ml with distilled water.

To begin an experiment, the ghosts were added at 0.67% hematocrit to solutions at 22°C containing 20 mM NaCl, 10 mM KCl, 20 mM Pipes-$Tris_2$, 89 mM choline chloride, 5 mM Na_2EDTA and either 5.5 mM $MgCl_2$ (zero free Ca, 500 μM free Mg); 1.844 mM $CaCl_2$, 3.678 mM $MgCl_2$ (25 μM free Ca, 500 μM free Mg); or 3.785 mM $CaCl_2$, 1.794 mM $MgCl_2$ (50 μM free Ca, 500 μM free Mg). The concentrations of free Ca and Mg were calculated for pH 7.3 from the total concentrations of Ca, Mg and EDTA using equilibrium constants corrected for 37°C and an ionic strength of 0.17 M [6,7]. After the ghosts had been added to the solutions, an aliquot of each solution was removed to determine the initial ATP content. The respective solutions were then put in a shaker bath at 37°C. 5 min later an aliquot of each suspension was removed to determine the initial ^{22}Na content. Then a small volume of concentrated ionophore A23187 in absolute alcohol was added to each flask to give a final concentration of 3 μM. Approx. 18 and 36 min thereafter, aliquots of the suspensions were removed for the determination of the ^{22}Na and ATP content. Aliquots for ^{22}Na were centrifuged 5 min at 4°C at $20000 \times g$, the supernatants removed and counted in a gamma counter (Packard, Model 5260, Downers Grove, IL). The percent ^{22}Na released at 18 and 36 min was calculated after subtracting off the initial ^{22}Na released. The rate constant for ouabain-sensitive ^{22}Na efflux at a given concentration of free Ca was calculated from the difference in the percent ^{22}Na released with and without 0.1 mM ouabain [8].

Aliquots removed for determining ATP were immediately placed in a boiling water bath for 5 min. After subsequent cooling, these samples were frozen at −20°C and later analyzed by means of the firefly method [9].

The concentration of free intracellular Ca (Ca_i) and Mg (Mg_i) after the addition of ionophore A23187 to the ghosts was determined by measuring Ca- and Mg-sensitive changes in absorbance of entrapped arsenazo III at the dual wavelength pair of 630–700 nm (ΔAB_{630}) and 655–700 nm (ΔAB_{655}) in a DW-2a spectrophotometer (American Instruments, Urbana, IL). Free Ca_i and free Mg_i were calculated from Eqns. 1 and 2, respec-

tively

$$[\text{free Ca}]_i = \left(\frac{K_{dCa_2D_2} \cdot [Ca_2D_2]}{[D]^2} \right)^{0.5} \quad (1)$$

$$[\text{free Mg}]_i = \frac{K_{dMgD} \cdot [MgD]}{[D]} \quad (2)$$

where Ca_2D_2 is a 2:2 complex of Ca and dye, MgD is a 1:1 complex of Mg and dye, D is free dye, and $K_{dCa_2D_2}$ ($2 \cdot 10^{-14}$ M) and K_{dMgD} ($1.25 \cdot 10^{-3}$ M) are the appropriate dissociation constants [6].

Since the concentration of CaD in these experiments was small compared to Ca_2D_2 and MgD,

$$[D]_T = [D] + 2[Ca_2D_2] + [MgD] \quad (3)$$

where $[D]_T$ is the total dye concentration. In this case,

$$\Delta AB_{630} = \Delta\epsilon_1 \cdot [Ca_2D_2] + \Delta\epsilon_2 \cdot [MgD] \quad (4)$$

and

$$\Delta AB_{655} = \Delta\epsilon_3 \cdot [Ca_2D_2] + \Delta\epsilon_4 \cdot [MgD] \quad (5)$$

where $\Delta\epsilon_1$ to $\Delta\epsilon_4$ are the appropriate molar extinction coefficients for a Ca- or Mg-sensitive change in absorbance [6]. D is calculated from Eqn. 3 after determining $[Ca_2D_2]$ from Eqn. 6

$$[Ca_2D_2] = (\Delta AB_{655} - \Delta\epsilon_4 \cdot [MgD])/\Delta\epsilon_3 \quad (6)$$

and [MgD] from Eqn. 7

$$[MgD] = \left(\Delta AB_{630} \frac{\Delta\epsilon_1 \cdot \Delta AB_{655}}{\Delta\epsilon_3} \right) / \left(\frac{\Delta\epsilon_4 \cdot \Delta\epsilon_1}{\Delta\epsilon_3} + \Delta\epsilon_2 \right) \quad (7)$$

where $\Delta\epsilon_1$ to $\Delta\epsilon_4$ have been multiplied by the percent hematocrit and divided by 100 [6].

Increasing free Ca_i from 0 to 50 μM inhibits the rate of ouabain-sensitive Na efflux in ghosts made with and without incorporated hemolysate (Fig. 1). In both sets of ghosts, substantially more of the activity was inhibited by increasing free Ca_i from 0 to 25 μM than from 25 to 50 μM. The pattern of inhibition in this range suggests that the Na^+,K^+ pump is being slowed by more than one process, one of which has a much higher sensitivity to Ca than the other (Fig. 1). Incorporating hemolysate increases the inhibitory effect of Ca and appears to do so by increasing the magnitude of this high-sensitivity component (Fig. 1).

Since the hemolysate incorporated into the ghosts must have included calmodulin which would have stimulated the Ca^{2+}-ATPase [4,10], increased inhibition of the Na^+,K^+ pump observed in the presence of the hemolysate could be caused by a decrease in available ATP. To try and keep the concentration of ATP constant during the measurement of ouabain-sensitive Na efflux, an ATP regenerating system with a capacity of 60 mM ATP was incorporated into the resealed ghosts (see above). The fractional change in the initial ATP concentration of approx. 1 mM for both kinds of ghosts was then assayed throughout the period in which Na efflux was measured. The ATP content in most of the ghosts stayed constant throughout the measurement of Na efflux and in all cases the ghosts at the end of the flux period had greater than 80% of their initial ATP (Fig. 2). The hemolysate, therefore, did not increase inhibition of the Na^+,K^+ pump by decreasing the available ATP via stimulation of other ATPases.

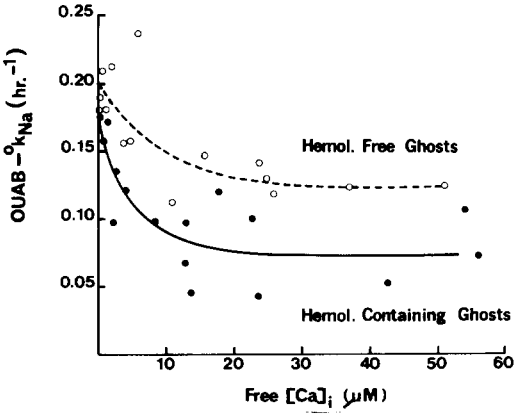

Fig. 1. The rate of ouabain-sensitive Na efflux as a function of intracellular Ca (Ca_i) in resealed ghosts made with and without incorporated hemolysate. The experiment, calculations, and the measurement of free Ca_i by means of incorporated arsenazo III are described in the text. The results are from eight separate experiments, four experiments with ghosts containing hemolysate (●———●) and four without (○———○). Each point is the rate constant for ouabain-sensitive ^{22}Na efflux (ouab-$^{o}k_{Na}$) which is the difference in the rate constant for ^{22}Na efflux for three samples with and three samples without ouabain. The concentration of free Ca_i in a given set of ghosts remained constant throughout the measurement of Na efflux.

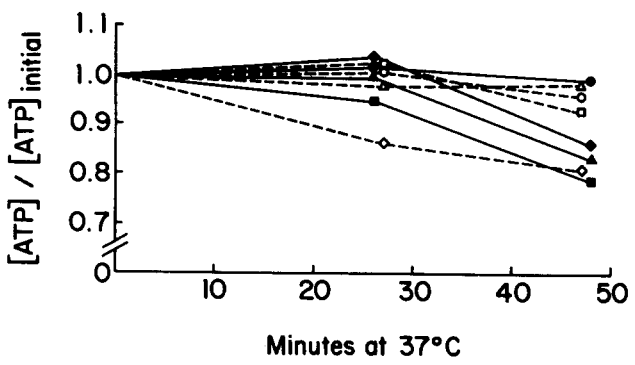

Fig. 2. Relative changes in ATP content of ghosts with and without incorporated hemolysate at the four concentrations of extracellular free Ca at which the ghosts were suspended. The values were obtained by sampling the suspension of ghosts as a function of time during the measurement of ^{22}Na efflux in flasks without ouabain. The initial concentration of ATP ($[ATP]_{initial}$) was measured less than 2 min after the ghosts were suspended in the flux solutions at 22°C and was approx. 1 mM for all ghosts. Then as explained in the text the flasks were put at 37°C, and the percent ^{22}Na released from the cells was measured 5, 23 and 41 min thereafter. Shortly after the second and third time points, the suspensions of ghosts were sampled for ATP content. The ratio of the ATP at these time points compared to the initial ($[ATP]/[ATP]_{initial}$) was determined separately for each incubation flask. Each of the points is the mean of the values in two separate experiments, each of which was determined from three separate values from three incubation flasks. Ghosts in separate experiments suspended in solutions with the same free Ca did not necessarily have the same intracellular free Ca. Open symbols are ghosts without incorporated hemolysate and closed symbols are those containing hemolysate, with both suspended at 0 (○,●), 10 (□,■), 25 (△,▲) and 50 (◇,◆) μM free Ca.

By showing that the hemolysate increases the inhibitory effect of Ca on the Na^+,K^+ pump, this study supports previous observations that the hemolysate of human red blood cells contains a factor that increases the inhibitory effect of Ca on the $(Na^+ + K^+)$-ATPase [4]. Such a protein could play an important role in the regulation of the Na^+,K^+ pump by Ca_i.

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