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Studies on the Cation Permeability of Human Red Cell Ghosts

Characterization and Biological Significance of Two Membrane Sites with High Affinities for Ca

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Summary. Net K movements in reconstituted human red cell ghosts and the resealing of ghosts to cations after osmotic hemolysis of red cells have been studied as functions of the free Ca ion concentration. The Ca-dependent specific increase in K permeability was shown to be mediated by a site close to the internal surface of the membrane with an apparent dissociation constant at pH 7.2 for Ca (K'_{D1}) of $3–5 \times 10^{-7}$ M, for Sr of $\sim 7 \times 10^{-6}$ M. Ba and Mg did not increase the K-permeability of the membrane but inhibited the Ca-mediated permeability changes. K'_{D1} decreased in a nonlinear fashion when the pH was increased from 6.0 to 8.5. Two different pK′ values of this membrane site were found at pH 8.3 and 6.3. The Ca-activated net K efflux into a K-free medium was almost completely inhibited by an increase in intracellular Na from 4 to 70 mM. Extracellular K antagonized this Na effect. Changes in the extracellular Na (0.1–140 mM) or K(0.1–6 mM) concentrations had little effect and did not change K'_{D1}. The Ca-stimulated recovery of a low cation permeability in ghost cells appeared to be mediated by a second membrane site which was accessible to divalent cations only during the process of hemolysis in media of low ionic strength. The apparent dissociation constant for Ca at this site (K'_{D2}) varied between 6×10^{-7} and 4×10^{-6} M at pH 7.2. Mg, Sr, and Ba could replace Ca functionally. The selectivity sequence was Ca > Sr > Ba > Mg. K'_{D2} was independent on the pH value in the range between 6.0 and 8.0. Hill coefficients of ~2 were observed for the interaction of Ca with both membrane sites suggesting that more than one Ca ion is bound per site. The Hill coefficients were affected neither by the ion composition nor by the pH values of the intra- and extracellular media. It is concluded that two different pathways for the permeation of cations across the membrane are controlled by membrane sites with high affinities for Ca: One specific for K, one unspecific with respect to cations. The K-specific "channel" has properties similar to the K channel in excitable tissues.

Two opposing actions of Ca on the Na- and K permeability of human red cells have been described. (1) Intracellular Ca enhances the K permeability without affecting the Na permeability. (2) Ca (or Mg) is re-

quired to reseal the ghost cells to K and Na after hypotonic hemolysis of erythrocytes at 37 °C (Lepke & Passow, 1966). In recent years much interest has been focused on the first of these two effects. The initial observations of Gárdos (1958) and Hoffmann (1962) were later confirmed and extended by the work of Lew (1970), Romero and Whittam (1971), Blum and Hoffman (1971), Kregenow and Hoffman (1972), Riordan and Passow (1973), and most recently Simons, 1976.

Intact red cells, which have a small passive Na and K permeability maintain a low level of intracellular Ca ($<10^{-6}$ M) by means of an ATP-dependent outward transport mechanism (Schatzmann, 1973). In order to incorporate an appreciable amount of Ca in intact cells the pump has to be inactivated and/or the low passive Ca permeability of the membrane has to be increased. Alternatively, Ca can be incorporated into red cell ghosts during osmotic hemolysis. The increase in K permeability which is initiated in these Ca-loaded cells is reversible upon reactivation of the pump and the subsequent decrease in the level of intracellular Ca (Romero & Whittam, 1971; Dunn, 1974). The exact mechanism of the selective action of internal Ca on K permeability is still unknown. Some authors observed an inhibitory action of ouabain, oligomycin and furosemide on the Ca-activated K efflux and concluded that in red cells the Na-K-activated ATPase in the presence of elevated levels of intracellular Ca might be transformed so as to provide a K-specific "channel" across the membrane (Blum & Hoffman, 1971; Hoffman & Knauf, 1973).

On the other hand the inhibitory action of Ca (or Mg) on K and Na permeability mentioned above under (2) has received much less attention. Even though it is generally assumed that a small amount of Ca which is tightly bound to membrane components may be essential for the low cation-permeability of the intact erythrocyte (*c.f.* Whittam, 1964) it is not clear what kind of binding sites are involved in this action of Ca. Moreover, it is unknown whether there exists an interrelationship between the Ca-activated K efflux and the Ca-dependent decrease in K permeability of red cell ghosts after reversal of hemolysis. The present experiments were designed to establish quantitative relationships (1) between Na and K movements across the membrane of resealed red cell ghosts and the cellular concentrations of alkaline earth cations and (2) between the yield of ghost cells sealing to Na and K and the concentrations of the same divalent cations in the hemolyzing medium. The results support the view that two separate pathways exist for the transmembrane passive movement of Na and K: one unspecific with respect to Na

and K and one specific for K. The two "channels" can be differentiated on the basis of the cation selectivity patterns and apparent pK values of the divalent cation-sensitive binding sites.

Materials and Methods

Fresh human red blood cells were supplied by the Swiss Red Cross Blood Transfusion Service in Bern. They were stored at 4 °C and used within 5 days after withdrawal of the blood. The cells were washed 3–4 times in isotonic NaCl-solution and then depleted of their endogenous energy stores either by an incubation for 3 hr in the presence of iodoacetate and inosine, according to Lew (1971) (soln II or II*a* in Table 1), or by a first incubation for 15 hr in isotonic substrate-free saline (soln I) which was then followed by a 3-hr period in iodoacetate and inosine. Cells of similar properties were obtained with both methods of starvation. The starved cells were washed 3–4 times in isotonic NaCl solution and ghosts were then prepared by reversal of osmotic hemolysis either at 0 °C, according to the method of Passow (1969), or at room temperature (20–23 °C). After restoration of isotonicity with 2 M buffered salt solution, the cells prepared at room temperature were immediately cooled down to 0 °C in an ice bath and kept there for about 40 min. This procedure helped to increase the yield of sealed ghosts from hemolysates at room temperature. Both types of ghosts (0 °C-ghosts and 22 °C-ghosts) were subject to an equilibration period of one hr at 37 °C and washed 3 times at 0 °C in isotonic Tris-buffer solution (soln III) of a pH somewhat higher than the pH of the hemolyzing medium before they were suspended in the experimental media. The elevated pH during the washing procedure was chosen to minimize the Ca-induced K loss. By the pH shift the free cellular Ca concentration could be lowered to inactive concentrations due to the pH dependency of the CaEGTA complex formation constant. Samples for the determination of the cellular Na, K, Ca, Sr or Mg concentrations were taken at suitable intervals during a 1- or 2-hr incubation period at 30 °C. The samples were treated essentially according to the method of Passow (1969), except that the washing medium (soln IV or V) had a pH of 8.2, again in order to reduce the intracellular free concentration of divalent cations below the threshold concentration required for these biological effects on membrane permeability. The cations were measured by atomic absorption and flame spectrophotometry using an IL 353 atomic absorption spectrophotometer or an IL 143 flame photometer with internal Li standard. To correct for the amount of extracellular ions carried over from the experimental solution into the sample sediment I used the Li method suggested by Passow (1969). In those experiments in which Na and K were measured by flame photometry Li had to be used as an internal standard. Therefore, as an extracellular marker Li was replaced by Sr-EDTA. Generally the Sr-EDTA space was somewhat smaller than the Li space, probably because a small fraction of the intracellular space (leaky ghosts?) was accessible to Li but not to Sr-EDTA.

The cellular ion content was always calculated on the basis of the hematocrit value of the ghost suspension at the beginning of the experiment. This calculation does not take into account volume changes which occured during the incubation period due to an asymmetry in the transmembrane cation fluxes. Therefore, the observed "apparent" changes of the cellular Na and K content under different experimental conditions reflected the net cation movements but did not necessarily correspond to the true intracellular concentration changes. Hence, in the context of this paper with respect to K and Na the expression "intracellular content" has been used rather than "intracellular concentra-

Table 1. Composition of solutions (mMoles/liter)

Solution No.	NaCl	KCl	Choline-Cl	Tris-Cl	HEPES	Me^{++}-buffer	Special additives	pH at 22 °C	Predominant use
I	129	5	–	25	–	–	2 µg/ml chloramphenicol	7.8	Starving of red cells
II	–	130	–	–	20	–	10 inosin 2 Na-iodoacetate	7.6	
II*a*	–	130	–	–	20	–	5 inosin 5 Na-iodoacetate	7.6	
III	–	–	–	175	–	–		7.6–8.6	Washing of reconstituted ghosts
IV	–	–	–	171	–	–	5 LiCl	7.4–8.6	Washing of samples from the experimental cell suspension
V	–	–	–	140	–	6 EDTA	2 $SrCl_2$ 15 LiCl	7.6–8.6	
VI	135	5	–	–	20	2 EGTA		6.5–8.5	Experimental incubation
VII	135	5	–	–	–	–	20 PIPES	6.0–7.0	
VIII	140	–	–	–	20	2 EGTA		7.2	
IX	140	–	–	20	–	2–3 EGTA		7.4	
X	–	–	140	–	20	2 EGTA		7.2	
XI	–	140	–	20	–	2 EGTA		7.4	
XII	–	1780	–	–	220	–		6.5–8.5	Reconstitution of ghosts after hypotonic hemolysis
XIII	–	1780	–	–	–	–	220 PIPES	6.0–7.0	

tion". In contrast, intracellular divalent cations were generally buffered using suitable complexing agents. The free ionic concentration of divalent cations were therefore independent of cell volume changes but were determined exclusively by the actual concentration ratio between cation-ligand complex and free ligand and the cation-ligand dissociation constant. An apparent initial rate of net K (or Na) efflux was estimated from the change in the cellular content between the 2nd and 20th min (=18 min) of incubation according to Eq. (1) or (2):

$$((K)i_1-(K)i_2)/18=\text{initial rate (mM/min/L ghosts)}; \quad (1)$$

$$100((K)i_1-(K)i_2)/(K)i_1\times 18=\text{initial rate (\% of initial content lost/min)}, \quad (2)$$

where $(K)i_1$ and $(K)i_2$ denote the intracellular K content after 2 and 20 min, respectively. The ion content *vs.* time curves of Fig. 1 show that the deviation from linearity within the first 20 min was tolerably small with Ca induced elevated K flux rates (curves II+III). Under control conditions (curve I), the slow phase of efflux which was linear in time was often preceded by a short-lasting rapid phase due to a small contribution of leaky ghosts (*c.f.* Bodemann & Passow, 1972).Therefore, the mean rate in the first 20 min calculated by the above equations tends to overestimate the efflux when the overall rate is slow.

To calculate the free Ca ion concentrations in the CaEGTA and CaHEDTA buffer solutions pK′ values (negative ^{10}log of the apparent dissociation constant) of 7.02 for the CaEGTA complex and 5.61 for the CaHEDTA complex at pH 7.2 were used. The respective constants used to calculate the Ba and Sr ion concentrations in BaEDTA and SrEDTA solutions were 4.66 and 5.53. The pK′ value for CaEGTA contains a small correction for temperature (37 °C) and ionic strength (0.16). To obtain the respective constants for the other elements the true pK values taken from Sillén & Martell (1964, 1971) were corrected only for the experimental pH value. Some uncertainty is introduced into the absolute figures which were found experimentally for Ca-membrane site dissociation constants by the choice which had to be made for the Ca-EGTA complex dissociation constant. The constant, reported by Ogawa (1968; pK=10.11) provides values for the Ca ion concentration at a given Ca/EGTA ratio which differ almost one order of magnitude from the values obtained with the constant given by Anderegg (pK=10.97) (cited by Sillén & Martell, 1971). However, the results obtained using the latter constant agreed reasonably with those from control experiments in which CaEDTA or CaHEDTA buffer systems had been used. For experiments at different pH the corresponding pK′ values were calculated adcording to the procedure of Portzehl, Caldwell & Rüegg (1964).

A more detailed description of the methods used in the present study is given by Porzig (1975).

The chemicals used for the preparation of the solutions were of the highest purity that was commercially available. All solutions were prepared with de-ionized doubly quartz-distilled water. The stock solutions of the divalent cations were prepared from the chlorides except the Ca standard solution which was prepared from $CaCO_3$. The $BaCl_2$, $MgCl_2$ and $SrCl_2$ content of the respective stock solutions was checked by chloride titration with a Radiometer CMT 10 instrument. In the text most solutions are identified by their respective number in Table 1. The varying compositions of the hemolyzing solutions is given in the legends to the figures.

The following abbreviations are used:

Me^{++}-buffers EGTA: ethyleneglycol-bis-(β-aminoethyl ether)-N,N′ tetraacetic acid
HEDTA: N′-(2-hydroxyethyl)-ethylenediamine-N,N,N′triacetic acid
EDTA: Ethylenediamine-N,N,N′,N′-tetraacetic acid

H^+-buffers HEPES: N-2-hydroxyethylpiperazine N′-2-ethanesulfonic acid
PIPES: Piperazine-N,N′-bis (2-ethanesulfonic acid)
Tris: Tris (hydroxymethyl)-aminomethane
$[Ca^{++}]$, $[Sr^{++}]$, $[Ba^{++}]$ denote the free ion concentration of the respective cations, Na_o and K_o represent the total extracellular concentrations, and Na_i and K_i the intracellular content of Na and K. Generally the symbols Na and K are used for sodium and potassium without specification of the respective free ion concentrations. Hct denotes hematocrit.

Results

The Effects of Ca on the K Permeability of Ghosts Resealed at 0 °C or at 22 °C

The present study was designed to examine in some detail the two different actions of Ca on the K permeability of human red cell ghosts which are illustrated in Fig. 1. In the experiment shown in graph A,

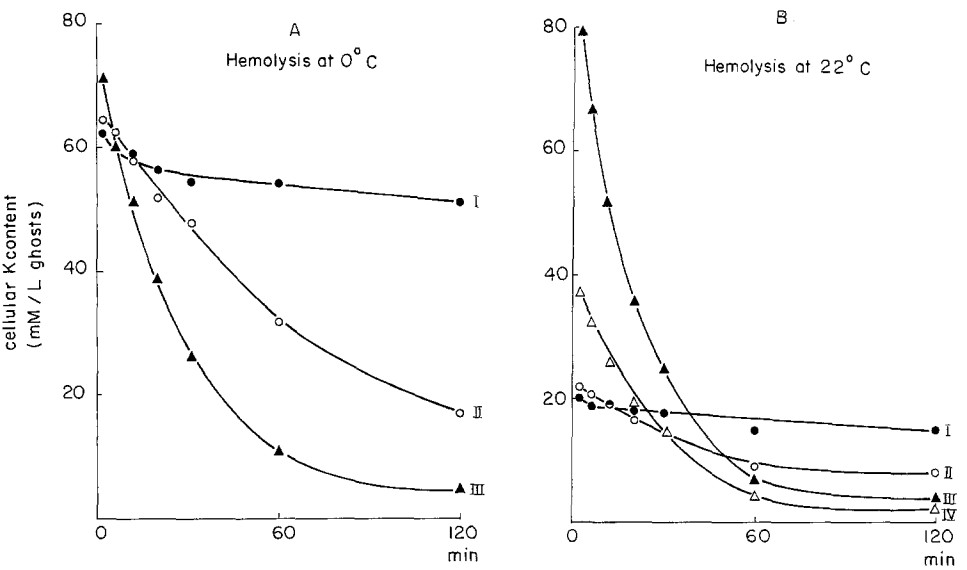

Fig. 1. Time course of K efflux from reconstituted red cell ghosts prepared by hemolysis at 0 ° and 22 °C in the presence of different Ca ion concentrations. (*A*): Hemolysis at 0 °C and pH 7.2 in 10 mM HEPES solution containing Ca ion concentrations of $< 5 \times 10^{-8}$ M (I), 2.9×10^{-7} M (II) and 1.8×10^{-6} M (III). (*B*). Hemolysis at 22 °C and pH 7.2 in 5 mM HEPES solution containing Ca ion concentrations of $< 5 \times 10^{-8}$ M (I), 5.4×10^{-7} M (II), 8.6×10^{-7} (IV) and 1.8×10^{-6} (III). The free Ca concentrations were established by mixing adequate amounts of 2 mM EGTA and 2 mM CaEGTA solution. Reconstitution of ghosts with KCl (soln XII). Incubation in a NaCl medium (soln VI). Hct 4.4–6.3%. Similar results were obtained in 7 experiments with 0 °C-ghosts and 8 experiments with 22 °C-ghosts

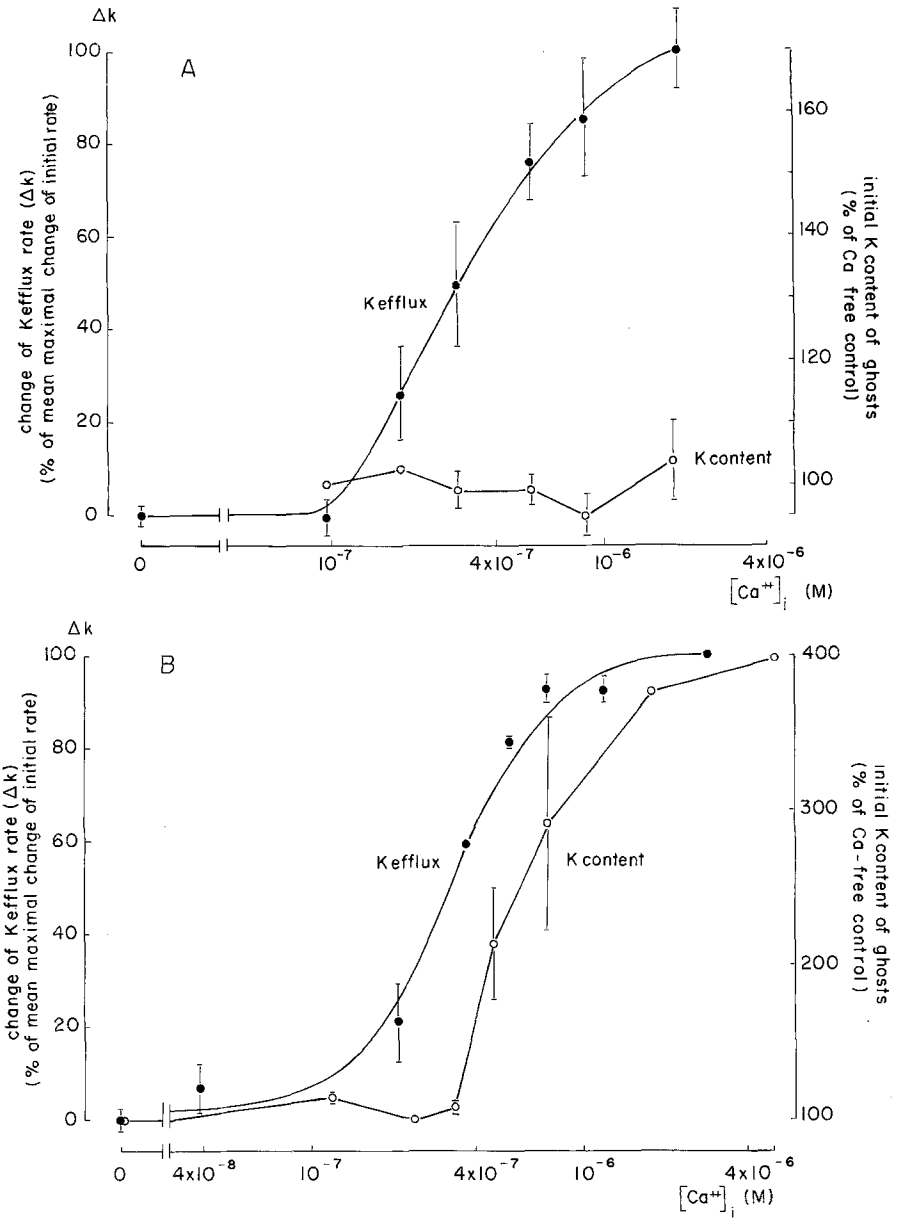

Fig. 2. Effect of Ca on the rate of K efflux and the initial cellular K content of reconstituted ghosts observed at two different temperatures of hemolysis. The change in the initial rates of K efflux, normalized with respect to the mean maximal change of rate (●–●, left ordinate) and the % increase in the K content of the ghosts at the beginning of the incubation period (○–○, right ordinate), are plotted against $[Ca^{++}]$. Same experimental procedure as in Fig. 1. (*A*): Hemolysis at 0 °C and pH 7.2. Incubation in a NaCl medium (soln VI) at pH 7.2. Data from 7 experiments. Individual points represent 3–6 measurements ±SEM. Hct 5–8%. (*B*): Hemolysis at 22 °C and pH 7.4. Incubation in a NaCl medium (soln IX) at pH 7.3. Data from 9 experiments. Individual points represent 2–8 measurements. Points without indication of SEM are the mean of 2 measurements. Hct 5–8%

EGTA-buffered free Ca concentrations ranging between 10^{-8} and 2×10^{-6} M were incorporated into red cell ghosts during osmotic hemolysis at 0 °C. The rate of K efflux during subsequent incubation at 30 °C in a NaCl medium increased as the cellular Ca ion concentration ($[Ca^{++}]_i$) was raised. However, the intracellular K content at the beginning of the experiment was almost independent of $[Ca^{++}]_i$ (*see also* Fig. 2*A*). Graph *B* is based on a similar experiment, the only difference being the temperature of hemolysis which was 22 °C instead of 0 °C. Under this condition only a small fraction of cells resealed to K if the Ca concentration in the hemolyzing medium was kept below 10^{-8} M by means of EGTA-buffering (curve I). When $[Ca^{++}]$ was raised gradually, first the rate of K efflux from the small fraction of sealed ghost cells was enhanced (curve II). Higher Ca concentrations, however, simultaneously increased the initial cellular K content and the rate of K efflux upon incubation at 30 °C (curves III and IV). Since the ghost cell preparations were all subject to a standardized washing procedure in Na- and K-free solution (*see* methods) which removed K from leaky cells, the amount of K retained by the cells at the beginning of the experimental incubation is a direct measure for the fraction of cells having sealed initially to K.

In Fig. 2*A* and *B* the rate of K efflux and the initial cellular K content in 0 °C-ghosts (*A*) and 22 °C-ghosts (*B*) are both plotted as functions of $[Ca^{++}]_i$. The two cell preparations did not differ significantly with respect to the Ca concentrations which were required for half maximal activation of K net efflux ($3–4 \times 10^{-7}$ M). Simons (1976) recently reported a similar value for half maximal stimulation of Ca-induced K equilibrium exchange. However, in contrast to the observations of Simons, no decrease in the rate of net K efflux was detected when $[Ca^{++}]_i$ was raised beyond 3×10^{-6} M. In a Hill plot of the data in Fig. 2, slopes of 1.8 (0 °C ghosts) and 2.3 (22 °C ghosts) were found for the Ca-mediated K outflow. Individual experiments in some occasions yielded slopes close to 4. This analysis suggests that at least two Ca ions have to interact with the respective membrane site to initiate an increase in K permeability. Correspondingly, linear fits were obtained in the Lineweaver-Burk plot only when $1/v$ and $1/[Ca^{++}]^2$ rather than $1/[Ca^{++}]$ were chosen as parameters (*see* Fig. 3*B*). On the basis of the present data no distinction can be made between different cooperative models for the Ca-membrane site interactions. Fig. 2*B* shows in addition that somewhat higher Ca concentrations were required to improve the resealing of 22 °C-ghosts towards K than to enhance net K efflux. In

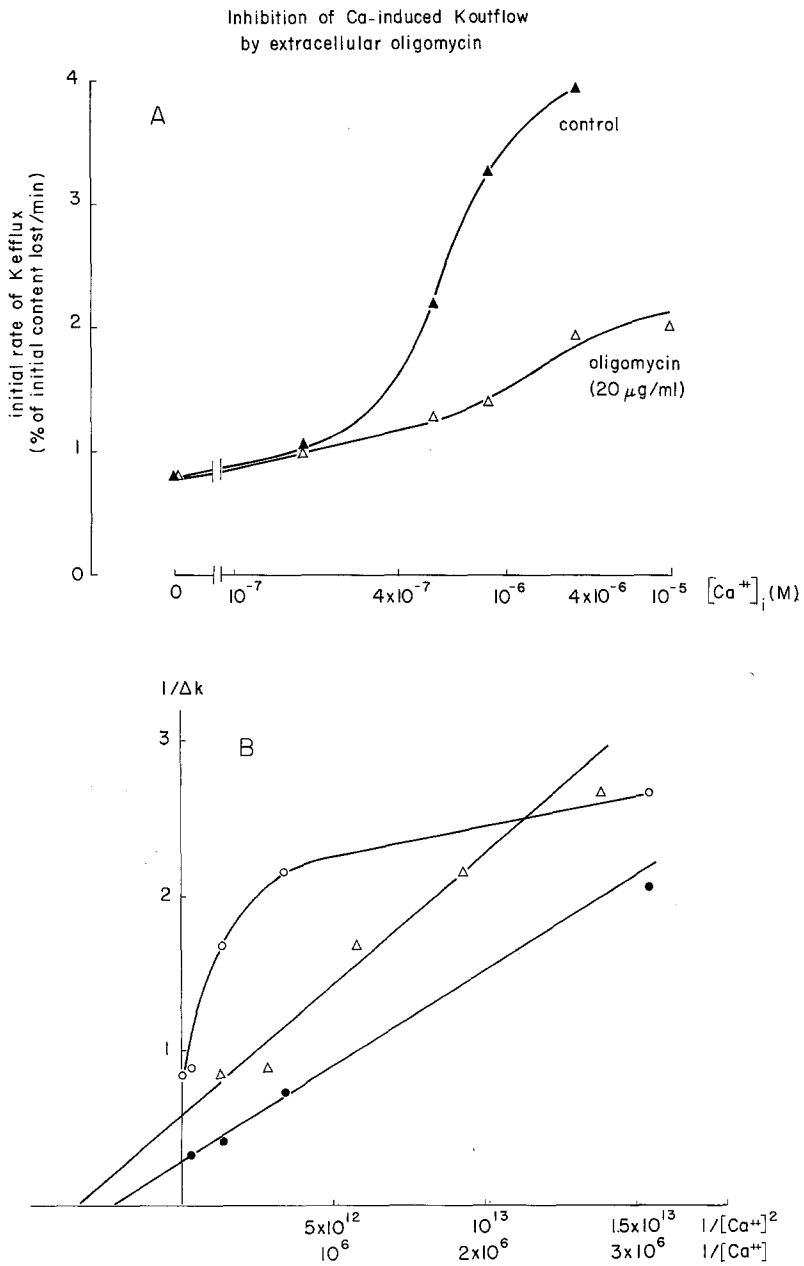

Fig. 3. Effect of oligomycin on the rate of K net efflux from reconstituted ghosts. Hemolysis at 22 °C and pH 7.2 in 5 mM HEPES solution containing EGTA (2 mM)-buffered Ca ion concentrations between $<5\times10^{-8}$ and 4×10^{-6} M. Reconstitution of ghosts with 2 M KCl. Incubation at pH 7.2 in a NaCl-medium (soln VIII) in the absence and presence of 20 μg/ml oligomycin (om.). Mean values from 4 experiments. Hct 5–8%. (*A*): Plot of the rate of K efflux as a function of $[Ca^{++}]_i$. (*B*): Double reciprocal plot of the data in (*A*). Δk is the change in initial rate at each particular $[Ca^{++}]_i$. ●–● = $1/\Delta k$ *vs.* $1/[Ca^{++}]^2$, no om.; ○–○ = $1/\Delta k$ *vs.* $1/[Ca^{++}]^2$, om. added; Δ–Δ = $1/\Delta k$ *vs.* $1/[Ca^{++}]$, om. added

this series of experiments a half maximal effect on resealing was obtained at about 6×10^{-7} M[Ca^{++}]. The small but significant difference between the two curves suggests that two different membrane sites correspond to the two opposing effects of Ca on K permeability.

The experiments which will be described in the following paragraphs were designed to answer the following questions: (1) What is the effect of oligomycin, which is known as an inhibitor of Ca-activated K efflux, on the apparent affinity and efficacy of Ca at its effector sites? (2) How is the relationship between $[Ca^{++}]_i$ and K efflux affected by changes in the intra- and extracellular Na and K concentrations? (3) What are the apparent pK values of the membrane sites with high affinity for Ca? (4) Where are these sites located in the membrane? (5) What is the affinity of the Ca-sensitive sites for other divalent alkaline earth cations?

The Effect of Oligomycin

Blum and Hoffman (1971) and Riordan and Passow (1973) reported that oligomycin is an effective inhibitor of Ca-activated K outflow. Fig. 3*A* shows the effect of extracellular oligomycin (20 μg/ml) on the rate of K efflux from 22 °C-ghosts which were loaded with Ca ion concentrations between $<10^{-8}$ (="0") and 4×10^{-6} M. Oligomycin decreased the apparent affinity for Ca of the K efflux site and strongly diminished the maximal response to intracellular Ca. The Lineweaver-Burk analysis of the data from Fig. 3*A* in Fig. 3*B* showed that in the presence of oligomycin the data could no longer be fitted by a straight line in a plot of $1/v$ against $1/[Ca^{++}]^2$ M but could be linearized in a plot of $1/v$ *vs.* $1/[Ca^{++}]$. It can be inferred from Fig. 3*B* that oligomycin is not only a noncompetitive inhibitor but may change also the stoichiometry or cooperativity of the Ca activation of K efflux. The resealing of ghost cells was not inhibited when oligomycin was present in the hemolyzing medium (not shown in Fig. 3). Hence, the drug seems to interact only with those Ca sites which are involved in the activation of K efflux.

The Effect of Monovalent Cations on Ca-Mediated K Outflow

The investigations of Blum and Hoffman (1971) and particularly of Passow and coworkers [see Riodan & Passow (1973) and Knauf, Riordan, Schuhmann & Passow (1974)] have clearly shown that low

concentrations of extracellular K activate the Ca-dependent K efflux, whereas internal Na strongly inhibits the effect of Ca. However, the mechanism by which the monovalent cations exert their effects is still unknown. One possible explanation is an action of K and Na on the affinity for Ca of the Ca-specific site on the inside of the membrane. The activation of K efflux as a function of $[Ca^{++}]_i$ was assessed in the presence of 0.2–5 mM K on the outside and in the presence or absence of extracellular Na (Na being replaced isotonically by choline-Cl). Furthermore, the Ca activation of K efflux was measured in cells which were loaded with KCl alone, with equal concentrations of K and Na or with equal concentrations of KCl and choline-Cl. This approach is illustrated by Fig. 4*A* and *B*. For the experiment represented in Fig. 4*A*, K-loaded ghosts were prepared at 0 °C and incubated in isotonic NaCl-HEPES solution (soln VIII; curve I), or in a NaCl-HEPES solution which contained 5 mM K (soln VI; curve II). With control cells containing less then 10^{-8} M$[Ca^{++}]$ the K concentration in the K-free incubation media measured at the end of the 60 min incubation period was 0.245 mM. However, the K concentration in the medium rose to 0.748 mM during the incubation period when the cells contained 3×10^{-7} M $[Ca^{++}]$. Half maximal activation of K efflux under the three conditions required very similar free Ca concentrations (around 1.5×10^{-7} M). The maximal initial rate of K efflux which could be elicited by Ca, depended on K_o but was independent of Na_o (not shown in Fig. 9*A*). The cells lost K at the same rate when the NaCl medium was replaced by a choline-Cl medium. As pointed out by Knauf *et al.* (1975), the reduction in the rate of Ca-induced K efflux in the presence of 5 mM KCl (curve II in Fig. 4*A*) is only partially explained by the decrease in driving force and may represent in addition some true inhibition of K efflux by external K concentrations above 4 mM.

From the experiment shown in Fig. 4*B*, it can be inferred that the replacement of half of the internal K by choline-Cl also did not introduce a major change in the Ca activation of K efflux (curve I), whereas intracellular Na strongly inhibited the effect of Ca on K loss (curve II). The inhibition by Na was not due to a shift of the activation curve towards higher Ca concentrations but must have been caused by some other mechanism. In addition the present experiments confirmed the finding of Knauf *et al.* (1975) that the inhibitory action of internal Na is inversely related to the extracellular K concentration. A stepwise reduction of K_o from 5 to 0.5 mM caused a gradual decrease in the maximal rate of Ca-induced K outflow but never a shift of the activation curve

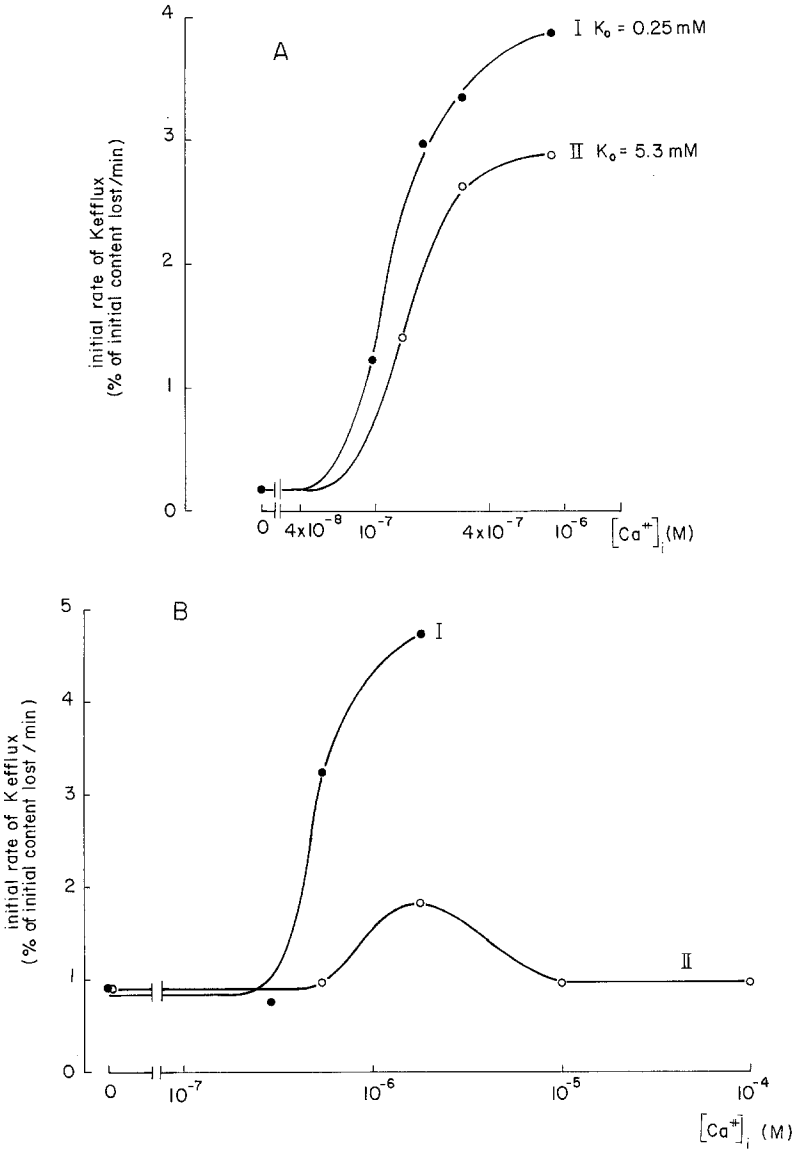

Fig. 4. The influence of extracellular K and intracellular Na on the Ca-mediated increase in K efflux. Hemolysis at 0 °C and pH 7.2 in 10 mM HEPES solution containing EGTA(2mM)-buffered Ca ion concentrations ranging from 10^{-8} to 10^{-5} M. (*A*): KCl-loaded cells were incubated in a K-free NaCl medium (soln VIII, curve I) or in a K containing NaCl medium (soln VI, curve II). The K concentration in the supernatant at the end of a 60 min incubation period was 0.25 mM (I) and 5.3 mM (II). Hct 0.32–0.46%. One of 4 similar experiments. (*B*): Reconstitution of ghosts in 2 M salt solution containing equal parts of KCl and choline-Cl (curve I) or equal parts of NaCl and KCl (curve II). The incubation medium contained isotonic choline-Cl (soln X). The K concentration in the supernatant at the end of a 120 min incubation period was between 0.926 and 1.364 mM (I) and between 0.509 and 0.967 mM (II). One of 4 similar experiments. Hct 4.1–5.9%

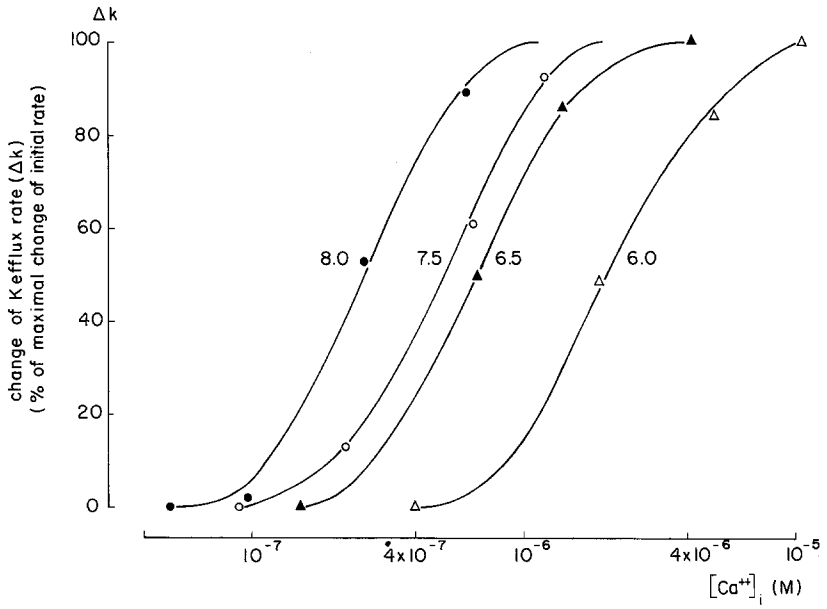

Fig. 5. Effect of the pH on the Ca-mediated increase in the rate of K net efflux. Hemolysis at 0 °C in solutions of different pH values containing either 10 mM HEPES (pH 8.0 and 7.5) or 10 mM PIPES (pH 6.5 and 6.0) and HEDTA (4 mM)-buffered Ca ion concentrations ranging between 5×10^{-8} M and 10^{-5} M. Reconstitution of ghosts with KCl (soln XII and XIII). Incubation in a NaCl medium (soln VI or VII) the pH values of which corresponded to the values in the hemolysis medium. One of 4 similar experiments. Hct 3.6–4.8%

along the concentration axis. Moreover, the activation curve changed from a simple S-shaped function to a bell-shaped curve (see curve II of Fig. 4*B*). The difference in $[Ca^{++}]_i$ causing half maximal activation in Fig. 4*A* and curve I in Fig. 4*B* is probably due to the fact that the cells were obtained from two different donors. In view of the variation of the activation curves, differences of this size can only be substantiated if they arise in the same cell population.

The Influence of pH on Ca-Dependent K Efflux

The effect of pH on the Ca-dependent activation of K efflux was studied in an attempt to identify possible dissociable groups as part of the Ca-sensitive site. Fig. 5 gives an example of the type of experiment that has been performed. Red cell ghosts were prepared at 0 °C and loaded with a suitable range of buffered Ca concentrations. The pH

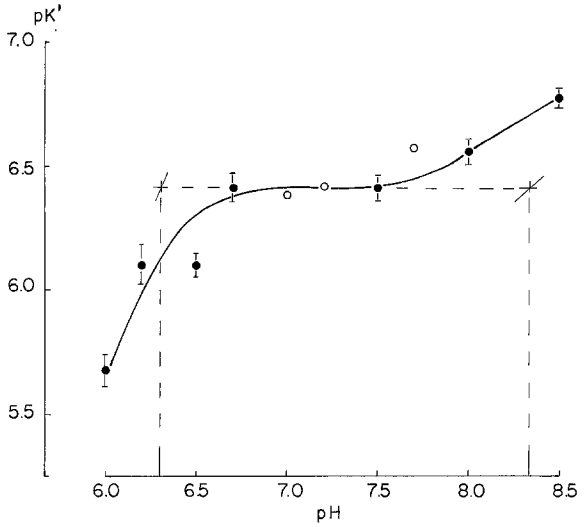

Fig. 6. Effect of pH on the apparent pK value for Ca at the site where it initiates the increase in K permeability. The negative log of the apparent dissociation constant, which was extracted from experiments similar to the one in Fig. 5, is plotted against the pH. The filled circles give mean values from 3–5 different experiments ±SEM. The open circles represent single values. Note that the actual curve misses the intersection points of the extrapolated neighboring straight parts by a vertical distance of log 2

during hemolysis and reconstitution, as well as during the incubation period was adjusted to values ranging from 8.5 to 5.8. In most experiments, HEDTA rather than EGTA was used to buffer $[Ca^{++}]$. The complex formation constant of CaHEDTA is lower than that of CaEGTA by about two orders of magnitude. Therefore, the range of Ca concentrations which can be buffered effectively by this complexing agent extends beyond the range covered by EGTA. This is particularly useful at elevated pH where the apparent K_{diss} of CaEGTA is so low that the range of Ca concentrations which can be buffered barely reaches the threshold of Ca-activated K efflux. At lower pH where EGTA and HEDTA buffer equally well similar results were obtained with the two buffer systems.

Apparently a decrease in pH caused a parallel shift of the activation curve for K efflux along the abscissa towards higher intracellular Ca concentrations. However, this shift did not appear to be a linear function of pH. From the curves presented in Fig. 5 (as well as from those of similar experiments) the apparent dissociation constants (K'_D) for Ca at different pH were evaluated. These values correspond to the $[Ca^{++}]_i$ at the point of half maximal activation of K efflux. The curves in Fig. 5

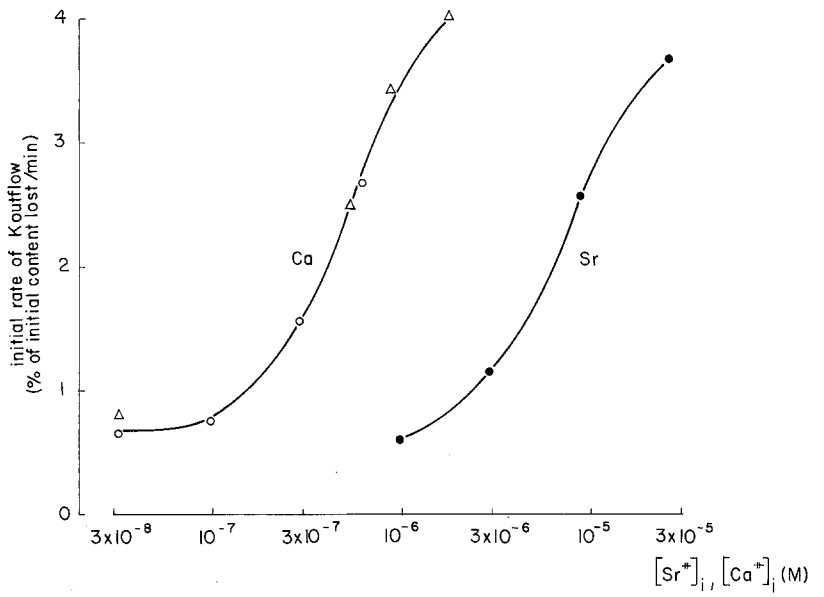

Fig. 7. Comparison of the effects of $[Ca^{++}]$ and $[Sr^{++}]$ on the rate of net K efflux from ghost cells. Hemolysis at 22 °C and pH 7.2 in 5 mM HEPES solution containing EDTA (4 mM)-buffered Ca or Sr ion concentrations ranging from 3.2×10^{-8} to 1.8×10^{-6} M for Ca and 9.8×10^{-7} to 2.6×10^{-5} M for Sr. Reconstitution of ghosts with 2 M KCl. Incubation in a NaCl medium (soln VI). The Ca curve was drawn using data from two different experiments (symbols ○ and △). One of 3 similar experiments. Hct 5–7.9%

yielded values for K'_D of 2.6×10^{-7}, 5.2×10^{-7}, 6.8×10^{-7} and 2×10^{-6} for pH 8.0, 7.5, 6.5 and 6.0, respectively.

It is important to note that in spite of the strong pH dependency of K'_D the slopes of the curves in Fig. 5 are very similar, indicating an unchanged stoichiometry of the Ca activation of K efflux. Moreover a Lineweaver-Burk plot of $1/[Ca^{++}]^2$ against the reciprocal of the initial rate of K efflux at different pH values gave straight lines which intersected at the $1/v$ axis indicating competitive inhibition of the Ca effect by protons. Fig. 6 summarizes this type of experiments in a plot of pK'_D versus pH. The graph includes the results with both 22° C cells and 0 °C cells which did not show significant differences. There was a pronounced increase in K'_D in the region between 8.5 and 7.5 and again between 6.8 and 6.0, whereas almost no change was observed between 7.5 and 6.8. The two marked inflection points of this curve indicate pK'_D values of about 8.3 and 6.3 for two ionizable groups in the Ca-sensitive membrane site. At pH values below 6 the fraction of cells which resealed to K after hypotonic hemolysis progressively decreased.

Moreover, the sealed cells were rather insensitive to changes in $[Ca^{++}]_i$. In one experiment at pH 5.8 more than 10^{-5} M $[Ca^{++}]_i$ was required to induce a moderate increase in K permeability.

A shift of the experimental pH from 8.5 to 6.0 per se tended to decrease the rate of K efflux from K loaded ghosts. Nevertheless the Ca-induced net change in the rate of K efflux was almost of similar magnitude within this pH range.

The Effect of Sr, Ba, and Mg on Ca-Dependent K Efflux

Of the three alkaline earth cations only Sr was found to share the properties of Ca in promoting K efflux from resealed red cell ghosts. Fig. 7 shows an experiment in which the initial rates of K outflow from 0 °C-ghosts were measured as functions of the intracellular ion concentrations of Ca or Sr. Compared to Ca, Sr is less effective by more than one order of magnitude. The K'_D, which can be derived from the activation curves in Fig. 7, was 5×10^{-7} M for Ca and 6×10^{-6} for Sr. Similar values were reported recently by Simons (1976). The two other cations did not enhance K efflux from resealed ghosts but strongly inhibited the Ca-mediated increase in K flux. Fig. 8 *A* and *B* show the effects of 4 mM Mg or 1 mM Ba in representative experiments. 0 °C-ghosts were prepared to contain EGTA-buffered Ca concentrations between 10^{-7} and 2×10^{-6} M either alone or together with 4 mM Mg or 1 mM Ba. Mg in this experiment decreased K'_D from about 2×10^{-7} to about 4.5×10^{-7} and reduced the maximal rate of efflux but did not significantly change the slope of the activation curve. The inhibition is of the mixed competitive-noncompetitive type. Ba, on the other hand, blocked almost completely any Ca-dependent increase in K permeability. This block could not be reversed even by increasing $[Ca^{++}]_i$ to 2 mM, i.e., twice the concentration of Ba. In Fig. 9 the inhibition of Ca-activated K efflux by Ba is plotted as a function of the Ba concentration in the hemolyzing medium. 0 °C-ghosts were prepared in the presence of 2 mM EGTA, 2 mM Ca and Ba concentrations ranging from 5×10^{-5} to 2×10^{-3} M. The free Ca concentration under these conditions, i.e., when all of the complexing agent is occupied by Ca at pH 7.2, amounts to about 10^{-5} M and is sufficient to activate the K efflux maximally in the absence of Ba. Since almost no free EGTA is present and since the affinity of Ba towards EGTA is more than two orders of magnitude lower than the affinity of Ca, complexing of Ba to EGTA will not significantly

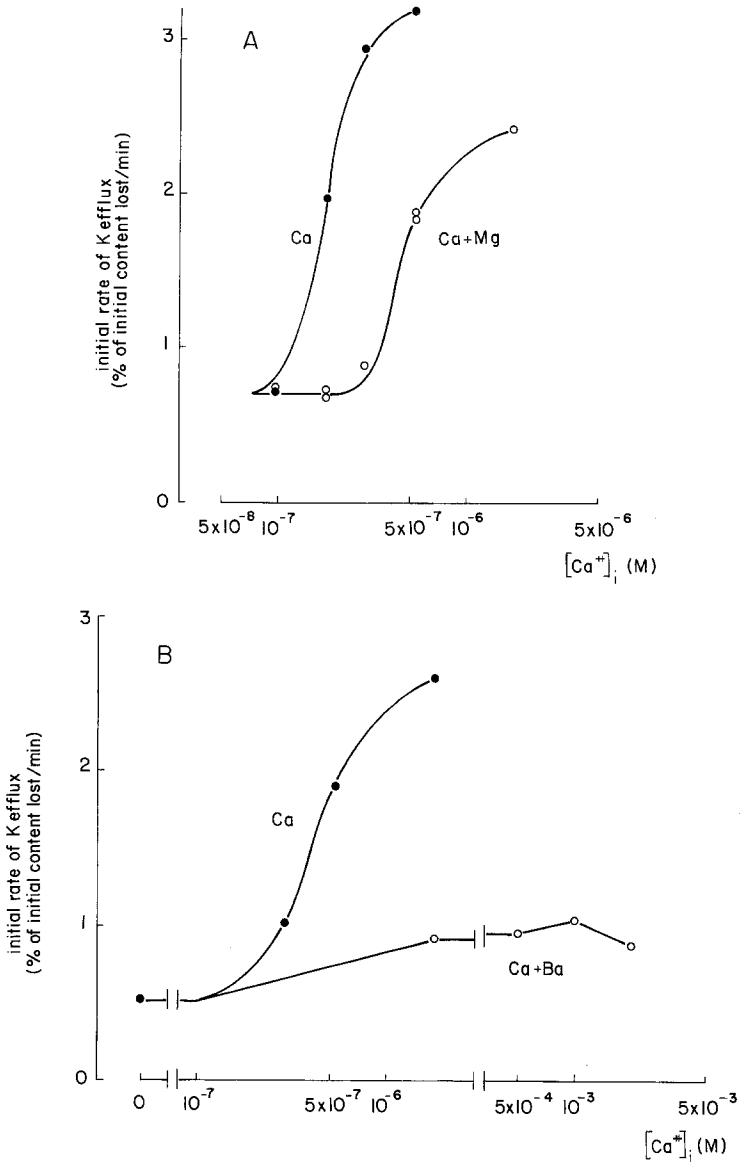

Fig. 8. The inhibitory effect of Mg and Ba on the rate of Ca-activated net K efflux. The initial rates of K efflux in the absence or presence of Mg or Ba are plotted against $[Ca^{++}]_i$. (*A*): Hemolysis at pH 7.2 in 5 mM HEPES solution containing 9.5×10^{-8} to 5.4×10^{-7} M EGTA (2 mM)-buffered free Ca and either none or 4 mM $MgCl_2$. Reconstitution of ghosts with 2 M KCl. Incubation in a NaCl medium (soln VI) at pH 7.2. The Mg data are from two different batches of cells. One of 3 experiments. Hct 8–10%. (*B*): Hemolysis in 10 mM HEPES solution containing 10^{-8} to 1.8×10^{-6} M$[Ca^{++}]$ or 1.8×10^{-6} to 2×10^{-3} M$[Ca^{++}]$together with 1 mM $BaCl_2$. Reconstitution and incubation as above. One of 3 experiments. Hct 3–5%

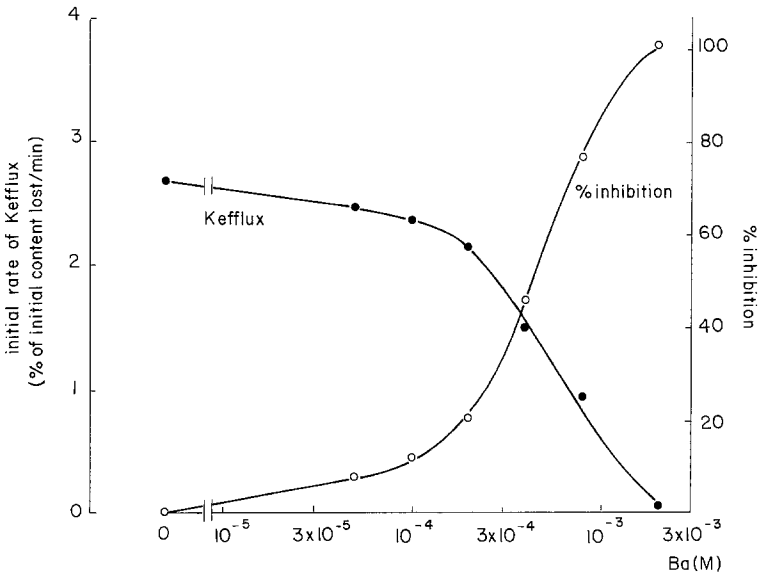

Fig. 9. The inhibition of Ca-dependent K net efflux as a function of the cellular Ba concentration. Initial rates of K outflow (●–●, left ordinate) and % inhibition of K outflow (○–○, right ordinate) are plotted against the cellular Ba concentration. Hemolysis in 10 mM HEPES solution containing 2 mM CaEGTA (corresponding to $\sim 10^{-5}$ M[Ca^{++}]) and 5×10^{-5}–2×10^{-3} M $BaCl_2$. Reconstitution of ghosts with KCl (soln XII). Incubation in a NaCl medium (soln VI) at pH 7.2. One of 4, but less complete, experiments. Hct 4.3–4.9%

decrease the effective Ba concentration in this experiment. The rate of K efflux decreased rapidly in the presence of more than 10^{-4} M Ba, half maximal inhibition being reached around 4×10^{-4} M Ba. In Ca-free cells intracellular Ba concentrations between 1 and 4 mM reduced the the K outflow well below the control level, whereas under similar conditions up to 10 mM Mg caused almost no change in the rate of K outward movement. In contrast, Ba in concentrations between 0.1 and 4 mM had no effect on K efflux from Ca-containing red cell ghosts when it was added to the extracellular medium after completion of the resealing period.

The Effect of Ca and of pH on the Yield of Sealed Ghosts

The experiments plotted in Fig. 2*B* had shown that Ca at a given pH increased the yield of 22 °C ghosts sealed to K. The study by Lepke and Passow (1972) provided evidence that the percentage of ghosts sealing to cations after hypotonic hemolysis is a function of the pH in the

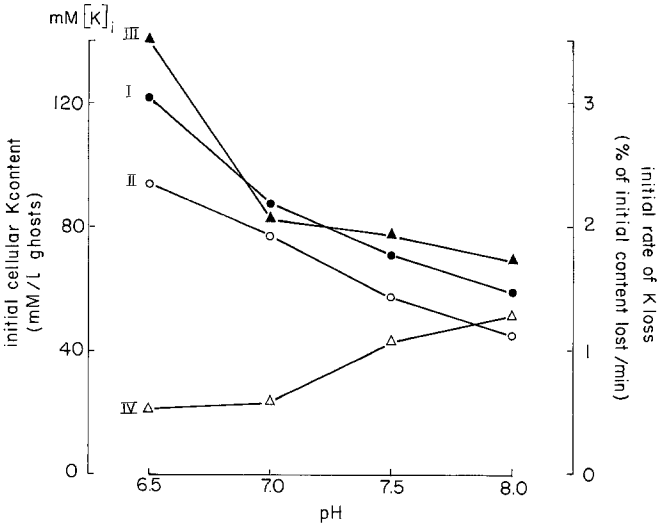

Fig. 10. Effect of pH and of intrinsic membrane Ca on the initial cellular K content and on the initial rate of net K loss from K-loaded ghosts. Hemolysis at 23 °C in 10 mM HEPES solution buffered to pH values between 6.5 and 8.0, which contained either no EGTA (curves I and III) or 0.5 mM EGTA (curves II and IV) but no added Ca. Incubation was in a NaCl medium (soln VI). The cellular K content at the beginning of the incubation period (left ordinate) in the absence (I) and presence (II) of EGTA, as well as the initial rate of K efflux (right ordinate) in the absence (III) and presence (IV) of EGTA, are plotted as functions of the pH value. One of 5 similar experiments. Hct 4.8–9%

hemolyzing medium. Experiments were, therefore, designed to test whether the effects of pH and of Ca involve interactions with the same membrane site or whether they are independent phenomena. Fig. 10 shows the result of a pertinent experiment in which 22 °C-ghosts were prepared and incubated at pH values ranging from 6.5 to 8.0. The hemolyzing medium contained either 10 mM HEPES-buffer alone or 10 mM HEPES with 0.5 mM EGTA to keep $[Ca^{++}]_i$ below 10^{-7} M. Isotonicity was restored with KCl. After the usual washing procedure, the cells were incubated in a NaCl medium at 30 °C (soln VI). The cellular K content of the control cells at the beginning of the incubation period as a measure of the yield of resealed cells dropped from 122 mM/liter ghosts at pH 6.5 to 59 mM/liter at pH 8 (curve I). The initial K content of the EGTA-cells, though lower than in the controls by an almost constant amount, was similarly dependent on the pH (curve II). However, the control cells lost a large fraction of their initial K upon incubation in NaCl solution (curve III). The size of this fraction, as well as the rate of loss, appeared to be pH-dependent. In contrast, K efflux from

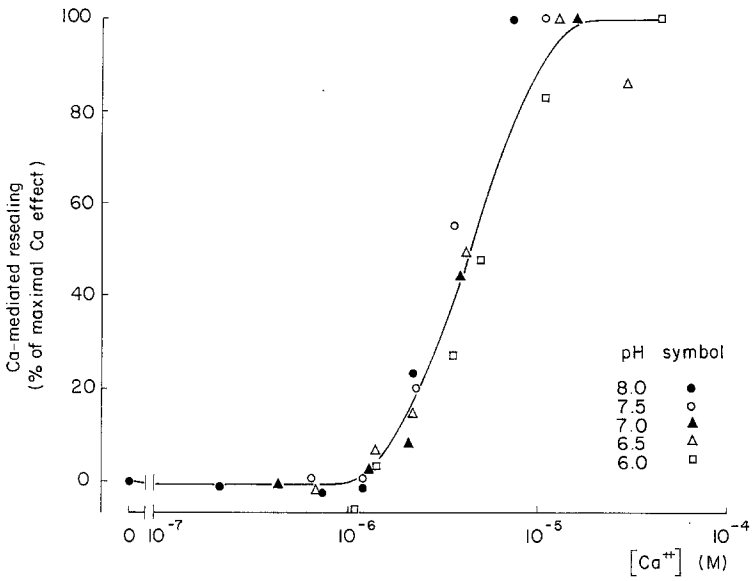

Fig. 11. Ca-mediated resealing of ghost cells to K at different pH values of the hemolyzing solution. Hemolysis at 22 °C in 10 mM HEPES-(pH 8.0, 7.5, 7.0) or PIPES solution (pH 6.5, 6.0) containing HEDTA (4 mM)-buffered Ca ion concentrations ranging from 5×10^{-8} to 3×10^{-5} M. Reconstitution of ghosts with KCl (soln XII or XIII). The cellular K content was measured after repeated washings at the beginning of an incubation period in NaCl media (soln VI or VII). The maximal Ca-dependent increase in the yield of sealed cells at each particular pH was set to 100%. One of two similarly complete experiments. Hct 2.6 to 8.8%

EGTA-cells was generally much slower and *increased* when the pH was raised (curve IV). The Na uptake which did not differ in both types of cells increased almost linearly with increasing pH (not shown in Fig. 10). In Fig. 11 (data taken from a different experiment) the initial K_i at pH values ranging from 8.0 to 6.0 are plotted as functions of $[Ca^{++}]$ in the hemolyzing medium. The cells were hemolyzed at 22 °C, washed and incubated for 1.5 to 2 min in NaCl media of the same pH. The experimental points were normalized with respect to the maximal effect of Ca at each individual pH. Evidently the Ca concentration which enhanced the resealing of ghosts was almost independent of the pH at which the resealing took place. The apparent dissociation constant for this effect of Ca varied between 2 and 4×10^{-6} M when CaHEDTA buffer-solutions were used. In a Hill plot the data of Fig. 11 were fitted by a regression line with a slope of 2.66 ($r^2 = 0.88$). This value suggests that the resealing effect requires more than one Ca ion to combine with each membrane site. Several conclusions can be drawn from the

observations presented in Fig. 10 and 11. (1) The Na and K permeability of Ca-free ghosts rises with increasing pH. (2) The strong pH effect on the resealing of ghosts to cations does not depend on the presence of Ca. (3) The effect of Ca on the resealing is pH-independent and hence, unlike the Ca-induced K efflux, does not involve a membrane group with a pK value in the pH range between 6 and 8. (4) The increase in the rate of K efflux at pH values below 8 is probably mediated by Ca because it could be blocked completely by EGTA. It is possible that membrane-bound Ca is released as the pH is shifted in the acid direction, thus increasing $[Ca^{++}]_i$ above the threshold level for the activation of K efflux.

Accessibility of the Ca-Sensitive Membrane Sites

The experiments of Lepke and Passow (1972) which were mentioned above suggested that the effect of pH at the moment of hemolysis on the permeability properties of the resealed ghosts is irreversible. The relevant ionizable group appeared not to be accessible in the reconstituted cell. The use of reversal of hemolysis as a means to incorporate Ca into the cell makes it very difficult to decide, whether the Ca ion concentration of the hemolyzing medium or the actual concentration in the resealed ghosts or both are in equilibrium with the Ca bound to the relevant permeability-controlling membrane sites. However, a possible way to manipulate $[Ca^{++}]_i$ in resealed cells is provided by the strong pH dependency of the Ca-EGTA dissociation constants. In the experiment shown in Fig. 12, 22 °C ghosts were prepared at pH 7.2 in the presence of 8.6×10^{-7} M $[Ca^{++}]$ and incubated at pH values ranging from 7.2 to 8.5. A shift of the pH towards more basic values will cause the intracellular $[Ca^{++}]$ to drop since the apparent dissociation constant of the Ca-EGTA complex becomes smaller (log K' increases by 0.2 per 0.1 pH unit). In fact the rate of K outflow which was maximal at pH 7.2 with 8.6×10^{-7} M $[Ca^{++}]$ was reduced to the level of the Ca-free control when the pH of the incubation medium was kept at 8.2 resulting in a $[Ca^{++}]_i$ close to 10^{-8} M. In other experiments it could be shown that cells which were loaded at pH 8 with Ca concentrations well below the threshold for the activation of K efflux at that pH, started to lose K only at lower pH where their calculated level of $[Ca^{++}]_i$ reached the threshold value. The described behavior cannot be explained by a pH-dependent shift of the threshold for $[Ca^{++}]$ to activate K efflux. In contrast to what was observed experimentally (Fig. 6) this would

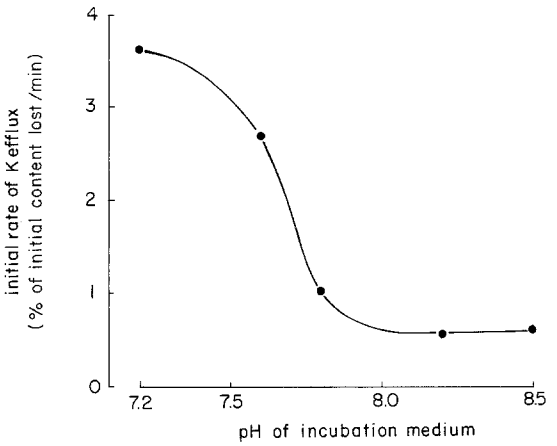

Fig. 12. Reversal of the Ca-dependent increase in K efflux rate by a pH-mediated reduction in $[Ca^{++}]_i$. Hemolysis at 22 °C and pH 7.2 in 10 mM HEPES solution containing 8.6×10^{-7} M EGTA (2 mM)-buffered free Ca. Reconstitution of ghosts with 2 M KCl. Incubation in a NaCl medium (soln VI). The pH of the incubation medium was adjusted to values between 7.2 and 8.5. Due to the pH dependency of the CaEGTA complex formation constant $[Ca^{++}]_i$ will decrease as the pH is increased. One of 9 similar experiments. Hct 4.1–7.8%

require an increase in the threshold $[Ca^{++}]$ with increasing pH. These results demonstrate that the Ca concentration in the hemolyzing medium did not predetermine irreversibly the K permeability of the resulting ghosts. Hence, an equilibrium seems to exist between the actual intracellular concentration of Ca ions and the amount of Ca bound to the site at which the increase in K permeability is initiated. One has to assume a localization of this site in the membrane close to its inner surface.

In contrast, Ca that is bound to the membrane site which promotes the resealing process ("resealing site") apparently is not in equilibrium with $[Ca^{++}]_i$ in the resealed cell. This can be inferred from experiments in which 22 °C-ghosts were prepared at pH 7.2 in the presence of Ca concentrations sufficient to enhance their initial K retention by at least 50% over the value found in Ca-free controls. The cells were then incubated at pH 8.2 in order to reduce $[Ca^{++}]_i$ by about one order of magnitude. At the new $[Ca^{++}]_i$ a considerable fraction of the initial cellular K should leak out of the cells due to a partial reversal of the sealing effect. However, this was not observed. The normalized rate of K efflux was the same in Ca-free and in Ca-treated cells. Hence, the resealing could not be reversed by lowering $[Ca^{++}]_i$. Since an extracellular $[Ca^{++}]$ of less than 10^{-8} M did not induce a K leak it must be concluded that this particular Ca binding site in resealed ghosts or in

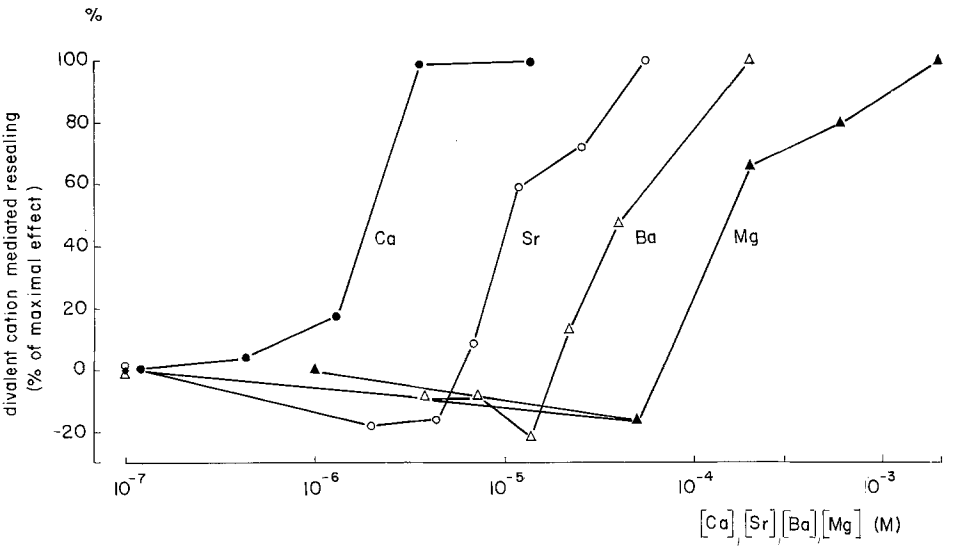

Fig. 13. Alkaline earth cation-mediated resealing of red cell ghosts to K. Determination of the selectivity sequence. Hemolysis at 22 °C and pH 7.2 in 5 or 10 mM HEPES solution containing HEDTA (4 mM)-buffered free Ca concentrations ranging from 10^{-7} to 1.4×10^{-5} M or EDTA (4 mM)-buffered Sr concentrations ranging from 10^{-7} to 5.6×10^{-5} M or EDTA (4 mM)-buffered Ba concentrations ranging from 10^{-7} to 2×10^{-4} M or unbuffered Mg concentrations ranging from 10^{-6} to 2×10^{-3} M. Reconstitution of ghosts with KCl (soln XII). Determination of the initial K content as in Fig. 11. The effects of Sr and Ba were measured in the same experiment. The data for Ca and Mg come from two separate experiments. Hct 4–10%. The resealing to K, mediated by alkaline earth cations other than Ca, was studied in 4(Ba), 5(Sr) and 7(Mg) experiments

intact cells is not accessible from either side of the membrane but is buried within the membrane during the process of resealing.

The Effects of Sr, Ba, and Mg on the Resealing of Ghosts

It has been known for some time that Mg in millimolar concentrations increases the yield of ghost cells sealed to K after hypotonic hemolysis at 22 °C (Hoffman, 1962; Colombe & Macey, 1974). As shown above, Ca in much smaller concentrations is also capable of improving the resealing toward cations (Fig. 11). The fact that Ca and Mg have similar effects on the resealing but antagonistic effects on the activation of K efflux from resealed cells suggested a marked difference in the selectivity sequence for divalent cations of the two relevant membrane sites. Since the effect of divalent cations on the yield of resealed cells was much more pronounced in 22 °C-ghosts than in 0 °C-ghosts, the cells in this set of experiments were hemolyzed at 22 °C. In Fig. 13 Ca, Sr, Ba, and

Mg are compared with respect to their efficiency in improving the resealing of red cell ghosts to K. About the same maximal effects on K retention were obtained with each individual cation. In Fig. 13 these maximal effects were taken as 100%. The graph combines the results of three experiments to show simultaneously the effects of the four cations. In control experiments pairs of cations with neighboring affinities were always tested to verify the differences measured. Of the four cations, only Mg was applied without a metal-buffering system because the high concentrations which had to be used were likely to prevent any significant decrease in the free concentration by binding to cell components. The selectivity sequence Ca > Sr > Ba > Mg could be established. The ratios of the concentrations required for a half maximal effect in the experiments of Fig. 13 were 1:5:20:75. A small decrease in the yield of sealed cells with cation concentrations slightly lower than those which promoted the resealing was a frequent observation with all cations tested. No ready explanation can be given for this behavior.

The determination of the efficiency of resealing as a function of divalent cation concentration in the case of Ca and Sr was complicated by the fact that the same concentrations which enhanced resealing, by some separate mechanism induced a K leak. Therefore, in control experiments the resealing to Na was measured as a function of the divalent cation concentration. The ghost cells were found to seal towards Na and K at about the same free cation concentration. With Ca the rate of net K inward movement into these Na-loaded cells was enhanced as was the K efflux under comparable conditions, but reversed Na-K gradients. This finding suggested that the Ca stimulation of K movements affected symmetrically the inward and outward fluxes.

Effects of Divalent Cations on Na Movements

Na Uptake. K-containing red cell ghosts prepared at 22 °C were used to study the influence of divalent cations on Na uptake. All alkaline earth cations tested were found to decrease Na uptake. Fig. 14 shows the result of an experiment with Ba. Ghosts were loaded with EDTA-buffered Ba ion concentrations ranging from 3.7×10^{-6} to 2×10^{-4} M. The Na uptake is plotted as a function of incubation time in isotonic NaCl solution (soln VI).

Up to 1.4×10^{-5} M intracellular Ba did not affect the Na uptake significantly, whereas higher concentrations strongly inhibited the inward

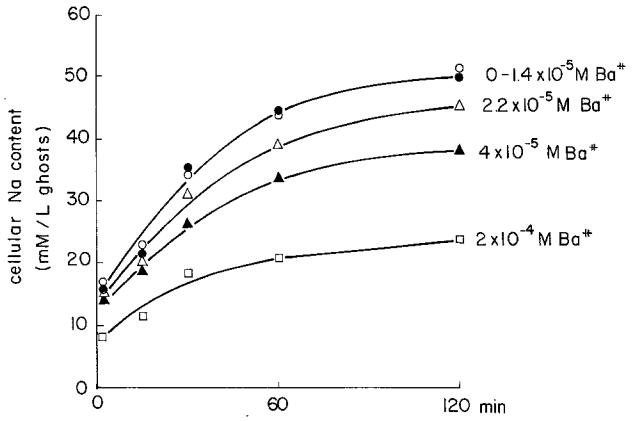

Fig. 14. Time course of Na uptake in reconstituted ghosts in the presence and absence of intracellular Ba. Hemolysis and reconstitution of ghosts as in Fig. 13. Incubation in a NaCl medium (soln VI). Hct 6.7–9.9%). One of 4 experiments

movement of Na. However, it should be noted that in influx experiments the uptake into incompletely sealed ghosts always constituted a considerable fraction of total cation uptake, whereas in efflux measurements the contribution of leaky ghosts could be neglected (*see* Fig. 1).

At the end of the washing procedure preceding the experimental incubation, the K-loaded ghosts in the present experiment contained about 4 mM Na because NaEDTA was used to prepare the metal-buffer solutions. Ba-free cells from the first sample which was withdrawn 2 min after the incubation was started, already contained 16 mM Na. This was more than 25% of their total Na uptake in 120 min. After the initial rapid phase the rate of uptake decreased until it reached an almost constant value after about 60 min. The initial uptake into leaky ghosts was subtracted from the total uptake if the effect of different divalent cations was to be compared (*see* below). Nevertheless, the Na influx into cells containing $0\text{–}4 \times 10^{-5}$ M Ba was much larger than the K efflux in the same period of time even when the initial Na uptake was disregarded. In the experiment of Fig. 14 the K efflux of ~ 5 mM/liter ghosts within the first 60 min remained essentially constant up to a cellular Ba concentration of 4×10^{-5} M. The ratio Na uptake to K outflow changed from 5.7 in the absence of Ba to 3.4 in the presence of 4×10^{-5} M Ba. The ratio was smaller than 1 only with 2×10^{-4} Ba. Consequently, the volume of the low-Ba-cells increased by about 20% during the incubation period, whereas the high-Ba-cells shrunk. Even though the osmotic behavior of the cells that took up Na suggested that they were at least

partially sealed to cations it is not entirely clear whether this finding indicates that their Na permeability was indeed higher than their K permeability. If the Na and K concentration gradients were reversed, i.e., if Na and K fluxes were measured in Na-loaded, Ba-free cells which were incubated in isotonic KCl-solution, the flux ratio was also found to be reversed. Therefore, it is possible that with 22 °C-ghosts the cell population involved in efflux measurements is not completely identical to the one which is involved in cation uptake.

In comparative experiments the Na inflow during an incubation period of 60 min at pH 7.2 was measured as a function of the cellular Ca, Sr, Ba or Mg concentration. With all divalent cations the Na uptake could be inhibited by 30 to 50%. The selectivity sequence for this effect was $Ca > Sr > Ba > Mg$ and, thus, was the same as the one established for the efficiency of resealing after hypotonic hemolysis (*see* Fig. 13).

Na Efflux. For a direct comparison of the action of alkaline earth cations on Na efflux with their action on K efflux 22 °C ghosts were prepared to contain Na instead of K as major osmotic constituent together with buffered free concentrations of either Ca or Ba or Sr. The cells were then incubated in an isotonic KCl medium (soln IX). In Fig. 15 the Na efflux from Na-loaded cells within 60 min is plotted against $[Ca^{++}]_i$. Between 3×10^{-7} and 1.2×10^{-6} M $[Ca^{++}]_i$ the Na efflux decreased by more than 50% of its value in the absence of Ca. Also shown in Fig. 15 is the initial cellular Na concentration. As discussed above, this parameter is a function of $[Ca^{++}]$ at the time of hemolysis. Note that the decrease in Na efflux and the increase in the yield of sealed cells occurred in the same range of Ca ion concentrations. In 0 °C-ghosts the Na efflux sometimes showed a distinct maximum at the same $[Ca^{++}]_i$ or $[Sr^{++}]_i$ which initiated an increase in K efflux in K loaded cells, whereas higher concentrations of Ca or Sr (like Ba and Mg) usually depressed the outward movement of Na.

The reduction in Na permeability by divalent cations was only partially reversible when $[Ca^{++}]_i$ was lowered by means of a pH shift (*see* p. 337). It seemed that $[Ca^{++}]$ during hemolysis as well as $[Ca^{++}]_i$ during the incubation period determined to a certain extent the Na permeability of the ghosts.

Ca, Sr, Ba, and Mg reduced Na efflux in the same order of selectivity as was reported above for Na uptake. The close relationship between the alkaline earth cation-mediated decrease in Na permeability and their effect on the yield of sealed ghosts strongly suggests that an interaction with the same membrane site might be responsible for both effects.

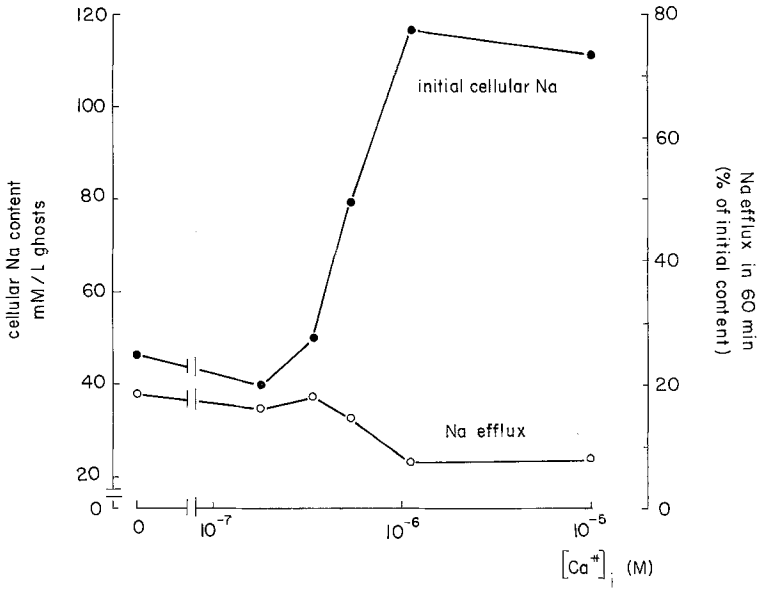

Fig. 15. The effect of Ca on the resealing of ghosts to Na and on the Na efflux from resealed ghosts. Hemolysis at 22 °C and pH 7.4 in 5 mM Tris solution containing either 2 mM EGTA alone or EGTA (2 mM)-buffered free Ca concentrations ranging from 1.8×10^{-7} to 10^{-5} M. Reconstitution of ghosts with 2 M NaCl. Incubation in a KCl medium (soln IX). Single experiment. Hct 5.1–7.3%. 6 similar experiments have been performed

Discussion

In the present study two actions of Ca on the cation permeability of human red cell ghosts were examined in some detail: (1) the increase in K permeability which follows a rise in $[Ca^{++}]_i$ and (2) the dependence of the yield of ghost cells sealed to Na and K on the Ca ion concentration in the hemolyzing medium. The results indicate that the two effects are caused by the interaction of Ca with two independent membrane sites controlling two separate pathways for the permeation of cations across the cell membrane. This conclusion is based on findings which demonstrated differences in the accessability, pH dependency, and monovalent as well as divalent cation selectivity of the two presumed membrane sites.

The site at which Ca initiated a selective and reversible increase in K permeability was shown to be accessible to intracellular Ca ions in resealed ghosts (Porzig, 1975; Simons, 1975). The availability of this site to Ca was demonstrated also in ATP-depleted and in ATP-containing human erythrocytes (Romero & Whittam, 1971; Dunn, 1974). Recently

Simons (1976), in a detailed kinetic study, showed that K equilibrium exchange is similarly affected by intracellular Ca, Sr, and Mg, as is the net flow of K, described in this paper. Hence, it seems that a component of the inner surface of the intact cell membrane is transformed by Ca into some kind of a K-specific "channel". Blum and Hoffman (1971) and Hoffman and Knauf (1973) working with ATP-depleted red cells provided evidence for an inhibition of the Ca-mediated K efflux by ouabain, oligomycin and furosemide and, therefore, favored the idea that the Na-K-ATPase might be the target for this action of Ca. The inhibitory effect of high concentrations of oligomycin has been confirmed by Riordan and Passow (1971) and is also shown in the present study (Fig. 3A). However, oligomycin is not considered a specific inhibitor of the membrane Na-K-ATPase. Unfortunately, the more crucial effect of ouabain remains controversial. In my own experiments on starved red cells, ouabain was ineffective. Lew (1974) presented evidence that ouabain had no effect on the Ca-mediated K efflux when the cellular ATP was lowered to less than 10^{-6} M. Therefore, he suggested that ouabain might act indirectly, by conserving cellular ATP as a consequence of the inhibition of the ATP-consuming Na-K-pump. Therefore, more ATP will be available to remove intracellular Ca via the Ca-pump. This seems to be a more likely explanation than a direct action of ouabain on the Ca-activated K efflux.

Moreover, the available data on Ca-mediated changes in the kinetic and biochemical parameters of the Na-K-ATPase do not yet allow a quantitative comparison with the results of the present study. Apparently, Ca binds to components of the Na, K pump apparatus (Schön, Schönfeld, Menke & Repke, 1972; Tobin, Akera, Baskin & Brody, 1973) and inhibits strongly the normal function of the pump in ATP-containing red cells (Dunn, 1974). But apparent Ca dissociation constants of these Na, K pump components have not been determined in terms of free ionic concentrations. Therefore, the present data neither prove nor disprove the "Na-pump hypothesis". More complete data are available for the interaction of Ca with the Ca activated ATPase involved in active Ca transport (Wolf, 1972; Schatzmann, 1973). It is interesting to note that the pK′ values of the two ionizable groups which were found in this enzyme at pH 8.2 and 5.8 correspond closely to the values reported here for the site which mediates the Ca-dependent increase in K efflux (8.3 and 6.3). This might well be a coincidence and by no means proves any direct interference of the Ca-pump enzyme with the activation of K efflux but suggests a similarity in the functional groups involved.

Wolf (1972) suggested an imidazol-nitrogen and an α-amino group of an amino acid as the most likely candidates for ionizable groups with pK′ values around 6 and 8.2, respectively.

Recent studies by Passow and his coworkers (Riordan & Passow, 1973; Knauf *et al.*, 1975) have shed some light on the interrelationship between the Ca-mediated increase in K permeability and the monovalent cation concentrations on both sides of the membrane. In the absence of extracellular K, intracellular Na was shown to inhibit Ca-dependent K efflux but not the Ca-activated K influx. However, the inhibitory action of Na_i was virtually abolished when K_o was raised to 2–5 mM. These observations were confirmed and extended in the present investigation (*see* Fig. 4*A* and *B*). Such properties make the Ca-induced K "channel" in erythrocytes strikingly similar to the pores that conduct K ions across nerve membranes. In squid axons the cation selectivity of the K pores has been thoroughly tested by Bezanilla and Armstrong (1972). They observed a rectifying effect of Na_i on K movements in that Na_i preferentially inhibited the movement of K in the outward direction. Moreover, extracellular K counteracted the effect of Na_i. Bezanilla and Armstrong envisage a pore with a wide and rather unselective inner opening and a narrow selective part towards the external membrane surface. The inhibitory action of internal Na in this model results from the entering of Na into the inner nonselective part of the channel. External K, by moving passively into the cell, would sweep Na out of the inner part of the channel and, hence, remove its inhibitory action on K efflux. If this type of model applies to the situation in red cells, divalent cations would have two possibilities to interfere with the Ca-induced net outward movement of K. They may either compete with Ca for the "channel-inducing-site" or compete with K for the occupancy of the inner nonselective part of the channel. The inhibitory effect of Ba, which was apparently not due to competitive interaction with Ca (*see* Fig. 8 *B*), might be representative for the latter type of interference. Mg on the other hand may interact at both sites since it caused competitive inhibition of the Ca effect as well as a decrease in the maximal rate of Ca-induced K efflux (*see* Fig. 8 *A*).

Difficulties with this interpretation arise from the accelerating effect of a change in K_o from 0.1 to 2 mM on the rate of Ca-activated net K efflux in the presence of low Na_i (2–4 mM) (Knauf *et al.*, 1974).

The model would not explain this effect unless additional assumptions are made. However, the present results strongly suggest that a change in K_o from 0.5 to 5 mM did not affect significantly the rate of K efflux

from K loaded ghosts if induced by well-defined and constant intracellular Ca ion concentrations (*see* Fig. 4*A*). This seems to be in contrast to the results of Knauf *et al.* However, the authors worked with K-loaded resealed ghosts which were exposed to a constant extracellular Ca concentration of 8 mM. The inflow of Ca under this condition was not quantified but was sufficient to initiate a maximal increase in K efflux in the presence of extracellular K. With 8 mM Ca on the outside $[Ca^{++}]_i$ within 60 min will increase by not more than 0.01 mM even in thoroughly starved cells (Porzig, 1974, and *unpublished observations*). Moreover, Knauf *et al.* prepared ghosts from nonstarved erythrocytes and, hence, the cells contained sufficient ATP to support a limited activity of the Ca-ATPase. Therefore, a stationary state value of $[Ca^{++}]_i$ well below 0.01 may be a more realistic estimate. Consequently, a small drop in Ca uptake, e.g., caused by a decrease in K_o, would be sufficient to maintain $[Ca^{++}]_i$ below the threshold concentration required for the initiation of K efflux. For these reasons the data of Knauf *et al.* by no means rule out the possibility that the pore model suggested by Bezanilla and Armstrong for the K channels in squid axons may apply also to the Ca-induced K-channels in red cells.

Ca-inducible K channels apparently exist in a number of different tissues including cat motoneurons (Krnjević & Lisiewicz, 1972) and snail neurons (Meech, 1974) but they are absent in squid axons (Begenisich & Lynch, 1974) as well as in erythrocytes of cattle or goat (Jenkins & Lew, 1973). It is completely unknown what factors determine their absence or presence in a particular membrane. Certainly the presence of the Na-K-ATPase is not the only requirement since this enzyme is a component of all the membranes mentioned above.

The second Ca-sensitive site ("resealing site") in the red cell membrane was accessible neither in the intact membrane nor during hemolysis at 0 °C. Since Ca complexing agents prevented the resealing of ghosts if the hemolysis was carried out at 20 °C or higher temperatures, one can infer that under this condition intrinsic Ca was removed which was bound to this site in the native membrane. The evidence presented in this paper indicates that the Ca-sensitive "resealing site" and an H^+- sensitive site described by Lepke and Passow (1972) are separable entities even though both affect the reconstitution of low cation permeability and exhibit the same type of restricted accessability (*see* Figs. 10 and 11). The functional connection of the two sites has still to be worked out.

Since resealing to both Na and K was dependent on the Ca ion

concentrations of the hemolyzing medium, the pathway which is controlled by the resealing site appears to be unspecific with respect to cations. In fact, the work of Palek, Curby and Lionetti (1971) suggests that it may not discriminate between charged and neutral particles. Moreover, since Ca can be replaced by Sr, Ba and Mg, different types of Ca-membrane site interaction may take place at the specific site mediating K efflux and the unspecific resealing site. The formation of a chelate complex could account partly for the Ca-Sr specificity of the former site, whereas at the latter site Coulombic interactions (defined as the forces acting between point charges) between Ca and negative fixed charges in the membrane may predominate.

The theory of Sherry (1969) predicts that only 7 out of 24 possible permutations of the four alkaline earth cations Ca, Sr, Mg, and Ba should be observed as equilibrium selectivity sequences for the interaction with negatively charged membrane sites of different field strengths. The selectivity sequence Ca > Sr > Ba > Mg, which was observed in the present study, is not represented in the Sherry series but seems closely related to his sequence III (Ca > Ba > Sr > Mg), which reflects an interaction at closely spaced sites (the spacing accounting for the prevalence of divalent over monovalent ions) of rather weak field strength. This conclusion is compatible with the rather small pH dependency of the Ca-dependent resealing between pH 6 and 8 (*see* Fig. 7). The pK′ value depends on the field strength of the site. A weak anionic site will have a pK′ value in the acidic range far below 6 (*see* Diamond & Wright, 1969). However, the possibility that non-Coulombic forces play a major role in the interaction of divalent cations with the resealing site cannot be ruled out definitely.

The same selectivity sequence reported above for the resealing effect was observed by Palek *et al.* (1971) for the Ca-induced shrinkage of osmotically hemolyzed human red cell ghosts. The threshold Ca concentration for this effect was 2×10^{-6} M. The authors provided evidence that a small initial decrease in cell volume preceding the restoration of isotonicity reflected a contraction of a membrane-associated contractile protein, whereas the later phase of isotonic shrinkage was osmotic and due to a cation-dependent decrease in Na permeability. These findings suggest that an interaction of Ca with the same site is responsible for the resealing effect, as well as for the shrinkage, and may point to some contractile membrane protein as target for these two effects of Ca.

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