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Kinetic studies of human erythrocyte membrane resealing

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Following lysis in hypotonic media, human erythrocyte membranes will spontaneously reseal and regain their original low permeability for polar solutes. It is generally accepted that resealing will only occur when the membranes are heated above a critical temperature, and that the membrane lesions are stable under cold conditions. Contrary to these prevailing notions, a detailed investigation of the temperature dependence of resealing kinetics over the temperature range 0–22°C revealed that resealing occurs at measurable rates at temperatures as low as 0°C, even in buffers of low ionic strength. At all temperatures studied, initial resealing rates were approximately first-order, and Arrhenius plots of these rates revealed a sharp, singular discontinuity at approx. 7°C.

Introduction

Hypotonic lysis of red cells leads to an abrupt loss of hemoglobin and other cytoplasmic proteins, through pores that form transiently in the erythrocyte plasma membrane. These pores result from ruptures created when the internal pressures generated by rapid osmosis become too large to be sustained by the membrane. Kinetic measurements made following lysis of human erythrocytes by an applied electric field pulse have suggested that the membrane disruption follows a stepwise mechanism: leakage of ions leading to an osmotic imbalance which in turn causes a colloid hemolysis of the membrane [1], and the formation of the pores which have a mean diameter of less than 500 Å [2]. Although cytoplasmic contents are lost upon bursting, indicating that the membrane permeability barrier has been destroyed, the cytoplasmic membrane nevertheless retains much of its overall geometric integrity and can spontaneously regain its barrier properties ('resealing') over a very broad range of temperatures. If lysis takes place in warm buffer, the lifetime of the pores is very short (a few seconds), but at 4°C they have a mean half-life of several hours. To account for this role of temperature in the resealing process, it has been postulated that the pores represent regions of discontinuity (phase separation boundaries) in the lipid matrix of the erythrocyte membrane [3]. The resealing is thus thought to be brought about by a disorganization of the phase separation, as the membranes are warmed, leading to lipid mixing and membrane reconstitution. The hypothesis that resealing should occur only when membranes are heated above a critical temperature is supported by the experimental observation of temperature-dependent lipid phase separations in both intact erythrocytes and in erythrocyte ghosts [4,5]. Nonetheless, the occurrence of definite phase transitions above 0°C still seems to be a debated issue, and in view of the considerable uncertainty surrounding membrane resealing at low temperatures we undertook a careful analysis of resealing rates

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for freshly prepared human erythrocyte membranes over the temperature range 0–22°C.

Materials and Methods

Bovine serum albumin was supplied by Calbiochem (San Diego, CA). ^{125}I-labeled protein was prepared by lactoperoxidase-catalyzed iodination performed at 4°C, essentially as described by Sefton et al. [6]. Labeled protein was purified by gel permeation chromatography over Bio-Rad P-10 prior to use. Greater than 97% of the radioactivity was associated with bovine serum albumin, as judged by precipitation in the presence of 10%, w/v trichloroacetic acid at 4°C.

Membrane preparation. Human blood was obtained by venipuncture. Typically, 20 ml of blood was aspirated into a heparin-treated tube. The blood cells were washed once in Alsever's solution [7] and twice more in 5 mM potassium phosphate buffer (pH 7.4) containing 150 mM sodium chloride (phosphate-buffered saline). The buffy coat (leukocytes) was removed by careful aspiration and the residual erythrocytes (approx. 8 ml packed cell volume) were lysed by diluting them in 320 ml ice-cold 5 mM sodium phosphate buffer (pH 7.4). From this point on, great care was taken to mainten the membranes at 0°C; all samples, buffers, glassware and centrifuge tubes were stored in an ice-water slush bath.

After 5 min incubation in the lysis buffer, the erythrocyte membranes were pelleted by centrifugation (30 000 × g, 15 min, 2°C) and resuspended in phosphate-buffered saline (approx. 8 ml) containing bovine serum albumin (1 mg/ml). After 5 min equilibration at 0°C, ^{125}I-labeled bovine serum albumin (typically $5 \cdot 10^6$ cpm) was added to the membrane suspension which was then mixed well before being stored carefully at 0°C.

Membrane resealing; kinetics. To initiate membrane resealing, the membrane preparation was transferred to a reaction vessel maintained at a specific temperature (zero time). The vessel consisted of a water-jacketed glass beaker connected to a circulating water bath. Temperature was maintained at the determined value to an accuracy of 0.1°C, and was monitored continuously using a platinum resistance thermometer coupled to a chart recorder. Throughout the experiment, the membrane suspension was agitated very gently using a slow-speed magnetic stirrer.

At specified time intervals, triplicate 100-μl samples were taken from the reaction vessel, transferred to 1.5 ml plastic centrifuge tubes and spun for 5 min at 18 000 × g to pellet the membranes. The pellets were washed once with 0.5 ml phosphate-buffered saline, and the radioactivities of the washed pellets and combined supernatants were determined.

Membrane resealing; temperature dependence. Membranes were prepared, as above, in double quantity, yielding approx. 16 ml (maintained at 0°C). This suspension was distributed, in 60-μl aliquots, into 260 glass tubes (5 × 40 mm) which had been precooled to 0°C. These tubes were fitted with small rubber O-rings near the tops and suspended in a sheet of plastic which had been drilled with a 16 × 16 array of 6 mm diameter holes. The entire assembly was placed over an ice-water slush bath so that the tubes were immersed to a depth of at least 30 mm.

A small water bath was equilibrated to the desired temperature and four of the small glass tubes, containing ice-cold membranes, were immersed in this bath and incubated at the specified temperature for exactly 15 min. At the end of this time, the tubes were removed from the reaction bath and immersed in ice-water. Ice-cold phosphate-buffered saline (0.5 ml) was added to each tube and, after approx. 10 min at 0°C, the tubes were centrifuged at 18 000 × g for 5 min, as described above. Membrane pellets were washed with phosphate-buffered saline, and the radioactivity of the combined supernatants and individual pellets was determined as noted above. This procedure was repeated for each experimental temperature, from 0 to 10°C at 0.5 deg. C intervals and from 10 to 20°C at 1 deg. C intervals. Temperatures were monitored, with a platinum resistance thermometer.

Discontinuities in Arrhenius functions of this data were determined by computer analysis of multiple overlapping segments of the Arrhenius function (e.g., 0–10, 5–15 and 10–20°C) [8]. For a given segment, an initial inspection involved simultaneous testing for equality of slopes and intercepts; if these values were inconsistent with a single line for a given segment ($P < 0.05$), a

curve-splitting routine was applied to obtain minimum residual sum of squares. (The program (PASCAL) for curve-splitting is available on request). Individual *t*-tests for equality of slopes and equality of intercepts were performed to determine if intervals obtained by curve-splitting differed significantly ($P < 0.05$).

Results

Fig. 1 shows the results of a representative resealing kinetics experiment in which measurements were made at 7.9°C. The inset presents the results in the form of a first-order plot, from which a first-order rate constant of $3.3 \cdot 10^{-4}$ s^{-1} was derived. That the resealing kinetics approximate first-order kinetics over the initial part of the reaction should not be taken as evidence that the kinetics are, in reality, first-order. Indeed, there is considerable deviation from linearity during the later stages of the resealing process, and it would probably be misleading to ascribe a reaction order to a process as ill-defined and complex as membrane resealing. Nevertheless, an assumption of log linearity during the early phase of resealing was of obvious practical utility in the subsequent stages of our study.

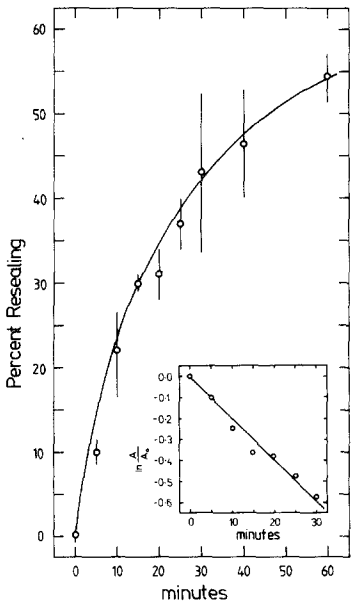

Fig. 1. Kinetics of membrane resealing. Resealing of erythrocyte ghosts was measured in 5 mM potassium phosphate buffer (pH 7.4) containing 150 mM sodium chloride and 1 mg/ml ^{125}I-labeled bovine serum albumin at 7.9°C. The degree of entrapment of albumin was determined at time intervals and used to calculate the percentage resealing over time. The inset shows the data plotted according to a first-order rate equation where A_0 and A are the relative proportions of ghosts unsealed at zero time and time t, respectively.

Resealing kinetics were studied at several temperatures over the range 0–35°C, and also under various conditions of buffer ionic strength. We found no significant difference in resealing rates of membranes suspended in low and high ionic strength buffers (Table I). Low concentrations of calcium ions had no significant effect on resealing either, but resealing was somewhat slower in 0.02 M Ca^{2+} (Table II).

In our initial measurements of the degree of temperature dependence of resealing rates, we monitored resealing kinetics, as described in Fig. 1, at a number of discrete temperatures. These initial observations indicated that there was a strong temperature dependence for the resealing process, and also showed that there was an appreciable rate of resealing even at 0°C ($k = 2.75 \cdot 10^{-5}$ s^{-1}, half-time 7 h). Temperature dependencies of membrane-associated processes are normally revealed by plotting data according to the Arrhenius equation. With this type of plot, however, it is essential that sufficient data points be accumulated to allowed intelligent line extrapolation and to permit the identification of line discontinuities. Since it was not feasible to collect the required number of data points by direct measurement of sealing kinetics at each temperature, we modified the assay conditions as described under

TABLE I

EFFECT OF IONIC STRENGTH ON RESEALING RATES
The half-time (minutes) of membrane resealing was measured at five temperatures in 5 mM potassium phosphate buffer (pH 7.4) (5P), or in the same buffer containing 150 mM sodium chloride (phosphate-buffered saline (PBS)).

Temperature (°C)	Resealing half-time (min)	
	PBS	5P
0	> 500	> 500
5	46	75
10	6.1	6.1
15	5.1	4.7
20	5.8	4.7

TABLE II

EFFECT OF CALCIUM ION CONCENTRATION ON RESEALING RATES

The half-time of membrane resealing was measured at 8°C in 5 mM potassium phosphate buffer (pH 7.4) containing 150 mM sodium chloride and varying concentrations of calcium chloride. The results are presented as half-times (minutes) for maximum resealing.

$[Ca^{2+}]$ (M)	Resealing half-time (min)
0	29
10^{-3}	29
10^{-2}	35

Materials and Methods. This enabled us to obtain enough data points for an accurate analysis of the temperature dependency. Fig. 2 shows the results of our study plotted according to the Arrhenius equation. Clearly, the data cannot be fitted to a single straight line. Above 7°C, the slope of the plot yields an apparent activation energy for resealing of 104 kJ · mol^{-1}; below 7°C, the activation energy appears to be approx. 550 kJ · mol^{-1}. The discontinuity in the line was located objectively with the aid of a computer program [8].

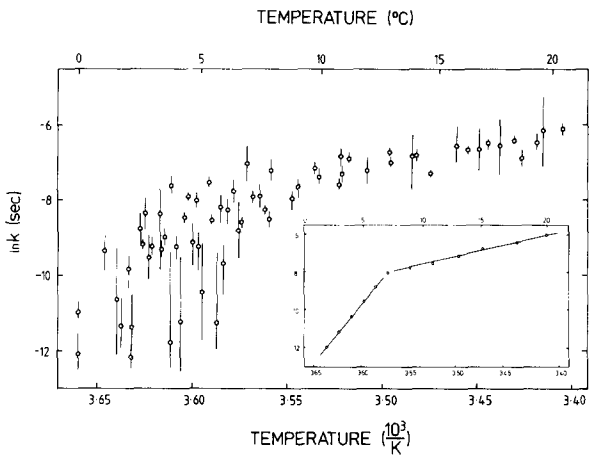

Fig. 2. Temperature dependence of membrane resealing. Initial rates of resealing were measured at different temperatures, as described under Materials and Methods. The rates are plotted according to the Arrhenius equation which yields an apparent activation energy (E_a) above 7°C of 104 kJ · mol^{-1}, and below 7°C of 550 kJ · mol^{-1}. Bars show standard deviations from the mean of at least four determinations. The inset shows the results of a simulation in which E_a is assumed to be 104 kJ · mol^{-1} (and constant) above 7°C. Below this temperature, E_a is assigned an inverse temperature dependence of 1.43 kJ · mol^{-1} resulting in a uniform increase from a value of 104 kJ · mol^{-1} at 7°C to 114 kJ · mol^{-1} at 0°C. The simulation matches the experimental data and shows how a sharp discontinuity can arise from the initiation of a gradual, progressive change in membrane properties.

Because of the length of time needed to monitor resealing rates over such a large temperature range, it was necessary to include appropriate controls. All determinations of trapped protein (measurement of resealing) were corrected for nonspecific protein adsorption, and were also corrected for changes in the maximum degree of resealing attainable at different times throughout the experiment. Adsorption was estimated by measuring the amount of radioactive bovine serum albumin copelleting with unsealed membranes after a 5 min incubation at 0°C. Maximum attainable trapping was determined after incubation of bovine serum albumin with membranes for 15 min at 37°C. These control determinations were made at several points during the resealing experiments in order to detect any changes in the behavior of the membranes that might have been caused by the prolonged incubation at 0°C in 5 mM phosphate buffer.

Discussion

The temperature dependence of resealing is an interesting, and experimentally accessible phenomenon. Intact erythrocyte membranes have been shown to undergo a variety of temperature-dependent changes in physical state [9–11], and there have been a number of investigations into the effects of temperature on resealing [3,12–15]. There seems to be no clear consensus regarding the mechanism of resealing or the factors regulating it. It has variously been suggested that resealing kinetics show a long lag period at temperatures below 20°C [14] or, alternatively, that resealing will not take place at all below 5°C, under conditions of low ionic strength or after extensive proteolysis of the membranes [3]. However, we have found that resealing readily occurs at temperatures as low as 0°C (Fig. 2) and will also occur spontaneously under conditions of reduced ionic strength (Table I). These inconsistencies with previous reports deserve some comment. The Arrhenius plot in Fig. 2 shows a marked discontinuity at approx. 7°C, leading to extremely low resealing rates be-

low 4°C. However, resealing certainly occurs at measurable rates in the cold and it is inappropriate to consider this discontinuity as marking a cut-off point for the resealing process. Reports implying such a cut-off presumably stem from the difficulties inherent in making measurements of the slow rates involved. The first-order kinetics for resealing at 7.9°C (Fig. 1) are qualitatively similar to those reported by Johnson for resealing at 37°C [14]. We find no evidence for a lag period, during which resealing does not occur, at any temperature above 0°C.

We have previously interpreted breaks in Arrhenius plots of membrane processes as a consequence of the inherent energy-entropy compensation at the lipid phase transition [16]. The large increase in apparent activation energy (E_a) for resealing which occurs at 7°C presumably stems from a change in the temperature dependency of E_a, and 550 $kJ \cdot mol^{-1}$ is, as a consequence, likely to be an overestimate of the true energy barrier for resealing. The inset to Fig. 2 shows a simulation of the consequences of temperature dependence of E_a below a critical temperature, designated 7°C. Above 7°C, E_a is assumed to be 104 $kJ \cdot mol^{-1}$ and independent of temperature. Below 7°C, E_a has been assumed to be inversely dependent on temperature (1.43 $kJ \cdot mol^{-1} \cdot deg^{-1}$). This assumption results in a simulated plot which closely matches the experimental data presented in the main part of Fig. 2. The apparent E_a calculated from this plot is 550 $kJ \cdot mol^{-1}$ below 7°C, but note that this value, determined from the slope of the Arrhenius function, has been derived from a simulated plot in which E_a progressively increases from a value of 104 $kJ \cdot mol^{-1}$ at 7°C to 114 $kJ \cdot mol^{-1}$ at 0°C. Clearly, literal interpretation of Arrhenius functions of membrane processes must be made with some degree of caution [16]. The simulation shows that a sharp discontinuity can arise from the initiation of a gradual, progressive change in membrane properties.

In our experiments, we were careful to store unsealed membranes on wet ice at all times prior to sealing measurements. The temperature of the resealing reaction vessel was allowed to equilibrate and at zero time glass tubes containing samples of the membranes were plunged into the vessel, incubated for an appropriate time interval, then returned to the ice bath. Consequently, at time zero the membranes were at 0°C, whereas the reaction vessel was at some higher temperature. The finite time required for temperature equilibration (which was never greater than 1 min) and the consequences of this equilibration time have been taken into account during our analysis of the data. Initially, we had hoped to be able to equilibrate membrane samples to the reaction temperature in low ionic strength buffer, and then initiate resealing by adjusting the sample to 150mM sodium chloride. However, our observation (Table I) that resealing rates appear to be relatively insensitive to moderate changes in ionic strength meant that this approach had to be abandoned in favor of the temperature shift described above.

Some earlier reports [14,15] have suggested that resealing does not occur in low-ionic strength buffer. In these earlier studies, membranes were stored for at least 60min in low ionic strength calcium-free buffer (during membrane washing procedures) at 4°C. Subsequently, the membranes were incubated in warm low ionic strength buffer for 2 min prior to the addition of marker solute for monitoring resealing. At any given temperature, only about half the ghost membranes appeared to reseal during the subsequent incubation in the presence of marker solute (hemoglobin). No explanation was given for this behavior; a reasonable interpretation is that a significant degree of resealing takes place before the addition of marker solute, an interpretation which is strongly supported by our own experimental observations. A decisive role for buffer ionic strength in controlling resealing would imply a parallel role for membrane proteins in regulating the process since these are likely to be the components most likely to be affected by moderate variations in buffer salt concentration. Since we found very little effect of ionic strength on resealing rates over a range of temperatures, we conclude that resealing is predominantly a phenomenon of lipid behavior.

Although there is no convincing evidence for the specific involvement of particular proteins in the formation or resealing of the pores that mediate the exit of hemoglobin and other cytoplasmic components from the cell, the membrane lipids themselves are not the sole components governing the recovery of membrane integrity. The red cell mem-

brane is supported by a complex of proteins, the membrane skeleton [17], which provides a mechanical support for the bilayer, and which presumably prevents the complete collapse of the membrane during the trauma of hypotonic lysis. We might draw the analogy of the red cell as a canvas tent which can sustain several fabric tears without losing its shape in contrast to a balloon which cannot. The major component of this membrane skeleton is spectrin, a high molecular weight protein which can self-assemble into very large aggregates [18,19]. Spectrin seems to be confined to a discrete zone close to the inside surface of the plasma membrane to which it is attached through a high-affinity association with integral membrane proteins, principally syndein [20].

Treatment of unsealed erythrocyte membranes, at 4°C, with the proteinases bromelain or thermolysin dramatically diminishes subsequent resealing efficiency (data not shown), and this is in agreement with earlier studies on the effect of proteolysis on ghost resealing [3]. However, in our view, the results of our present study are entirely consistent with a process of resealing which depends primarily on lipid behavior. Hemolysis cannot be initiated by changes in temperature, only by osmotic shock, so it seems unlikely that the membrane lesions are created by temperature-dependent lipid phase separations. In contrast, resealing is exquisitely sensitive to temperature, and undoubtably involves the lipid phase. The probable role of the membrane skeleton, as a matrix preventing disintegration of the membrane during hemolysis, is illustrated in Fig. 3. Upon lysis, the plasma membrane suffers extensive rupturing as the osmotic forces generated by the movement of water during hypotonic shock become greater than the (mainly hydrophobic) forces maintaining the phospholipid molecules in a continuous two-dimensional array. The large membrane fragments are held to the underlying spectrin network in an orientation which allows them to fuse together again, forming a single continuous surface, once the osmotic forces have been dissipated. The subsequent resealed ghosts appear to have the same trapped volume to surface area ratio as the original erythrocytes. In the absence of an intact membrane skeleton, the membrane fragments are less likely to be in close juxtaposition after lysis, and

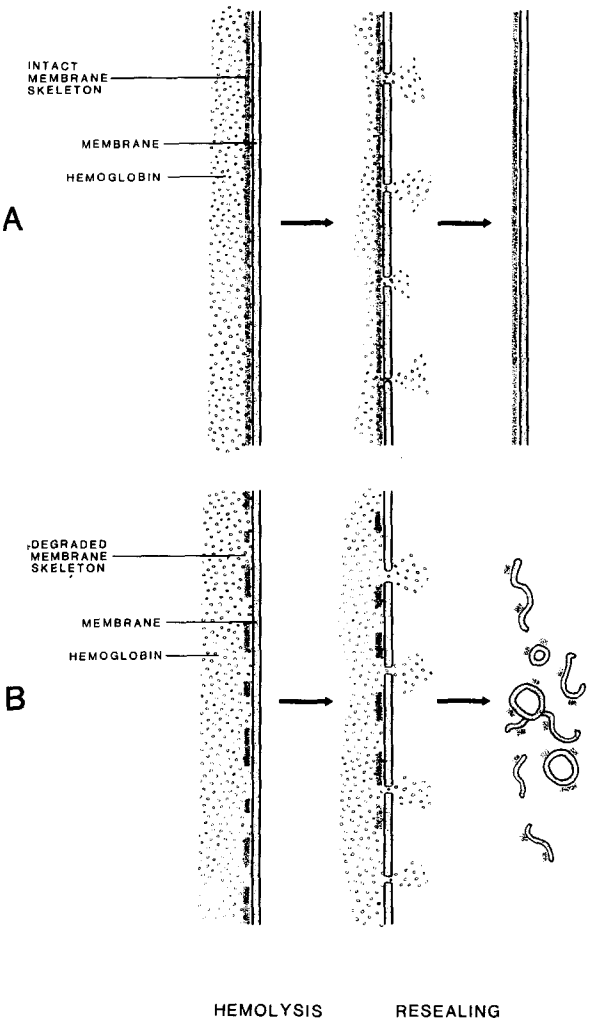

Fig. 3. Role of the membrane skeleton in resealing. (A) Membrane fragments, generated during hypotonic lysis of the erythrocyte membrane, are held to an underlying spectrin network allowing them to fuse together again once osmotic forces have been dissipated. (B) In the absence of an intact membrane skeleton, the membrane fragments are liberated during lysis and are unable to reseal as a continuous bilayer, instead tending to form inverted membrane vesicles.

are correspondingly less likely to be capable of resealing as a single sheet, instead tending to form small membrane vesicles having a greatly diminished volume to surface area ratio. The implication is that during moderate osmotic shock, the membrane skeleton is sufficiently elastic to absorb the forces leading to lysis of the bilayer to which it is attached. However, treatment of membranes

with proteinase, or hemolysis of erythrocytes in very low ionic strength buffer (e.g., 0.5 mM sodium phosphate), leads to the partial disintegration of the spectrin skeleton, liberating membrane fragments which reseal (presumably in a temperature-dependent manner) as small inverted vesicles [21]. At low temperatures, membranes appear to be remarkably resistant to low calcium conditions. At higher temperatures, low calcium promotes loss of spectrin [15] leading to a diminished resealing ability; however, it is significant that the process of membrane resealing, monitored in cold conditions, is not calcium-dependent (Table II).

Our study shows that erythrocyte resealing represents an easily accessible model system for studying both intramembrane fusion and membrane skeleton integrity. A careful analysis of the rates of resealing reveals fundamental features of phospholipid behavior within the complex environment of a eukaryotic plasma membrane. In this regard, the model is a useful adjunct to the more conventional assays of intermembrane (cell-cell) fusion used to study the effects of fusogenic agents such as lysophosphatidylcholine, saturated fatty acids, dimethyl sulfoxide or sorbitol. However, if our interpretation of resealing as illustrated in Fig. 3 is correct, then the system can also be used to examine the effects of conditions which disturb the integrity of the membrane skeleton, such as changes in divalent cation concentration. Our results suggest a sharp distinction between the independent roles of protein and phospholipid in the maintenance and recovery of membrane integrity which will need to be clarified before a more detailed analysis of resealing mechanisms can be proposed.

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