Biochimica et Biophysica Acta, 553 (1979) 320—325

BBA 78372

UNMASKING OF A POTASSIUM LEAK IN RESEALED HUMAN RED BLOOD CELL GHOSTS

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(Received August 28th, 1978)

Key words Erythrocyte ghost, Chelator, K^+ leak

Summary

A selective potassium leak is observed in resealed, human red blood cell ghosts when hemolysis is performed with distilled water at pH 6.5, 0°C. The leak, which has a maximum near pH 6.7, is suppressed when either magnesium or a chelating agent is present in the hemolysing medium. The potassium leak has the additional property that it can be suppressed after resealing by washing the ghost membranes in a medium containing a low concentration of ATP or EDTA. The data suggest that through the dilution of endogenous chelating agents at hemolysis a potassium leak may be unmasked.

Introduction

The resealed human red blood cell ghost is widely used to model the transport properties of the intact red blood cell. Therefore, it has been important to investigate conditions at hemolysis which can influence the transport characteristics of the resealed ghost membrane. A particularly sensitive aspect of the resealed ghost is the state of the cation barrier. The topic of this paper is the restoration of the physiologically significant K^+ barrier to a low permeability state in resealed ghosts prepared by hypotonic hemolysis.

Lepke and Passow [1] have tried to optimize the conditions at hemolysis in order to maximize the number of resealable ghosts and the tightness of the resealed ghost membrane to K^+. In this paper it is shown that deviations from their conditions can lead to a resealed ghost (type 2, in the nomenclature of Bodemann and Passow [2]) which is leaky for K^+ but not for Na^+. The expression of this specific leak for K^+ is prevented when either Mg^{2+} or a chelating agent is present in the hemolysing medium.

Abbreviation: Pipes, piperazine-*N*,*N*′-bis(2-ethane sulfonic acid).

Methods

Human, O^+, red blood cells, obtained from the Hessen Red Cross blood bank (used within 7 days after collection), preserved in standard acid/citrate/dextrose (U.S.P. XIX, fortified with adenine and guanosine), were washed three times in 166 mM NaCl and resuspended in either 166 mM Tris or Pipes (piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)) buffer, pH 6.5, at 0°C. Unless otherwise noted, the hematocrit of the cell suspension ranged between 40 and 50%. All solutions were prepared with quartz doubly distilled water. The cells were hemolysed by the addition of 1 vol. cell suspension to 20 vols. hemolysing medium. Hemolysis was performed at 0°C with distilled water or a solution containing either 5 mM $MgSO_4$, 5 mM citrate (sodium salt) or 1 mM EDTA (sodium salt) at pH 6.5. 5 min after hemolysis the ion concentration was restored to isotonicity with the addition of either 3 M KCl (K-ghosts) or 3 M NaCl (Na-ghosts). The K^+ (Na^+) concentration after the reversal step ranged between 140 and 150 mM. The ghosts were allowed to equilibrate at 0°C for 5 min and then transferred to a water bath and incubated at 37°C for 45 min to allow resealing.

Subsequently, in a typical experiment, the resealed K-ghosts (Na-ghosts) were then washed at room temperature twice in a medium containing 20 mM Tris and 146 mM KCl (NaCl) and, to deplete any ghosts which did not reseal, twice in a medium containing 20 mM Tris and 146 mM NaCl (KCl). Alternatively, Pipes buffer was used. The entrapped K^+ (Na^+) was measured by flame photometry as a measure for the percentage of resealed, type 2, ghosts. The yield of resealed ghosts was between 80 and 90%. Net K^+ (Na^+) loss was measured after resuspension of the ghosts into a medium identical to the last wash medium at 37°C. Usually, 100 µl ghost pellet was added to 2 ml medium. The K^+ (Na^+) content of the ghosts was measured after sampling the ghost suspension and centrifugation. The pellet was lysed with distilled water and assayed on an Eppendorf flame photometer in a background of 1 mM CsCl. The initial volume of ghosts present in the efflux suspension was calculated from the volume of ghost pellet added to the flux medium, the hematocrit of the pellet, and the volume of the efflux suspension. The cation content was calculated in terms of mM/l packed ghosts.

The rate of cation loss does not necessarily follow a single exponential throughout the period of measurement. However, the interval between 5 and 35 min may be used for such an approximation. The time interval was extended to 60 min, when very slow rates were encountered. A least-squares method was used to calculate the rate constant.

Results and Discussion

When hemolysis is performed with distilled water as the hemolysing medium at 0°C, pH 6.5, and in the absence of added alkaline earth metals or complexing agents, resealed (type 2) ghosts are formed. However, the resealed ghosts display a leak specific for K^+. In Fig. 1 the K^+ or Na^+ content of resealed ghosts, which had been previously loaded with either K^+ or Na^+ and resuspended in a pH 6.9 Na^+ or K^+ medium at 37°C, is plotted as a function of time. The esti-

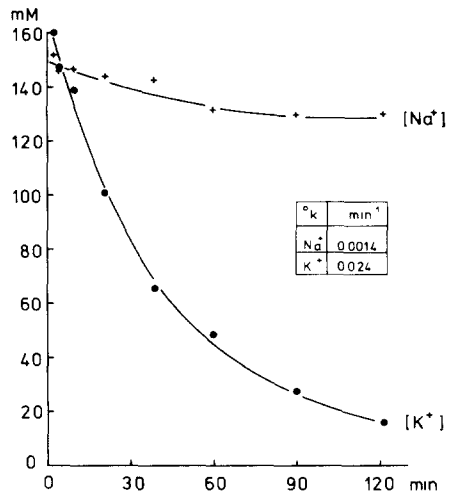

Fig. 1. The efflux of K^+ into Na^+ medium and the efflux of Na^+ into K^+ medium after hemolysis with distilled water. Washed red blood cells were resuspended to form a 41% hematocrit in 166 mM Pipes buffer, pH 6.5 (sodium salt for Na-ghosts and potassium salt for K-ghosts). The cells were hemolysed at 0°C with distilled water, 1 vol. cell suspension to 20 vols. distilled water. After reversal and resealing, the K-ghosts (Na-ghosts) were washed twice in a medium containing 161 mM KCl (NaCl) and 5 mM Pipes, and twice in a medium containing 161 mM NaCl (KCl) and 5 mM Pipes. The rate of cation efflux was measured at 37°C, pH 6.9, in a 3.1% ghost suspension. Ordinate: cation content in mM/l packed ghosts. Abscissa: time, min.

mated rate constant for net K^+ loss is 17 times that of net Na^+ loss. A selectivity of K^+ over Na^+ is also seen when efflux is measured into choline medium (unpublished observations).

The magnitude of the K^+ leak is highly pH dependent. Fig. 2a illustrates K^+ loss into Na^+ medium at several pH values. The rate constants calculated from the data in Fig. 2a and replotted in Fig. 2b indicate that a maximum for K^+ loss into Na^+ medium may be present near pH 6.7.

In contrast, when the cells are hemolysed in the presence of Mg^{2+} as described by Hoffman [3], Bodemann and Passow [2], and Lepke and Passow [1], the resealed ghost is tight for K^+. Fig. 3 indicates that the K^+ leak of the magnesium-resealed ghost is only slightly dependent on pH. Alkaline earth metal ions have been considered to be required for the restoration of membrane barriers when hemolysis was conducted at 20°C or above [3,4]. And alkaline earth ions can bind to the membrane, when they are present at hemolysis [4–6]. The data suggests that Mg^{2+}, even at 0°C, has a powerful effect on restoration of the K^+ barrier.

It is, therefore, surprising that metal ion-free chelators can also exert a similar restoring action. Hemolysis in the presence of a chelating agent can lead to the masking or suppression of the K^+ leak. Fig. 3 shows that citrate and EDTA can restore the ghost membrane to a low permeability state. The pH dependence of the K^+ leak in citrate and EDTA resealed ghosts is similar to that of the Mg^{2+}-resealed ghosts. Several other chelators have been tested, including ATP, and all display the restoring effect. They differ only in their relative potency.

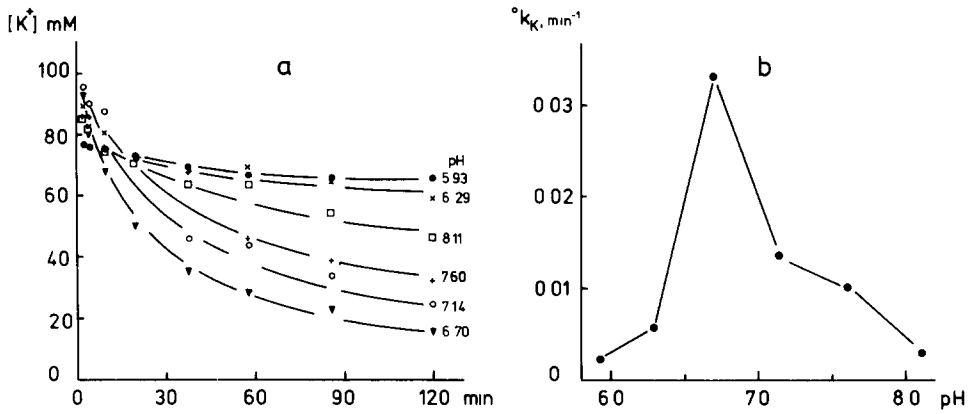

Fig. 2. (a) The efflux of K^+ into Na^+ medium as a function of pH after hemolysis with distilled water. Ghosts were prepared as stated in Methods after resuspension of washed cells into 166 mM Tris buffer, pH 6.5. The cells were hemolysed at 0°C with distilled water, 1 vol. cell suspension to 20 vols. distilled water. After reversal and resealing, the ghosts were washed twice in a medium containing 146 mM KCl and 20 mM Tris and twice in a medium containing 146 mM NaCl and 20 mM Tris. The efflux of K^+ into Na^+ medium was measured at the indicated pH, 37°C, in a 3.8% ghost suspension. Ordinate: K^+ content, mM/l packed ghosts. Abscissa: time, min. (b) The pH dependence of the estimated rate constant for cells hemolysed with distilled water. The data in (a) were used to estimate a rate constant for the rate of K^+ loss into Na^+ medium. Ordinate: rate constant, min^{-1}. Abscissa: pH at 37°C.

In the first successful attempts to prepare resealable ghosts, Teorell [7] utilized dilutions which left about 10% of the original cell content in the ghosts. In the later work of Bodemann and Passow [2] about 5% of the original cell content remained in the ghosts. Under their conditions, at 0°C hemolysis, Mg^{2+} was not absolutely essential for the restoration of the K^+ barrier. Unpublished observations have shown that even under similar conditions, in the absence of Mg^{2+}, a small pH-dependent leak is present. It is abolished when either Mg^{2+} or EDTA is present in the hemolysing medium. In the experiments described in the preceding paragraphs, the resealed ghosts contained about 2.5%

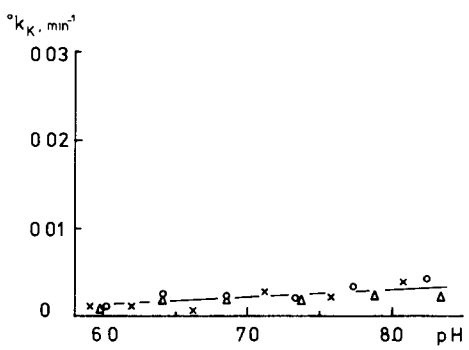

Fig. 3. The effect of Mg^{2+}, citrate, and EDTA on the pH dependence of K^+ efflux into Na^+ medium. Ghosts were prepared and handled as in Fig. 2, with the exception that the hemolysing medium contained either: o———o, 5 mM $MgSO_4$; X———X, 5 mM sodium citrate, or Δ———Δ, 1 mM EDTA (sodium salt). The rate of potassium loss was measured at 37°C in a 3.9% ghost suspension. Ordinate: rate constant, min^{-1}. Abscissa: pH at 37°C.

of their original cell content (as calculated from the dilutions used at hemolysis). In view of these observations, the question arises whether the dilution at hemolysis of some intracellular component leads to the uncovering of the K^+ leak.

To answer this question cells were hemolysed at constant final ion concentration, but at various dilutions of intracellular contents. Red blood cells were resuspended in buffer to a hematocrit between 12 and 66% prior to hemolysis. 1 vol. of cell suspension was hemolysed with 20 vols. of hemolysing medium. Fig. 4 shows the relationship between the rate of K^+ loss from the resealed ghost and the dilution achieved at hemolysis. When the cells are hemolysed with distilled water, the dilution of a cytoplasmic component unmasks a specific leak for K^+ in the resealed ghost. The K^+ leak, resulting from the dilution of the cellular factor, may be suppressed by the presence of either Mg^{2+} or EDTA in the hemolysing medium.

Chelating agents, including ATP, EDTA, and citrate, have the additional capacity of being able to close or mask the leak even after resealing at 37°C. Fig. 5 shows that the presence of as little as 10 μM EDTA in the wash media following resealing is sufficient to close the leak. In contrast, Mg^{2+} is effective only when present at hemolysis and has little or no effect after resealing.

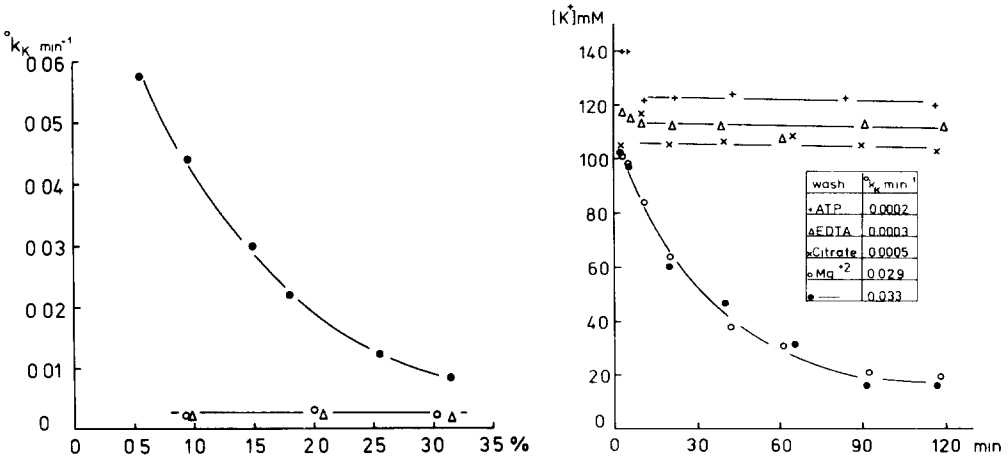

Fig. 4. The effect of dilution at hemolysis on the magnitude of the K^+ leak. Ghosts were prepared from cells resuspended in 166 mM Pipes buffer. The hematocrit of the suspension was adjusted to a value between 12 and 66%. 1 vol. cell suspension was hemolysed with 20 vols. hemolysing medium, which contained either: ●———●, distilled water only, ○———○, 5 mM $MgSO_4$, or △———△, 1 mM EDTA (sodium salt). After hemolysis, reversal and resealing, the ghosts were washed twice in a medium containing 146 mM KCl and 20 mM Pipes, and twice in a medium containing 146 mM NaCl and 20 mM Pipes. K^+ efflux was measured at 37°C, pH 7.0, in a 3.6% ghost suspension. Ordinate: K^+ content in mM/l packed ghosts. Abscissa: calculated dilution of original cell content, percent

Fig. 5. The effect of washing in the presence of magnesium or chelating agents on resealed ghosts. The data is taken from four experiments in which cells were suspended in 166 mM Pipes buffer, pH 6.5, 38% hematocrit. 1 vol. cell suspension was hemolysed with 20 vols. distilled water at 0°C. After reversal and resealing, the ghosts were washed twice in a medium containing 146 mM KCl and 20 mM Pipes and twice in a medium containing 146 mM NaCl and 20 mM Pipes (●———●). In addition, the wash media contained either: +———+, 5 mM ATP (sodium salt); ○———○, 5 mM $MgSO_4$, X———X, 5 mM sodium citrate, or △———△, 10 μM EDTA (sodium salt). K^+ efflux was measured at 37°C, pH 7.1, in a 3.7% ghost suspension. Ordinate: K^+ content in mM/l packed ghosts. Abscissa: time, min.

While it may be possible that the dilution of a cellular metal ion such as Mg^{2+} may be responsible for the uncovering of the K^+ leak, one should consider the alternative possibility that a cellular chelating agent may be the essential factor that is diluted. In the absence of an adequate concentration of the chelator at either the inner or outer surface a specific leak for K^+ can develop.

The intact red blood cell and resealed ghost can display a specific leak for K^+ under special conditions. Heavy metals such as lead can produce a K^+-specific leak [8]. In metabolically depleted cells and ghosts, added Ca^{2+} can induce a specific leak for K^+ [8–11]. In the present work, the K^+ leak differs from that of the Ca^{2+}-induced leak since: (a) no added Ca^{2+} was required at either hemolysis or after resealing at 37°C; (b) the pH dependence of the Ca^{2+}-induced K^+ leak does not display a maximum near pH 6.7. With increasing pH, the leak reaches a maximum near pH 7.5 and forms a plateau at higher pH [10]. Still one cannot say how exclusive are the two leak pathways. The observed K^+ leak in resealed ghosts may be the result of damage induced at the time of hemolysis to the membrane. Johnson and Kirkwood [12] have shown that ghosts can lose protein and the ability to reseal when the membranes are maintained at low ionic strength and elevated temperature. However, in the work presented here, the open membranes were in a low ionic strength medium for a short period of time and at 0°C. In addition it is unlikely that the loss of protein is the source of the leak, since the leak once present can be suppressed by washing in a number of chelating agents.

It may be more likely that through the depletion of native chelators, the state of the membrane or a membrane component is changed. And with the redistribution of membrane-bound metal ions at hemolysis, in the absence of chelators, a K^+ leak is induced. Hemolysis in the presence of chelators may protect the membrane through the sequestering action of the chelator.

Acknowledgement

The authors would like to thank gratefully Prof. H. Passow for his support and stimulating discussions.

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