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EFFECTS OF CALCIUM ON POTASSIUM AND WATER TRANSPORT IN HUMAN ERYTHROCYTE GHOSTS

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SUMMARY

Bulk water transport in reconstituted ghosts is statistically comparable to that in the parent red cells, and is unaffected by incorporation of Ca^{2+} over the range of 0.01 to 1 mM. Brief exposure of ghosts to *p*-chloromercuribenzene sulfonate results in a supression of osmotic water flow but leaves K^+ permeability unchanged. Incorporation of *p*-chloromercuribenzene sulfonate provokes extremely rapid K^+ loss which can be counteracted by simultaneous inclusion of Ca^{2+}.

Erythrocyte ghosts, when prepared with a small amount of Ca^{2+}, demonstrate recovery of normal impermeability to choline, sucrose, Na^+ and inulin and have an improved K^+ retention over Ca^{2+}-free preparations.

The rate of passive transport of K^+ from unwashed erythrocyte ghosts was measured during the initial few minutes of efflux. The initial rates vary in a bimodal fashion with the concentration of Ca^{2+} incorporated at the time of hemolysis. In low concentrations (0.01–0.1 mM), Ca^{2+} protects the K^+ barrier while at higher concentrations (0.1–1.0 mM) it provokes a K^+ leakage ranging from 7 to 50 times the normal rate of passive K^+ loss. The Ca^{2+}-induced K^+ leak is thus a graded response rather than a discrete membrane transport state. The transition from a Ca^{2+}-protected to a Ca^{2+}-damaged membrane occurs upon an increase in Ca^{2+} concentration of less than 50 μmoles/l.

INTRODUCTION

The erythrocyte ghost is used as a model system in the investigation of plasma membrane transport because it retains many of the attributes of normal erythrocyte membranes such as relative impermeability to cations [1–3], and functional enzyme systems associated with glycolysis [4] and the active transport of ions [2, 5–8]. In this paper, we have utilized ghosts prepared both in the presence and absence of Ca^{2+} and Mg^{2+} to measure (1) the passage of bulk water under conditions of normal and inhibited flow and (2) K^+ permeability, concentrating on the very rapid fluxes that occur during the first few minutes of efflux. Ca^{2+} and Mg^{2+} are believed to

Abbreviation: PCMBS, *p*-chloromercuribenzene sulfonate.

confer stability upon biological membranes [3, 9–12] although Ca^{2+} is known to have deleterious effects under certain conditions of altered cell metabolism [13–15]. Ca^{2+} is of special interest since it can affect the hydration and water permeability of artificial and biological membranes [16, 17] and induce a specific increase in K^+ permeability in erythrocytes and ghosts [3, 18–21]. We find that while Ca^{2+} influences K^+ permeability in a complex fashion, bulk water flow is essentially unaffected. The value of the hydraulic permeability coefficient (RTL_p) in ghosts is statistically comparable to that of intact red cells.

MATERIALS AND METHODS

Preparation of ghosts

Red cells were sedimented from heparinized blood that was freshly drawn, or stored for less than 4 days, and the plasma and buffy coat removed by aspiration. The pellet was resuspended in 150 mM choline chloride–10 mM Tris, pH 7.4, centrifuged for 5 min at $3000 \times g$ and the supernatant discarded. This resuspension–centrifugation procedure was repeated twice. The final pellet had a hematocrit of about 85 %. For hemolysis, red cells were resuspended dropwise into the hemolyzing medium at a ratio of 1 : 10 (cells to medium) after Hoffman [3]. The composition of the hemolyzing solution was adjusted to 60 mosmoles per l with mannitol and to an ionic strength of 0.024 with choline chloride. After 5 min of hemolysis on ice, a concentrated salt solution, usually 375 mM KCl plus 2625 mM choline chloride, was added to return the hemolysate to isotonicity (290 mosM). This solution adjusted the internal K^+ concentration of the ghosts to about 15 mM K^+, and thereby avoided the

TABLE I

MEAN CELL VOLUME BEFORE AND AFTER K^+ EFFLUX OF Ca^{2+}-TREATED GHOSTS RECONSTITUTED WITH 3 M KCl

Ghosts were hemolyzed by the procedure described in Materials and Methods in the presence of either 0.5 mM or 0 mM Ca^{2+}. Ghosts were reconsituted with 3 M KCl, sedimented, then resuspended, and incubated in the media indicated. Incubation Medium A: 120 mM KCl–24 mM choline chloride–10 mM Tris (pH 7.4), the incubation medium which results from addition of 3 M KCl to the hemolyzing medium. Incubation Medium B: 0.5 mM $CaCl_2$–120 mM KCl–21 mM choline chloride–6 mM mannitol–10 mM Tris (pH 7.4), the usual incubation medium for ghosts hemolyzed in 0.5 mM $CaCl_2$. Efflux Medium: 150 mM choline chloride–10 mM Tris (pH 7.4), the standard K^+-free medium used for K^+ efflux measurements. Mean cell volumes were measured after 1 h of resuspension. Values are the average of two determinations.

Cell type	Ca^{2+} concentration in hemolyzing medium (mM)	Resuspension medium	Mean cell volume (μm^3)
Ghosts	0.0	Incubation A	90.0
Ghosts	0.0	Efflux	64.2
Ghosts	0.5	Incubation B	89.5
Ghosts	0.5	Efflux	48.3
Erythrocytes	—	Incubation A	90.0
Erythrocytes	—	Incubation B	90.0
Erythrocytes	—	Efflux	88.7

large volume changes which result when K^+ efflux occurs into K^+-free medium from ghosts loaded exclusively with KCl (see Table I). The hemolysate was incubated at 37 °C for 60 min while rocked gently in a Dubnoff shaker. The ghost suspension was then centrifuged at $8500 \times g$ for 7 min. The supernatant was discarded leaving a pellet of dark pink ghosts. A red "button" of dense material at the tube bottom was also discarded. The pellets were kept on ice and used on the same day. All solutions and centrifugations were at 4 °C unless otherwise noted.

Osmotic water flow experiments

Osmotic water flow experiments were conducted by the method of Farmer and Macey [22]. The ghosts were diluted to a 2 % suspension. In most experiments, swelling or shrinking was induced by step changes in the concentration of choline chloride, and subsequent cell volume changes were monitored photometrically.

Following a small perturbation in osmotic pressure of the suspending solution, the cell volume V changes exponentially from an initial isotonic volume V_0 to a final volume V_∞ [22]. That is:

$$V = V_\infty + (V_0 - V_\infty)e^{-0.69t/T_{\frac{1}{2}}} \quad (1)$$

where t represents time and $T_{\frac{1}{2}}$ is the half-time. The osmotic water permeability coefficient denoted by RTL_p can be calculated directly [22] from $T_{\frac{1}{2}}$ and is given by:

$$RTL_p = 0.69\,(V_0 - b)C_0/AC_\infty^2 T_{\frac{1}{2}} \quad (2)$$

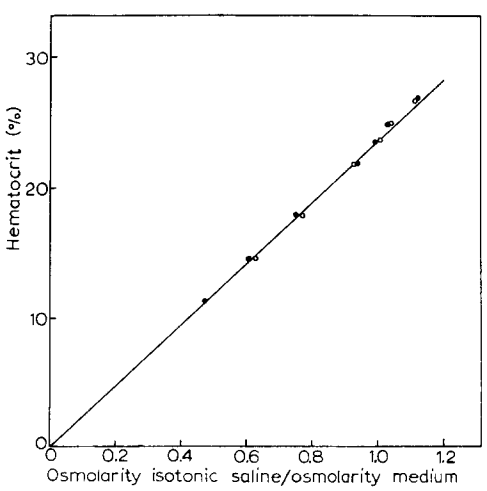

Fig. 1. Determination of non-osmotic volume (b) of ghosts prepared in the presence and absence of Ca^{2+}. Ghosts were prepared by hemolyzing red cells in a ratio of 1:10 (cells/medium) in ice-cold hemolyzing solution of 60 mosM/l. Ca^{2+}-free medium was 24 mM choline chloride–10 mM Tris (pH 7.4). The 1 mM Ca^{2+}-medium also included 6 mM mannitol–21 mM choline chloride–10 mM Tris (pH 7.4). After 5 min, a volume of hypertonic salt solution of 375 mM KCl–2625 mM choline chloride was added, sufficient to return the hemolysate to isotonicity. Ghosts were incubated for 1 h at 37 °C, sedimented, then resuspended in solutions of varying osmolarity and hematocrits measured. A plot of hematocrit of ghost suspension vs osmolarity of isotonic saline/osmolarity of suspending medium shows that the data points lie on a line which intercepts the origin, indicating the absence of non-osmotic volume [23]. ○—○, Ca^{2+}-free ghosts; ●—●, ghosts prepared in 1 mM Ca^{2+}.

where b represents the non-osmotic volume, C_0 is the initial osmotic concentration ($C \approx 0.290$ osM in these experiments), C_∞ is the osmotic concentration in the suspending medium following perturbation, and A is the area of the cell membrane. The value of b in ghosts is determined by measuring the hematocrit of reconstituted ghosts equilibrated in solutions of varying osmolarity [23]. A plot of hematocrit vs osmolarity of isotonic saline/osmolarity of medium, intercepts the origin (Fig. 1), indicating negligible osmotically inactive cell volume. A value of $b = 0.4\ V_0$ is used for red cells.

Application of this analysis to our data carries the implicit assumption that the cell membrane is impermeable to choline, i.e. the reflection coefficient, σ, is equal to 1. Support for this assumption was obtained by comparing results where sucrose was substituted for choline in the step-change of concentration of osmotic solute. Values for RTL_p using sucrose were comparable to choline. This implies that the value for σ was the same for both sucrose and choline; presumably the only feature common to both solutes is their impermeability.

Measurement of static cell volumes

Ghosts and red cells were counted in a Coulter particle counter, Model B (Coulter Electronics, Hialeah, Fla.), using a 100 μm aperture. The mean cell volume was designated to be that lower threshold setting at which half of the counts in a 0.5 ml aliquot of the particular sample were obtained. The instrument was calibrated using intact red blood cells as a standard and assuming a mean red cell volume of 90 μm^3. Ghost volumes were determined on a $1:1 \cdot 10^{-5}$–$2 \cdot 10^5$ dilution of the ghost pellet. The osmolarity of the sizing medium was identical to the final osmolarity of the water permeability experiment, thus making the measured ghost volumes equal to the final volume.

K^+ efflux procedure

An aliquot of the ghost pellet was rapidly diluted to 1 : 20 with room-temperature 150 mM choline chloride–10 mM Tris (pH 7.4) and a 10 ml aliquot transferred to a cuvette containing a magnetic stir bar. A K^+ electrode (Electronic Instruments Ltd, Model GKN33C) measured the K^+ entering the medium from the cells the electrode signal was calibrated by a set of K^+ standard solutions. Sampling was begun 1.5 min following contact of the suspension with the electrode giving ample time for electrode stabilization. After 5–6 min of efflux, the sample was removed, sonicated for 20–30 s with a Sonifier (Model S45, Branson Instruments, Inc., Stamford, Conn.) at setting "7", the K^+ content of the sonicate measured and designated the total K^+ of the sample. The extracellular K^+ of the remaining efflux suspension (unsonicated) was measured after 4 h and designated the asymptote of the efflux curve. While the first few minutes of K^+ efflux were often linear, there were cases where the efflux data showed significant curvature. For this reason, initial slopes were estimated by fitting the data to a second degree polynomial by the method of least squares. The initial slope obtained from the polynomial was designated the initial rate of K^+ efflux.

Extracellular volume measurement

[^{14}C]Inulin was introduced into the hemolysate at 0.05 μCi/ml suspension

at times indicated in the text (p. 235). Aliquots of 0.1 ml of supernatant and packed cell pellet were counted in 20 ml of counting gel in a liquid scintillation counter, in conjunction with appropriate ^{14}C-labelled standards to correct for quenching of counts by the different sample materials. The percentage of the pellet volume which was extracellular (%ECV) was calculated by:

$$\%ECV = \frac{\text{cpm/ml pellet}}{\text{cpm/ml supernatant}} \times 100$$

RESULTS

Osmotic water permeability of ghosts

The osmotic water permeability of the ghosts was not significantly different from that of the erythrocytes from which the ghosts were prepared. While the reconstituted ghosts and red cells had similar mean cell volumes at isotonicity, the ghosts required a relatively longer time for attainment of osmotic equilibrium (Table II).

TABLE II

WATER PERMEABILITY OF ERYTHROCYTES AND GHOSTS: HALF-TIMES ($T_{\frac{1}{2}}$) OF OSMOTIC EQUILIBRATION AND FILTRATION COEFFICIENTS (RTL_p)

Erythrocytes were hemolyzed in 24 mM choline chloride–10 mM Tris (pH 7.4). An aliquot of red cells was reserved unhemolyzed as a control. A separate sample of blood was used for each experiment and measurements of cell volume and $T_{\frac{1}{2}}$ and calculations of RTL_p were as described in the text. The values for $T_{\frac{1}{2}}$ and RTL_p are the mean and S.E. of eight determinations on a single sample of cells. A paired *t*-test was applied to the pairs of RTL_p values obtained for each experiment.

Experiment	Cell type	Mean cell volume (μm^3)	Medium osmolarity (mosM/l)	$T_{\frac{1}{2}}$ (s)	RTL_p ($cm^4 \cdot osM^{-1} \cdot s^{-1}$)
W-a	Erythrocytes	90.0	294	0.27	0.282
	Ghosts	86.8	294	0.36	0.338
W-b	Erythrocytes	90.0	264	0.30±0.02	0.332±0.02
	Ghosts	99.2	264	0.76	0.240
W-c	Erythrocytes	90.0	309	0.25±0.01	0.294±0.01
	Ghosts	80.8	309	0.33±0.01	0.330±0.01
W-d	Erythrocytes	90.0	289	0.24±0.01	0.340±0.01
	Ghosts	82.6	289	0.39±0.00	0.329±0.03
W-e	Erythrocytes	90.0	291	0.26±0.01	0.313±0.01
	Ghosts	69.1	291	0.38±0.01	0.277±0.01
W-f	Erythrocytes	90.0	270	0.34±0.02	0.279±0.02
	Ghosts	90.0	270	0.53±0.02	0.296±0.01
W-g	Erythrocytes	90.0	281	0.22±0.01	0.398±0.01
	Ghosts	91.2	281	0.35±0.00	0.429±0.04
W-h	Erythrocytes	90.0	281	0.21±0.01	0.420±0.01
	Ghosts	89.4	281	0.36±0.01	0.400±0.01

Average RTL_p erythrocytes 0.330±0.017
Average RTL_p ghosts 0.322±0.021 ($P > 0.5$)

This reflects the larger osmotic volume of the ghosts resulting from hemoglobin loss. The ghost non-osmotic volume, *b*, was determined to be near zero by hematocrit measurements made at various suspending solution osmolarities (see Methods and Fig. 1). The values of the filtration coefficient, RTL_p, of ghosts and erythrocytes were statistically comparable (Table II).

The possible effect of Ca^{2+} on water movement was examined in two ways; first by treatment of the interior surface of the membrane with Ca^{2+} by hemolyzing the red cells in dilute choline chloride solutions containing Ca^{2+} in concentrations from 0.01 to 0.5 mM, and second, by exposure of the exterior membrane surface of reconstituted ghosts to a similar range of Ca^{2+} concentrations. Following preparation of ghosts in the presence of Ca^{2+}, a reduction in isotonic cell volume occurs ranging from 10 to 40 % of the Ca^{2+}-free controls (Table III). However, while ghost volumes decrease with increasing Ca^{2+} concentration, the RTL_p of these cells remains essentially constant at a level about 10 % higher than the Ca^{2+}-free controls (Table III). The addition of Ca^{2+} to the resuspending medium of reconstituted Ca^{2+}-free ghosts or intact red cells did not alter the volume or RTL_p from control values, nor, conversely, did the removal of Ca^{2+} from the media of ghosts hemolyzed with Ca^{2+} affect the isotonic cell volumes or RTL_p values. These data strongly suggests that the site of action of Ca^{2+} is at the membrane interior.

The reduction in isotonic ghost volumes was found to depend upon both the particular alkaline earth metal present at hemolysis and the solute used to restore isotonicity to the hemolysate. When ghosts were prepared with Mg^{2+} substituted in identical concentrations for Ca^{2+}, volume reductions with Mg^{2+} ranged from 5 to

TABLE III

EFFECTS OF Ca^{2+} INCORPORATED INTO ERYTHROCYTE GHOSTS AT HEMOLYSIS UPON INITIAL RATE OF K^+ EFFLUX, MEAN CELL VOLUME AND BULK WATER PERMEABILITY (RTL_p)

Initial rates of K^+ efflux were calculated by a least squares fit of the data from the first 5–6 min of K^+ efflux from 0.5 ml of packed ghosts into 10 ml of room temperature 150 mM choline chloride–10 mM Tris (pH 7.4). Each value is the mean and S.E.; the number of determinations is given in parentheses. Mean cell volumes and osmotic water permeability coefficients (RTL_p) are expressed as a percentage of the Ca^{2+}-free control which is set equal to 100 %. Each value is the mean and S.E. for six experiments. Each experimental value for RTL_p is the mean of eight determinations made on the same ghost preparation. Mean cell volume and RTL_p were determined as indicated in Materials and Methods. The ghosts were prepared as outlined in Fig. 1 at the Ca^{2+} concentrations indicated.

Ca^{2+} concentration in hemolyzing medium (mM)	Initial rate of K^+ efflux (mmoles per l cells/h)	Mean cell volume (% of control)	RTL_p (% of control)
0.0	75±18 (16)	100	100
0.01	31±11 (3)	86±2	110±8
0.025	15±3 (9)	—	—
0.05	18±3 (14)	—	—
0.10	47±13 (14)	83±3	113±5
0.15	98±8 (12)	—	—
0.5	101±17 (10)	68±3	107±9
1.0	104±14 (6)	—	—

TABLE IV

COMPARISON OF ISOTONIC VOLUMES AND WATER PERMEABILITY OF GHOSTS HEMOLYZED IN THE PRESENCE OF Ca^{2+} OR Mg^{2+}

Ghosts were hemolyzed in the standard medium of choline chloride–10 mM Tris (pH 7.4) supplemented with mannitol as described in Materials and Methods. Ca^{2+} or Mg^{2+} was included in the hemolyzing medium at the concentrations indicated. Mean cell volume measurements and RTL_p calculations are the average of two determinations.

Alkaline earth concentration at hemolysis (mM)	Mean cell volume (% of control)		RTL_p (% of control)	
	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}
Control 0.0	100	100	100	100
0.01	93.3	83.5	94.0	110
0.10	90.9	70.7	95.3	102
0.50	88.5	58.7	90.4	92.6

20 % as compared with 20 to 40 % with Ca^{2+}. However, there was no appreciable difference in RTL_p values between the Ca^{2+} and Mg^{2+} preparations (Table IV). Reconstitution of the Ca^{2+}- and Mg^{2+}-treated ghosts was accomplished with choline as the major cation in most experiments, with the resultant isotonic volume changes noted above. Reconstitution of Ca^{2+}-treated ghosts with K^+ alone failed to evoke cell shrinkage (compare mean cell volume of Ca^{2+}-free and 0.5 mM Ca^{2+} ghosts in incubation medium, Table I) suggesting that the membranes can recover normal impermeability to choline but not to K^+.

Water transfer through red blood cells probably occurs through two mechanisms, a bulk flow possibly through aqueous pores and a diffusional flow through the lipid bilayer. Recent experiments indicate that the bulk flow can be inhibited by organic mercurials such as *p*-chloromericurbenzene sulfonate (PCMBS) [24]. Experi-

TABLE V

ALTERATIONS IN WATER PERMEABILITY OF GHOSTS AND ERYTHROCYTES BY THE COMBINATION OF PCMBS AND Ca^{2+}

Results are expressed as the ratio of half-times of osmotic equilibration of PCMBS-treated to PCMBS-free controls. These data constitute a representative experiment performed with a single blood sample. Ca^{2+} was added to the hemolyzing medium of ghosts or to the suspending medium of erythrocytes. Cells were incubated in PCMBS-containing solutions for 30 min before water permeability measurements were made. 50 mM sucrose was added to 1 and 2 mM PCMBS solutions to prevent hemolysis.

PCMBS concentration (mM)	Cell type	Ca^{2+} concentration of hemolyzing medium (mM)				
		0.0	0.01	0.10	0.5	1.0
0.3	Ghosts	1.6	1.6	1.2		1.3
	Erythrocytes	1.6				
1.0	Ghosts	4.5	3.6	3.3	2.5	
	Erythrocytes	7.6			5.4	
2.0	Ghosts	3.8	3.5	2.7	2.6	

ments using combinations of PCMBS and Ca^{2+} were performed to explore the possibility that any influence Ca^{2+} might have on the diffusional permeability through the lipid bilayer was masked by the massive bulk flow through pores. Exposure of reconstituted ghosts to PCMBS at concentrations of 1 to 2 mM extended the half-times to more than four times control values (Table V). Hemolysis in the presence of Ca^{2+} generally reduced the inhibitory effects of PCMBS on water transport in ghosts.

This interference with PCMBS action was obtained in both the presence and absence of Ca^{2+} in the external medium, suggesting that the interaction (direct or indirect) of Ca^{2+} and PCMBS is not a simple competition by ions in solution for sites on the external membrane surface.

Initial rates of K^+ efflux from ghosts

Measurements of the early time course of passive K^+ fluxes in ghosts were intended to serve in part as a positive control on the effects of Ca^{2+} on membranes since it has been shown by Hoffman and others that Ca^{2+} can change cation permeabilities of erythrocyte membranes. Fig. 2 illustrates the family of kinetic curves typical of K^+ efflux from ghosts hemolyzed in the presence of Ca^{2+}. Note the striking dependence of the apparent efflux asymptote on the Ca^{2+} concentration. At the higher Ca^{2+} concentration, the amount of K^+ appearing in the medium after 3 h corresponded to 90–100 % of the total cell K^+. In contrast, in low Ca^{2+} concentrations, the amount of K^+ lost asymptotically approached values as little as 10–20 % of cell K^+.

During the first 5–6 min of efflux, the passive transport of K^+ from unwashed, Ca^{2+}-treated ghosts occurred at rates from 7 to more than 50 times that of normal K^+ leakage from red cells (i.e. 2 moles per l cells/h, Fig. 2). The initial rates varied in a biphasic manner with Ca^{2+} concentration of the hemolyzing medium over a range of 0.01 to 1 mM Ca^{2+} (Table III and Fig. 4). Ca^{2+} concentrations from 0.01 to 0.1 mM depressed the K^+ efflux rates below the Ca^{2+}-free control level while from 0.1 to 1 mM Ca^{2+} increased the rates to a maximum which was about 30 % greater than the

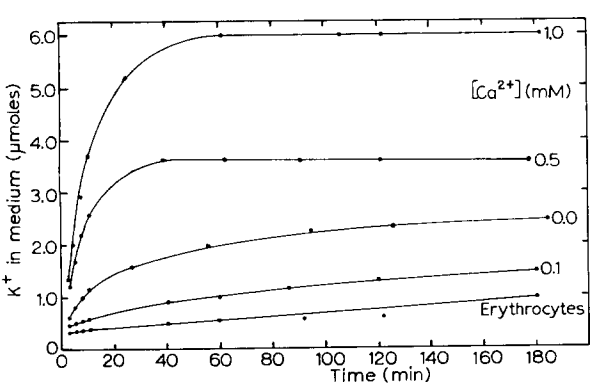

Fig. 2. K^+ efflux from ghosts treated with Ca^{2+} at hemolysis. The family of kinetic curves representing K^+ efflux from ghosts hemolyzed in the presence of Ca^{2+} at concentrations indicated and from intact red blood cells from the same donor. Ghosts were prepared as in Fig. 1 except the hemolysate was returned to isotonicity with 3 M KCl. K^+ efflux was from 0.1 ml of packed ghosts resuspended in 10 ml of 150 mM choline chloride–10 mM Tris (pH 7.4) at room temperature.

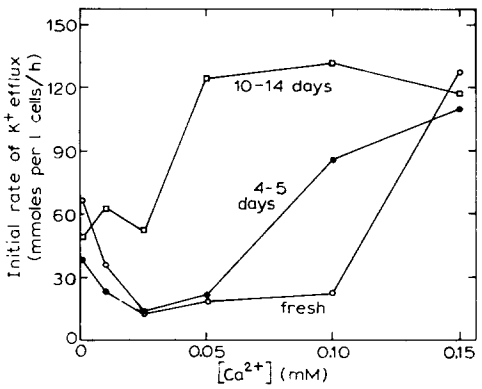

Fig. 3. Effects of cell aging upon initial rates of K^+ efflux from ghosts. Erythrocytes were stored in the cold in heparinized plasma for the number of days indicated, and then made into ghosts as in Fig. 1. Each point is the mean of three experiments. In each experiment, the same blood sample was followed over the two-week period. The transition point from protected state to leaky state occurs at successively lower Ca^{2+} concentrations as the blood ages.

controls. The transition from Ca^{2+}-induced protection to leakage was relatively abrupt, occurring upon an increase in Ca^{2+} concentration in the hemolysis medium of only 0.05 mM (Figs 3, 4 and 5). When parent red cells of the ghost preparation were stored in the cold for 1 and 2 weeks, the transition to K^+ leakage in the ghost preparations occurred at successively lower Ca^{2+} concentrations (as low as 0.01 mM) until, after 18 days of blood storage, the protective action of Ca^{2+} is no longer found (Fig. 3). The Ca^{2+}-associated efflux rate changes and effects of aging were specific

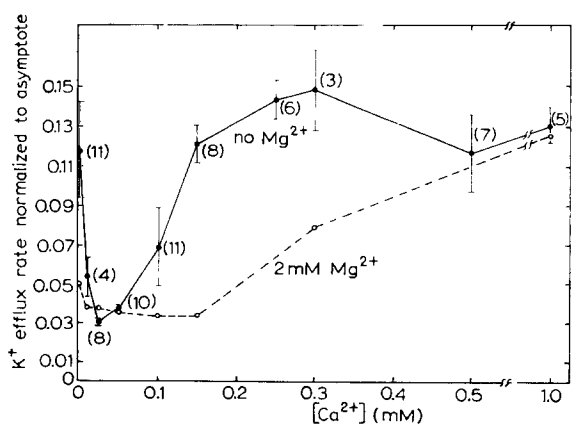

Fig. 4. Initial rates of K^+ efflux normalized to the asymptotes of the kineticcurves; effect of the addition of Mg^{2+} to the hemolyzing medium. The initial rates of K^+ efflux were normalized to the 4-h asymptotic values of the kinetic curves. This calculation normalized the rates to the varying K^+ efflux pool sizes. The biphasic nature of rate vs Ca^{2+} concentration remains, indicating that K^+ leakage can occur at different rates rather than at a single rate. Inclusion of Mg^{2+} in the hemolyzing medium delays the transition from depressed K^+ leakage (protection) to the leaky state. Ghosts were prepared by the procedures of Fig. 1.

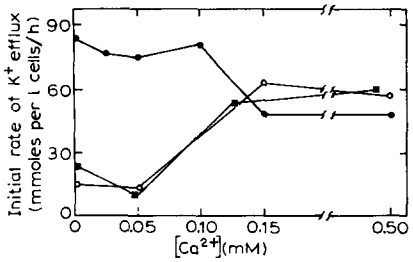

Fig. 5. Application of PCMBS to the interior and exterior of ghosts: effects on Ca^{2+}-induced K^+ leakage. A representative experiment illustrating the differential effects of a 1 mM solution of PCMBS on Ca^{2+}-induced K^+ leakage when introduced at hemolysis (●—●) or when added to the hemolyzed cells during the final 30 min of incubation at 37 °C (○—○). Ca^{2+} concentrations in the hemolyzing medium are as indicated. Data points for the controls are ghosts in PCMBS-free medium (■—■). Incorporated PCMBS promotes a rapid and immediate loss of K^+ which can be counteracted by including Ca^{2+} at concentrations greater than 0.1 mM.

for K^+ and were absent when the efflux of Na^+ was measured under the same conditions.

Both Mg^+ and PCMBS affect the initial rates of K^+ efflux from Ca^{2+}-treated ghosts. Inclusion of 2 mM Mg^{2+} in the hemolyzing medium extended the protected region of low Ca^{2+} to higher Ca^{2+} concentrations but became ineffective at greater than 0.3 mM Ca^{2+} (Fig. 4).

Brief exposure (30 min) of reconstituted ghosts to 1 mM PCMBS in the resuspending medium (the same experimental conditions for the water permeability measurements) had no effect on K^+ efflux from either Ca^{2+}-free or Ca^{2+}-treated ghosts. However, ghosts containing PCMBS incorporated by hemolysis lost K^+ 2–5 times faster than the PCMBS-free controls or than ghosts treated externally with PCMBS (Fig. 5). This exceedingly rapid escape of K^+ could be counteracted by the incorporation of Ca^{2+} at hemolysis in concentrations in excess of 0.1 mM. At 0.15 mM Ca^{2+}, rates were returned to expected values of K^+ efflux for that Ca^{2+} concentration.

Recovery of membrane integrity to other diffusable species

Ghosts are believed to regain the normal erythrocyte impermeability to hemoglobin very soon after hemolysis [25]. Evidence for a similarly rapid and spontaneous reconstitution of membrane impermeability to diffusable species larger than ions was obtained from measurements of the percentage of ghost pellet volume occupied by [^{14}C]inulin at various times during ghost preparation. When [^{14}C]inulin was added to an aliquot of the ghosts immediately following the reconstituting salt, the extracellular volume (ECV) of a pellet of ghosts prepared with 0.1 mM Ca^{2+} was 19.1 %. After 1 h incubation, the ECV of the ghost pellet from a second aliquot was 17.7 %. In contrast, incorporation of inulin at hemolysis gave an apparent extracellular space of 119 %, that is, 19 % more inulin in the pellet (cells plus ECV) than in the supernatant of that pellet. The inulin-marked ghosts were made in 0.1 mM Ca^{2+} and usually equilibrate at about 80 % isotonic cell volume. This suggests the possibility that the membrane became impermeable to the inulin tracer before salt movements had ceased, and the cells shrank thereby trapping and concentrating the inulin inside.

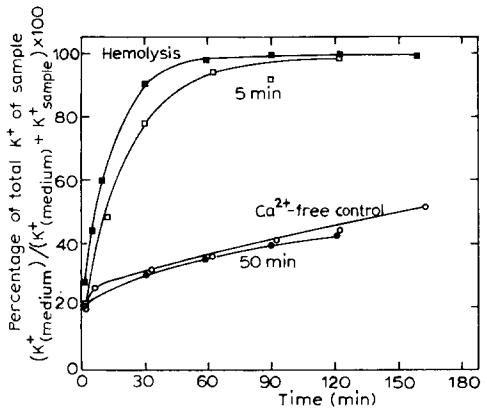

Fig. 6. The resealing of ghosts to Ca^{2+}. The ordinate shows the total K^+ in the medium divided by the total amount of K^+ of the entire sample (medium plus ghosts). Ca^{2+} (0.4 mM) was added to the ghosts at hemolysis, 5 min after hemolysis in conjunction with the reconstituting salt, or at the end of the 1-h incubation period. K^+ efflux from Ca^{2+}-free controls and from the 1-h addition of Ca^{2+} is similar indicating the resealing of the ghost membrane to Ca^{2+}. The kinetics of K^+ loss from 5-min addition of Ca^{2+} resemble those of Ca^{2+} incorporated at hemolysis.

When the previous experiment was repeated with 0.4 mM Ca^{2+} instead of [^{14}C]inulin, the ghost interior was still accessible to Ca^{2+} at the time of reconstitution with salt. The same rapid K^+ leakage occurred when 0.4 mM Ca^{2+} was added either at hemolysis or at the time of reconstitution with salt, while Ca^{2+} introduced after annealing for 1 h gives K^+ kinetics which resemble Ca^{2+}-free controls (Fig. 6), indicating a resealing of the membrane against external Ca^{2+}.

DISCUSSION

The preparation of ghosts by osmotic hemolysis does not alter the transport properties of bulk water through human erythrocyte membranes; the RTL_p values for ghosts and parent red cells are not significantly different. Moreover, the application of PCMBS to ghosts induces a large reduction in the passage of water similar to, although not quite as great as, that in red cells [24]. As previously established for erythrocytes [22], RTL_p in ghosts is independent of cell volume over a size range of 1.1–0.5 times isotonic volume.

Ca^{2+}, over a concentration range of 0.01 to 1 mM, has no major effect on bulk water transport whether applied to the membrane exterior of red cells and reconstituted ghosts or to the membrane interior by incorporation at hemolysis. However, incorporation of higher amounts of Ca^{2+} (0.5–1 mM) impairs the capacity of PCMBS to depress osmotic water movements. One possible interpretation of these results is that Ca^{2+} has increased the diffusional component of water movement and thus only apparently interferes with PCMBS. Alternatively, the inhibition of PCMBS by Ca^{2+} may occur by either direct or indirect competition for binding sites on or in the membrane [26]. In our ghosts, the drastic K^+ leakage induced by incorporation of 1 mM PCMBS can be counteracted by simultaneous inclusion of as little as 0.1 mM Ca^{2+}. This finding strengthens the notion that some of the Ca^{2+} reacts either directly

or indirectly with some membrane SH groups [26–28], and may account in part for the decreased efficiency of 1 mM PCMBS to depress bulk water flow.

The very rapid initial portion of K^+ efflux from Ca^{2+}-free ghosts reconstituted with KCl or with choline-KCl, is more than 15 times the rate reported for similarly prepared ghosts by Hoffman et al. [1]. Our omission from the Hoffman procedure of multiple washings was done so that the initial portion of K^+ efflux could be monitored. Thus, the initial rate measurements probably represent K^+ loss which was obscured by pre-efflux washings in the previous studies. The efflux curve for the Ca^{2+}-free ghosts in Fig. 2 shows that after 60–90 min of efflux, the rate approximated 5 mmoles K^+ per l cells/h [1].

The observed inverse relation between Ca^{2+} concentration and isotonic volumes of the ghosts can be interpreted as evidence of a successful recovery of normal impermeability to choline, the major cation of the hemolyzing and reconstituting solutions. A similar effect was noted by Palek et al. [5] in Ca^{2+}-treated ghosts reconstituted in NaCl and sucrose. The absence of small ghosts when isotonicity is restored by KCl is predictable by the K^+ efflux data which shows that the K^+ barrier is poorly recovered. The protective action of Ca^{2+} against excessive K^+ leakage further confirms that at extremely low concentrations Ca^{2+} can contribute to the reconstitution of membrane impermeability [29, 30].

The protective effect of a given concentration of Ca^{2+}, indicated broadly by Bodemann and Passow [29], is highly reproducible. The maximum inhibition of K^+ leakage from ghosts made from fresh blood occurs at about 0.05 mM Ca^{2+} with only small deviation from the rate of 15 mmoles K^+ per l cells/h (Table III). Note the very large standard error of the mean K^+ efflux rate from Ca^{2+}-free controls. Exposure of the membranes to low Ca^{2+} concentrations at hemolysis apparently "standardizes" the variable K^+ permeability of ghosts made from a variety of blood samples to a common protected state. The explanation of this effect is unknown but might involve a limited number of protective Ca^{2+}-binding sites on the membrane which are partially filled to varying degrees in different samples of fresh blood. In support of this notion is the finding of Tolberg and Macey [26] that the loss of bound Ca^{2+} is coincident with increased K^+ permeability which occurs at rates similar to those of the protected region (from 5 to 25 mmoles K^+ per l cells/h). The transition to leakiness produced by higher Ca^{2+} concentrations (or by aging) would follow if the protective sites became filled allowing the excess of Ca^{2+} to exert an opposing action at other sites.

Both the initial rates of passive K^+ transport and the size of the effluxing K^+ compartment are affected by the presence of Ca^{2+} and Mg^{2+} at hemolysis. Perhaps the simplest interpretation of these experiments assumes that the data reflects the K^+ loss from a portion of the total population of cells. Presumably, the leaky cells all have similar properties; the Ca^{2+} simply alters the proportion of cells which become leaky. In these terms, the 4-h asymptotes of the kinetic curves, expressed as a percentage of the total cellular K^+, would be an indicator of the portion of the total cell population that is leaky. On this basis, variations in initial efflux rates with Ca^{2+} would be expected because of the variation in the proportion of leaky cells, i.e. the apparently slow initial rates simply reflect the emptying of relatively small K^+ pools. If this interpretation were correct, then normalizing the initial efflux rates to pool sizes (asymptotes) would remove the dependence of initial rates on Ca^{2+}. Fig. 4

shows that this does not happen; the same biphasic character of K^+ loss versus Ca^{2+} dependence remains. The results suggest that there exist gradations in the K^+ permeability change. The notion that K^+ leakage occurs upon an absolute breakdown of the K^+ barrier leading to complete loss of cellular K^+ [15, 30, 32] is inapplicable to these data. Moreover, if the K^+ pool providing the rapid efflux rate measurements is coming from unsealed ghosts ("Type III", [2, 29]), then we would expect to see a similarly rapid loss of Na^+, which was not the case.

The production of ghosts in the presence of 0.05 mM Ca^{2+} and 2 mM Mg^{2+} produces fully hemolyzed cells with transport properties similar to the original erythrocytes, that is, essentially normal bulk water permeability, relatively good K^+ retention and low permeabilities to Na^+, choline, Ca^{2+} and inulin.

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