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Role of External Potassium in the Calcium-Induced Potassium Efflux from Human Red Blood Cell Ghosts*

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Summary. The exposure of red cell ghosts to external Ca^{++} and K^+ leads to a rapid net K^+ efflux. Preincubation of the ghosts for various lengths of time in the absence of K^+ in the external medium prior to a challenge with maximally effective concentrations of Ca^{++} and K^+ renders the ghosts unresponsive to that challenge with a half-time of about 7–10 min. Preincubation at a range of K^+ concentrations for a fixed length of time (60 min) prior to the challenge revealed that K^+ concentrations of about 500 μM or more suffice to maintain the K^+ channel in a maximally responsive state for at least 60 min. These K^+ concentrations are considerably lower than the K^+ concentrations required to make the responsive channel respond with a maximal rate of K^+ efflux. Thus, external K^+ is not only necessary to induce the permeability change but also to maintain the transport system in a functional state.

The presence of Mg^{++} or ethylenediamine-tetraacetic acid (EDTA) in the K^+-free preincubation media preserves the responsiveness to a challenge with Ca^{++} plus K^+. In contrast to external K^+, the presence of external Ca^{++} does not reduce but rather enhances the loss of responsiveness. An excess of EDTA prevents the effects of Ca^{++} while washes with EDTA after exposure to Ca^{++} do not reverse them.

In red cell ghosts that contain Ca^{++} buffers, the transition from a responsive to a nonresponsive state incubation in the absence of external K^+ is enhanced. The effects of incubation in the presence of Ca^{++} in K^+-free media are reversed; external Ca^{++} now reduces the rate at which the responsiveness is lost. The loss of responsiveness after incubation in K^+-free media prior to a challenge with external K^+ and internal Ca^{++} does also take place when K^+-efflux from red cell ghosts is measured by means of $^{42}K^+$ into media that have the same K^+ concentrations as the ghost interior. This confirms that the effects of K^+-free incubation are due to the modification of the K^+-selective channel rather than to an inhibition of diffusive Cl^--efflux.

Abbreviation used in text: TRIS = Tris (hydroxymethyl) aminomethan

In 1958, it was observed by Gárdos that under certain abnormal metabolic conditions, the addition of Ca^{++} to the medium causes a rapid K^+ efflux from human red blood cells. The high rate of K^+ efflux far exceeds the rate of the K:Na-pump-mediated influx and thus indicates the activation of a cation-permeable pathway that is not detectable in the untreated red cells. The pathway was found to be highly selective for K^+ as opposed to Na^+ (Passow, 1961, 1963, 1964; Kregenow, 1962; Kregenow & Hoffman, 1962, 1972) and to resemble in this respect the pathways opened by Pb^{++} in metabolically intact red cells and by Ca^{++} or Mg^{++} in fluoride poisoned red cells (Ørskov, 1935; Wilbrandt, 1937, 1940; *cf.* Passow, 1961, 1963, 1964 for reinvestigation and review of these findings in the context of the "Gárdos effect"). Later work has provided a considerable body of evidence which indicates that Ca^{++}, Mg^{++} and Pb^{++} do indeed activate the same pathway; for example, it was observed that oligomycin produces in all three cases a strong inhibition of the K^+ efflux (Blum & Hoffman, 1971; Riordan & Passow, 1971, 1974; Knauf, Riordan, Schuhmann, Wood-Guth & Passow, 1975).

* *This paper is dedicated to the memory of Walther Wilbrandt.*

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The site of action of Ca^{++} could be localized at the inner membrane surface (Whittam, 1968; Blum & Hoffman, 1971; Romero & Whittam, 1971) and it was shown that micromolar concentrations of Ca^{++} suffice to elicit a maximal response (Blum & Hoffman, 1971; Lew, 1974; Porzig, 1975; Simons, 1976*a, b*).

Among the three metal ion species that are capable of inducing the effect, Ca^{++} has received the most attention. In the intact red cell, it is present at a concentration of less than 1 μM. This is due to the combination of a low rate of passive Ca^{++} entry, a high rate of extrusion by the $Ca^{++}-Mg^{++}$-stimulated membrane ATPase and the complex formation with a large variety of metabolites and other intracellular substances. (For comprehensive recent reviews, *see* Lew & Ferreira, 1978; Lew & Beaugé, 1979).

Alterations that increase the Ca^{++} concentration inside the red cells to values above a few micromoles per liter enhance K^+ efflux across the K^+-selective pathway. For example, an increase of passive Ca^{++} influx by the addition of a suitable ionophore (Ferreira & Lew, 1976), or by the damage produced by the insertion of a micropipette (Lassen, Pape & Vestergaard-Bogind, 1976) or just by a sudden increase of the extracellular Ca^{++} concentration (Lassen et al., 1978) can no longer be balanced out by active Ca^{++} extrusion and leads to an enhancement of K^+ exit. Similar effects occur after the addition of certain drugs (Manninen, 1970; Porzig, 1975) or are evoked by substrate depletion or metabolic inhibitors (Gárdos, 1958*a, b*; Passow, 1961, 1963, 1964; Kregenow, 1962; Kregenow & Hoffman, 1962, 1972; Blum & Hoffman, 1971; Lew, 1971*a*; Romero & Whittam, 1971) which reduce the ATP concentration of the cells thereby depriving the Ca^{++} pump of its energy-yielding substrate and lowering the concentration of intracellular complexing agents. A particularly convenient method of rendering the cell membrane susceptible to the actions of Ca^{++} consists of diluting the cell contents by hemolysis and the subsequent preparation of resealed ghosts (Lepke & Passow, 1968; Blum & Hoffman, 1972).

In the present paper, we report on some observations of the actions of extracellular K^+ on the Ca^{++}-induced permeability change in resealed red cell ghosts. The paper is based on earlier work of Blum and Hoffman (1971) and Lew (1971*b*) who had shown that extracellular K^+ stimululates ^{42}K efflux from Ca^{++}-treated ghosts or cells. This stimulation is not simply due to an enhanced $^{42}K/K$ exchange since subsequent work from our laboratory demonstrated that even the induction of a net KCl efflux requires the presence of some K^+ in the external medium. In fact, the external K^+ was found to be as essential for the activation of the K^+-selective pathway as the internal Ca^{++} (Knauf et al. 1975; Simons, 1979). The experiments described below were performed to elucidate further the role of K^+ in eliciting the response. They show that external K^+ plays at least two different roles: at very low concentration it maintains the K^+ pathway in a state in which it remains capable of responding to Ca^{++}; at higher concentrations it cooperates with Ca^{++} inside the cells in converting the responsive pathway from a closed (inactive) to an open (activated) state. At even higher concentrations, external K^+ possibly plays a third role and produces an inhibition of the K^+-activated pathway.

Materials and Methods

All experiments were performed with Rh^+O blood from healthy donors. The blood was stored in acid-citrate-dextrose buffer and used usually after 3–4 days of storage.

Resealed ghosts were prepared essentially as described by Bodemann and Passow (1972) by hemolysis of the thrice washed red cells at 0 °C, pH 6.0. The hemolysis medium contained 4 mM $MgSO_4$ and 1 mM acetic acid, except in the experiments with citrate as a Ca^{++} buffer where the $MgSO_4$ was omitted. Hemolysis was performed by dilution of cells with hemolysis medium at a ratio of 1:40.

Five minutes after hemolysis ice-cold, concentrated solutions of choline chloride, KCl and TRIS-Cl were added to the hemolysate to give final concentrations of 70, 70 and 20 mM, respectively. Subsequently, the ghosts were allowed to equilibrate for 10 min. Resealing was achieved by transfer of the ice-cold ghost suspension to 37 °C and incubation at that temperature, pH 7.6, for 45 min. The resealed ghosts were sedimented and washed thrice in "choline medium" containing 144 mM choline-Cl, 20 mM TRIS-Cl, pH 7.6. Before the last wash, the ghosts were incubated at 37 °C, in the K^+-free choline medium for 30 min (hematocrit ≈ 1%). This served to achieve the complete release of K^+ from any ghosts that had not been successfully resealed (*see* Bodemann & Passow, 1972, p. 16).

After the last wash, the ghosts were resuspended at a hematocrit of 0.25% in the choline medium at 37 °C and incubated under the conditions specified in the text and in the figure legends. During such incubation periods, either K^+ or Ca^{++} or both were absent from the external media. At the end of these periods, the permeability change was induced by adding sufficient K^+ and Ca^{++} to produce a maximal response, which was achieved by bringing the final concentrations of K^+ and Ca^{++} up to 2.5 and 10 mM, respectively (zero time in the figures). Subsequently, measured aliquots of the suspension were taken, diluted with measured volumes of an ice-cold medium containing 94 mM choline-Cl, 50 mM LiCl and 20 mM TRIS-Cl, pH 7.6, and centrifuged. The sediment was dissolved in 1 mM CsCl and used for flame photometric determination of intracellular K^+. The corrections for extracellular K^+ in the sediment were based on determinations of the LiCl space as described by Passow (1969).

In the figures, the time course of release of KCl from the ghosts is represented by plotting the intracellular K^+ content per cell as a per cent of the K^+-content at zero time of the curves. The absolute values of the intracellular K^+ contents at zero time in the different runs that are represented by the different curves in the same figure agree to within less than 10%, except in Fig. 9 where the difference between the curve of 12.7 μM $CaCl_2$ and the highest value observed amounted to about 20%.

In a number of experiments, Ca^{++} buffers were used that contained besides the Ca^{++}, $MgSO_4$ (4 mM) and EDTA (1 mM). The concentration of free Ca^{++} was calculated as described by Perrin and Dempsey (1974) and adjusted by the addition of $CaCl_2$. The $MgSO_4$ was present at the time of hemolysis; Ca^{++} and EDTA were added during reversal together with the choline chloride, KCl and TRIS-Cl. For reasons which have not been explored further, the sequential addition of Mg^{++} and the other components of the Ca^{++} buffer give more reproducible results than the simultaneous addition. The response of the resealed Ca^{++}-containing ghosts was elicited by the addition to the external medium of 2.5 mM KCl (zero time in the figures).

In other experiments Ca^{++}-citrate buffers were used, containing 5 mM citric acid and suitable concentrations of $CaCl_2$.

For the determination of K^+ equilibrium exchange, the ghosts were prepared essentially as described above. However, they were resealed in the presence of ^{42}K in TRIS-buffered choline media in which increasing amounts of choline had been substituted by equivalent amounts of KCl. The resealed ghosts were subdivided into batches that were further processed as described above, either in the absence or presence of 1.0 mM KCl in 20 mM TRIS plus 140 mM choline chloride. After the second incubation period (1 hr, 37 °C), at a hematocrit of 0.25%, they were resuspended at that hematocrit in media that had the same composition as the media in which resealing had been performed. Except in the controls, to these media were added 2 μM of $CaCl_2$ plus 2.0 μM of the ionophore A 23187, yielding a total Ca^{++} concentration of 4 μM. The efflux of ^{42}K was measured by taking samples and counting the radioactivity in the supernatant. The time course of release of ^{42}K followed a single exponential from which rate constants were calculated by means of a least-squares technique.

All chemicals were of analytical grade. Choline chloride was obtained from Syntex, Springfield, Missouri, ^{42}K from Amersham-Buchler, the ionophore A 23187 from Dr. Hamill (Eli Lilly Research Laboratories, Indianapolis, Indiana) and all other chemicals from Merck, Darmstadt.

Results and Discussion

Induction of Net K^+-Efflux by the Addition of Ca^{++} and K^+ to the External Medium

Resealed red cell ghosts that contain KCl undergo a net KCl efflux after resuspension in media that contain Ca^{++} and K^+. In order to obtain easily measurable rates of efflux with little if any lag period preceding the onset of the permeability change, Ca^{++} concentrations of 0.1 to 10 mM are required at KCl concentrations in the medium up to 2.5 mM. The effect becomes maximal at 10 mM Ca^{++} and 2.5 mM K^+. Exceeding 10 mM Ca^{++} at the K^+ concentration mentioned produces no further augmentation of the rate of efflux. Elevating the KCl concentration beyond 2.5 mM leads to some inhibition of net K^+ efflux. This is only partially related to the decrease of the electrochemical potential difference for K^+ across the membrane (Knauf et al., 1975).

The response to the combined action of optimal concentrations of Ca^{++} and K^+ outside (10 mM and 2.5 mM, respectively) depends strongly on the pretreatment of the red cell ghosts. If the ghosts are not excessively diluted and washed after resealing, the response is maximal. It makes no difference whether Ca^{++} and K^+ are added simultaneously or if the Ca^{++} is added after preincubation in the presence of K^+ (Fig. 1). If, however, the washing protocol described in Materials and Methods is followed and an incubation period ("leaching period") is included at low hematocrit and elevated temperature in a strictly K^+-free medium, then subsequent exposure to the combined action of Ca^{++} and K^+ produces a less than maximal response (Fig. 2). If the length of the leaching period in the K^+-free medium is systemati-

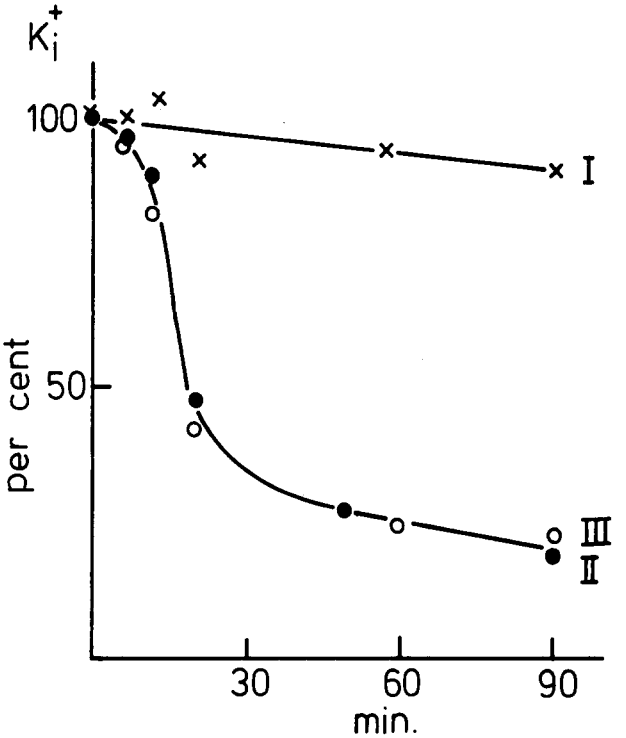

Fig. 1. Potassium efflux in response to the combined action of Ca^{++} and K^+ ("responsiveness") after preincubation in presence and absence of K_o^+. After resealing, washed ghosts were incubated at 37 °C for 30 min in K^+-free medium and washed again. Subsequently, they were resuspended at 37 °C. The media contained (in mM):

I		II		III		t (min)
K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	
0	0	(−)		2.5	0	−40
0	0	2.5	10.0	2.5	10.0	0

$t=0$: composition of the medium as established at zero time in the figure. −40 min: incubation at the compositions listed was begun 40 min before zero time in the figure. (−) indicates that the packed ghosts had been stored on ice until zero time. Hematocrit in this and all other experiments 0.25%. Ordinate (in this and all subsequent figures, except in Figs. 3*b*, 9*b* and 12): intracellular potassium content (amount per cell) as a percent of the initial value. Abscissa: time in minutes

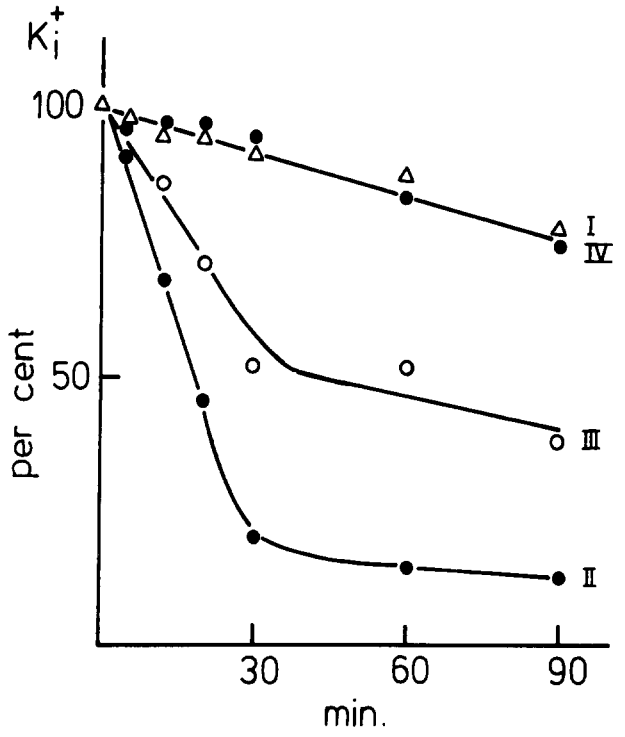

Fig. 2. Absence of K_o^+ leads to loss of responsiveness and presence of Ca_o^{++} in the K_o^+-free medium enhances the rate at which the loss of responsiveness occurs. After resealing, washed ghosts were incubated at 37 °C for 30 min in K^+-free medium and washed again. Subsequently, they were resuspended at 37 °C. The media contained (in mM):

I		II		III		IV		t (min)
K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	
0	0	2.5	0	0	0	0	0.5	−20
0	0	2.5	0	2.5	0	2.5	0.5	−10
0	0	2.5	10.0	2.5	10.0	2.5	10.0	0

$t=0$: concentrations in the external medium as established at zero time in the figure. −20 min: incubation at low hematocrit was started 20 min before zero time at the concentrations indicated in this row. −10 min: 10 min later the media were modified to obtain the concentrations listed here. Thus, the inititation of the permeability change by bringing the concentrations of K^+ and Ca^{++} up to the values listed at 0 min is preceded by two consecutive 10-min incubation periods in media of the compositions indicated in the Table

cally varied, and if the system is then challenged by the maximally effective concentrations of Ca^{++} and K^+, one finds that the response decreases with increasing time of leaching in the K^+-free media (Fig. 3*a*). An increase of the K^+ concentration in the challenging medium (at a fixed concentration of Ca^{++}) can partly, but not completely, restore the ability of the system to respond with an increase of K^+ efflux (not shown). If the time of preincubation in K^+-free media is made sufficiently long, the re-

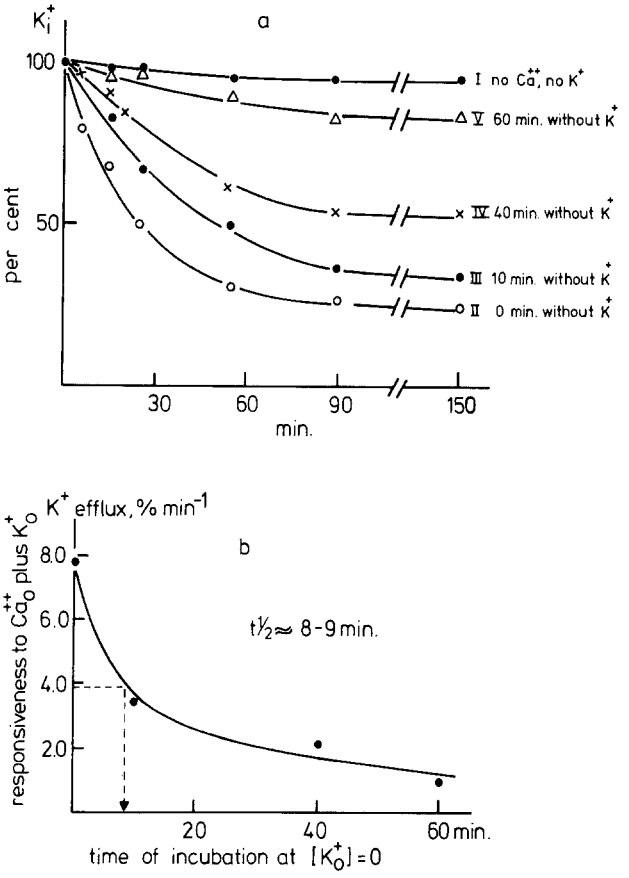

Fig. 3. (*a*): Increasing the time of preincubation in the absence of K_o^+ reduces the responsiveness (i.e. the K_o^+ efflux in response to a challenge by an increase of Ca_o^{++} and K_o^+ to concentrations that produce a maximal effect in ghosts that had never been incubated in K^+-free media). (*a*): After resealing washed ghosts were incubated at 37 °C for 30 min in K^+-free medium and washed again. The sedimented ghosts were then resuspended at 37 °C. The media contained (in mM):

I		II		III		IV		V		t (min)
K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	
(−)		(−)		(−)		(−)		0	0	−90
(−)		(−)		(−)		0	0	0	0	−70
0	0	2.5	0	0	0	0	0	0	0	−40
0	0	2.5	0	2.5	0	2.5	0	2.5	0	−30
0	0	2.5	10.0	2.5	10.0	2.5	10.0	2.5	10.0	0

$t=0$: concentrations in the external medium as established at zero time in the figure. (−) refers to packed cells stored at 0 °C until the times indicated. The times with the minus signs designate the time in minutes before zero time at which the ghosts were suspended at 37 °C in media containing K^+ and Ca^{++} at the concentrations listed. The times on the individual curves indicate the time periods of incubation in the absence of external K^+ corresponding to the data in the Table. (*b*): K^+ efflux as determined from the initital slopes of the curves in Fig. 3*a* is plotted against time of preincubation in absence of K_o^+. $t_{\frac{1}{2}}$ refers to the half-time of decay of sensitivity to exposure to K^+-free media in this particular experiment

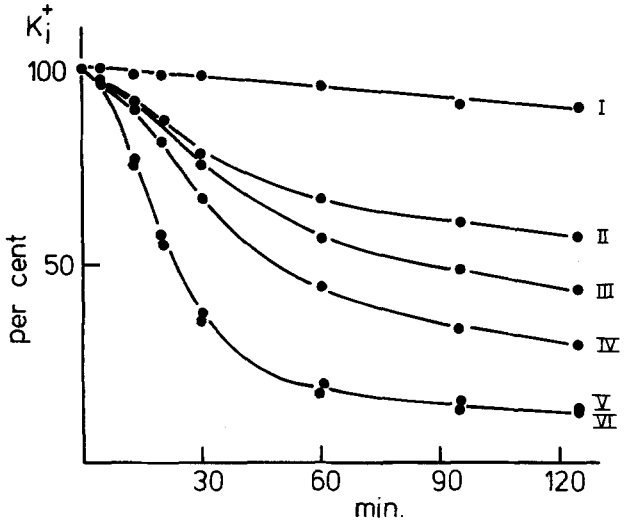

Fig. 4. Increasing K^+ concentrations during a fixed period of preincubation in the absence of Ca^{++} (60 min) lead to increasingly better maintenance of responsiveness to Ca_o^{++} plus K_o^+. After resealing, washed ghosts were incubated at 37 °C for 30 min in K^+-free medium and washed again. Subsequently, they were resuspended at 37 °C. The media contained (in mM):

I		II		III		
K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	
0	0	0.01	0	0.05	0	
0	0	2.5	0	2.5	0	
0	0	2.5	10	2.5	10	
IV		**V**		**IV**		*t* (min)
K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	
0.1	0	0.5	0	2.5	0	−90
2.5	0	2.5	0	2.5	0	−30
2.5	10	2.5	10	2.5	10	0

$t=0$: concentrations in the external medium as established at zero time in the figure. −90: incubation started 90 min before zero time at the concentrations indicated. −30 min: 60 min later, the compositions of the media were changed to those listed in the corresponding row

sponsiveness is irreversibly lost. The half-time of the loss of responsiveness is about 7–10 min (Fig. 3*b*).

The findings described are corroborated by the experiments in Fig. 4. Here, ghosts have been incubated after resealing and washing for a fixed period of time (60 min) at a range of K^+ concentrations. At the end of the incubation period, in all suspensions the KCl was brought up to a concentration of 2.5 mM. Thirty minutes later, Ca^{++} was added to give a final concentration of 10 mM. The ensuing K^+ efflux became lower, the lower the K^+ concentration was during the preincubation period. Thus, the effects of exposure to low $[K^+]$ are only partially reversible. Maximal responsiveness is only maintained if the K^+ concentration is about 500 μM or higher. It seems that after incubation at KCl concentrations around 1 μM or less for 60 min or more, a response can no longer be elicited.[1]

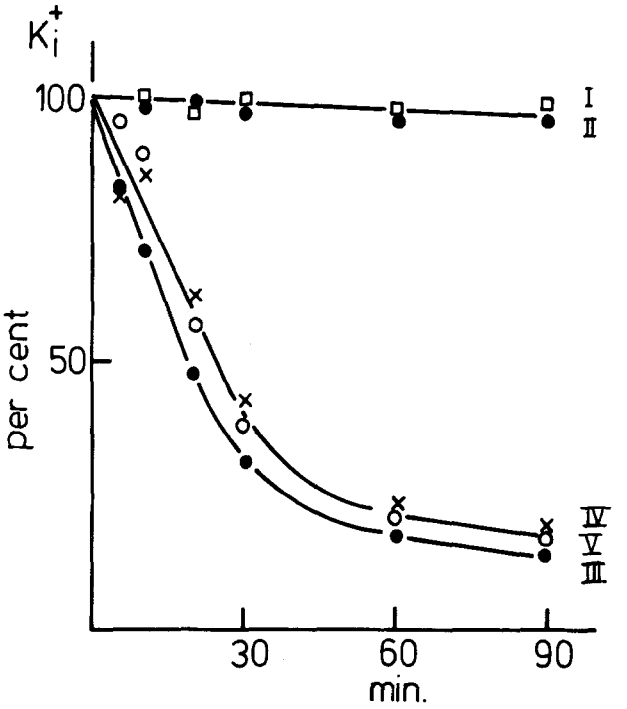

Fig. 5. EDTA maintains responsiveness to the combined action of Ca^{++} and K^+ when present during incubation in K^+-free media. After resealing, the washed ghosts were incubated at 37 °C for 30 min in a K^+-free medium and then washed again. Subsequently, they were resuspended at 37 °C. The media contained (in mM):

I			II			III		
K^+	Ca^{++}	EDTA	K^+	Ca^{++}	EDTA	K^+	Ca^{++}	EDTA
0	0	0	0	0	0	2.5	0	0
0	0	0	2.5	10	0	2.5	10	0

IV			V			*t* (min)
K^+	Ca^{++}	EDTA	K^+	Ca^{++}	EDTA	
0	0	1.0	2.5	0	1.0	−60
2.5	10	1.0	2.5	10	1.0	0

$t=0$: concentrations in the external medium as established at zero time in the Figure. −60 min: incubation was started 60 min before zero time at the concentrations indicated in this row

[1] Ghost suspensions usually contain mixtures of resealed and leaky ghosts. (For a review, *see* Schwoch & Passow, 1973.) The leaky ghosts seem to serve as a reservoir for the supply of traces of K^+ that keep populations of packed ghosts and concentrated ghost suspensions responsive for some time in the absence of added external KCl. Moreover, the release of traces of K^+ by hemolysis may also help in maintaining responsiveness.

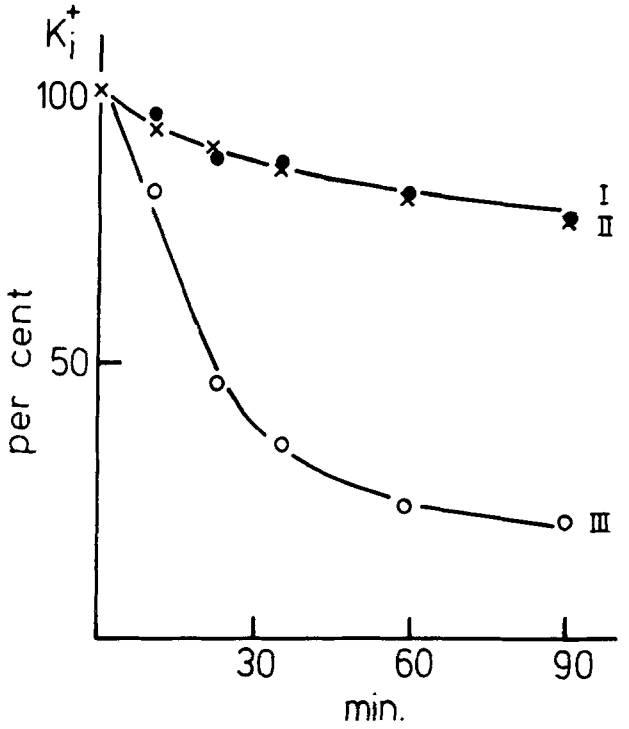

Fig. 6. Mg^{++} maintains responsiveness to the combined action of Ca^{++} and K^+ when present during incubation in K^+-free media. After resealing, washed ghosts were incubated at 37 °C for 30 min in K^+-free medium and then washed again. Subsequently, they were resuspended at 37 °C. The media contained (in mM):

I			II			III			t (min)
K^+	Ca^{++}	Mg^{++}	K^+	Ca^{++}	Mg^{++}	K^+	Ca^{++}	Mg^{++}	
0	0	0	0	0	0.4	0	0	8	−40
2.5	0	0	2.5	0	0.4	2.5	0	8	−10
2.5	10	0	2.5	10	0.4	2.5	10	8	0

$t=0$: concentration as established at zero time in the figure. −40: start of incubation, 40 min before zero time, at the concentrations indicated. −10: 30 min later or 10 min before zero time sufficient potassium was added to increase the K^+ concentration to 2.5 mM

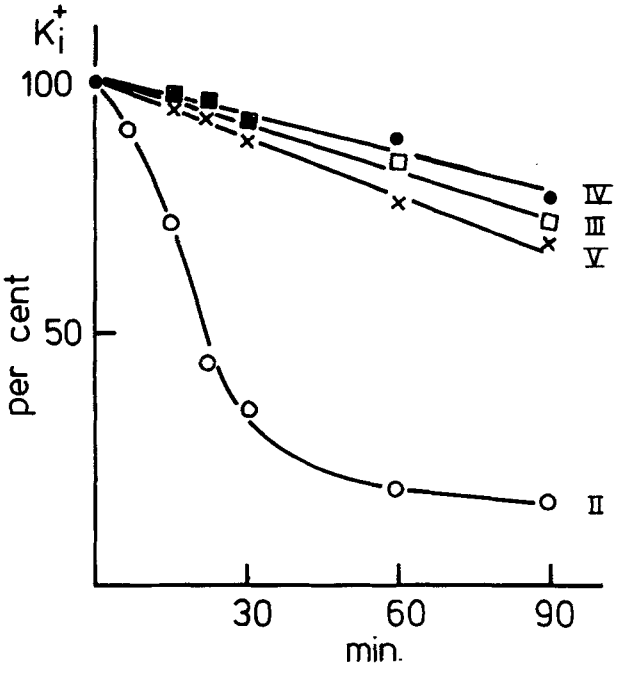

Fig. 7. Extracellular Ca^{++} in K^+-free media reduces the responsiveness to the combined action of subsequently added Ca^{++} and K^+. After resealing, the washed ghosts were incubated at 37 °C for 30 min in K^+-free medium and washed again. The sedimented ghosts were then resuspended at 37 °C. The media contained (in mM):

II		III		IV		V		t (min)
K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	
0	0	0	0.05	0	1.0	0	10	−20
2.5	0	2.5	0.05	2.5	1.0	2.5	10	−10
2.5	10.0	2.5	10.0	2.5	10.0	2.5	10	0

$t=0$: concentrations as established at zero time in the figure. −20: start of incubation in absence of K^+ or the Ca^{++} concentration indicated. −10: 10 min later, or 10 min before zero time, the potassium concentration was increased to 2.5 mM

The inhibition of the response to Ca^{++} plus K^+ by preincubation in the absence of K^+ can be reduced or prevented by agents other than K^+. If the K^+-free incubation that precedes the challenge with Ca^{++} plus K^+ is performed in the presence of 1 mM EDTA or 8.0 mM Mg^{++}, the system maintains its responsiveness to the same extent as during preincubation in media that contain 0.5 mM or more of KCl (Figs. 5 and 6, respectively).

In the previously described experiments K^+ efflux was elicited by the addition of Ca^{++} to red cells that had been in contact with KCl for a certain length of time under the conditions specified above. In the experiment in Fig. 2, we wanted to explore what would happen if Ca^{++} gained access to the transport system in the absence of external KCl. The red cells were incubated in K^+-free media in the presence of 0.5 mM $CaCl_2$ for 10 min. Subsequently, KCl was added to give a final concentration of 2.5 mM. Another 10 min later the system was completed by adjusting the Ca^{++} concentration to 10 mM. Although the ghosts are now exposed to conditions under which K^+ efflux is maximally stimulated when Ca^{++} and K^+ are added simultaneously, or when K^+ is added first and Ca^{++} subsequently, the K^+ channel is totally refractory. This inhibition is much more pronounced than after mere incubation for 10 min in the absence of K^+, without Ca^{++} present. The con-

version of the K^+ channel into a refractory state by external Ca^{++} becomes maximal when the Ca^{++} concentration in the K^+-free medium amounts to 50 μM or more (Fig. 7). If, after 10 min of incubation at this concentration, the Ca^{++} concentration is raised to 10 mM and the KCl concentration to 2.5 mM, i.e. to concentrations that normally produce a maximal increase of K^+ efflux, the rate of efflux is only a little higher than in the control that had not been challenged by the addition of Ca^{++} and K^+. This indicates that binding of Ca^{++} at very low concentrations renders the transport system refractory to subsequently added K^+ while previous binding of K^+ does not prevent the subsequent binding and action of Ca^{++}.

The loss of responsiveness after treatment with Ca^{++} in the absence of K^+ cannot be reversed by washing the treated ghosts with EDTA; the effect is irreversible. An excess of EDTA over Ca^{++} in the K^+-free media prevents the loss of responsiveness, indicating that EDTA competes more effectively for Ca^{++} than the K^+-transport system.

Induction of Net KCl Efflux by the Incorporation of Ca^{++} into the Ghosts and Addition of External KCl

In a series of experiments, Ca^{++} buffers containing either Ca^{++} and citrate or Ca^{++}, $MgCl_2$ and EDTA were incorporated into the ghosts. After resealing, the ghost suspensions were cooled to less than 4 °C, and the ghosts were washed at this low temperature. They were subsequently resuspended at 37 °C. If the medium was K^+-free, there was very little KCl efflux. This observation is interesting for the following reason: the ghosts had been resealed in media containing besides 70 mM choline chloride, 70 mM KCl plus the Ca^{++} buffer, i.e. under conditions under which the K^+-specific pathway is activated (*see* for example the work of Blum & Hoffman, 1971; Knauf et al., 1975; Simons, 1977*a, b*). Nevertheless, during and after removal of extracellular K^+ by washing at low temperature, the ghosts retained the intracellular KCl and remained fairly impermeable to KCl after resuspension at 37 °C in KCl-free media. This indicates that they were successfully resealed and that the previously activated K^+-transport system can be inactivated by simple removal of external KCl. Addition of extracellular KCl now induces, without much time lag, the efflux of KCl (Fig. 8; *cf.* Simons, 1979, Fig. 5). As in our previous work (Knauf et al., 1975), where the KCl efflux was initiated by the addition of extracellular KCl together with extracellular $CaCl_2$, the effect of the extracellular KCl reaches a maximum at 2.5 mM

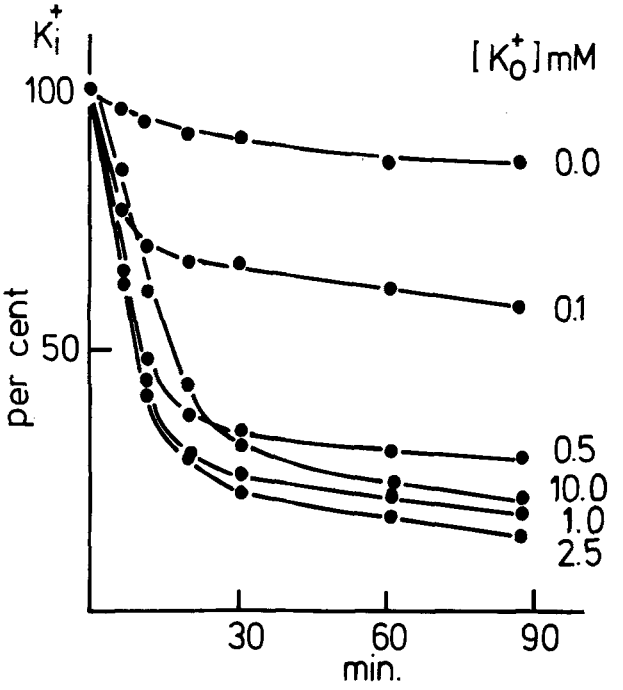

Fig. 8. Induction of net K^+ efflux from Ca^{++} containing (4 μM) resealed ghosts by the addition of varying concentrations of extracellular K^+. Ghosts were resealed in choline media containing Ca/citrate buffer, at a concentration of free Ca^{++} of 4 μM. The sealed ghosts were washed, incubated for 30 min, washed again (all procedures at 0 °C) and subsequently resuspended at 37 °C. The media contained (in mM):

I K^+	II K^+	III K^+	IV K^+	V K^+	VI K^+	*t* (min)
0	0.1	0.5	1.0	2.5	10.0	0

and produces some inhibition above this value. This strongly suggests that the induction of KCl efflux by traces of internal $CaCl_2$ is an essentially similar reaction as the induction of the loss by high concentrations of extracellular $CaCl_2$. The experiments described confirm in a very striking manner that the effect of external KCl does not simply consist of controlling the rate of Ca^{++} influx into the ghosts. They show that the presence of external KCl is as necessary for eliciting the response of the transport system as the presence of internal Ca^{++}. They also confirm again that the external KCl does not simply stimulate a K/K exchange but is a prerequisite for the activation of the transport system and the ensuing net KCl loss.

In the experiments described in this section, the action of intracellular Ca^{++} manifests itself at the same low concentrations that were found to stimulate K/K exchange (*see*, for example, Blum & Hoffman, 1971; Simons, 1976*a, b*). Fig. 9*a* indicates that the system reacts very strikingly to variations of internal

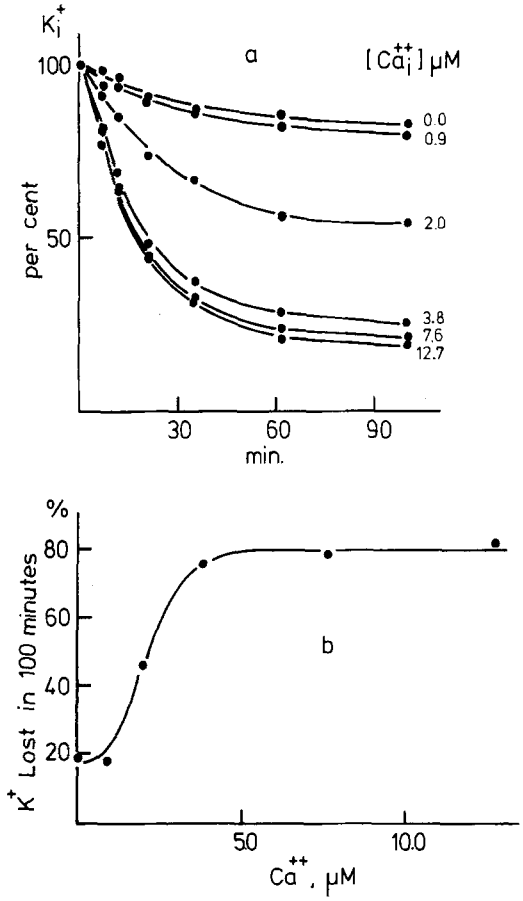

Fig. 9. (*a*): Induction of net K^+ efflux from ghosts that contain varying concentrations of free Ca^{++} by the addition of KCl (2.5 mM) to the external medium. Ghosts were resealed in choline medium containing Ca^{++}/Mg^{++}/EDTA buffers of varying concentrations of free Ca^{++}. The sealed ghosts were washed, incubated for 30 min, washed again (all procedures at 0 °C) and subsequently resuspended at 37 °C. The ghosts contained (in μM):

I	II	III	IV	V	VI	*t*
Ca^{++}	Ca^{++}	Ca^{++}	Ca^{++}	Ca^{++}	Ca^{++}	(min)
0.0	0.9	2.0	3.8	7.6	12.7	0

The potassium concentration in the external media was always 2.5 mM. (*b*): K^+ lost in 100 min as function of $[Ca_i^{++}]$. Data from Fig. 9*a*

Ca^{++} concentration at a maximally activating concentration of external KCl of 2.5 mM. Up to about 1 μM, Ca_i^{++} there is virtually no effect[2]. At about 4 μM Ca_i^{++}, the effect is nearly maximal (Fig. 9). This behavior suggests that cooperative interactions occur

[2] In the work of Simons (1976*a*), with human red cell ghosts, and of Dissing, Lassen and Scharff (1979) with *Amphiuma* red cells, the onset of K^+ loss was observed at Ca^{++} concentrations of more than 0.2 μM, i.e. at lower concentrations than in the present work. The reason for this difference is unknown.

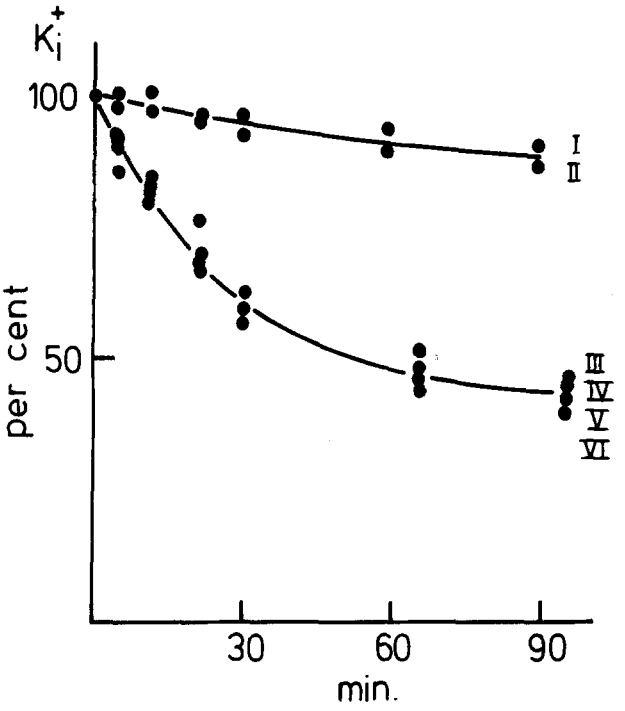

Fig. 10. (*a*): Irreversible loss of responsiveness after 10 min of incubation in K^+-free medium. (I–III). (*b*): Failure of short incubation (30 sec) in the presence of Ca_o^{++} to affect responsiveness (IV–VI). Ghosts were resealed in a choline medium containing Ca^{++}/Mg^{++}/EDTA buffer at a concentration of free Ca^{++} of 4 μM. The sealed ghosts were washed, incubated for 30 min, washed again (all procedures at 0 °C) and subsequently resuspended. The media contained (in mM):

I		II		III		
K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	
0	0	0	0	(−)		
0	0	0	0	2.5	0	
0	0	2.5	0	2.5	0	

IV		V		VI		*t* (min)
K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	
(−)		(−)				−10.0
0	0.1	0	1.0	0	10.0	−0.5
2.5	0.1	2.5	1.0	2.5	10.0	0.0

$t=0$: concentrations as established at zero time in the figure. −10.0: start of incubation 10 min before zero time in the figure at the concentrations indicated. −0.5: 9.5 min later, or 0.5 min before zero time, Ca^{++} was added (IV–VI) to give the final concentrations indicated. (−) Packed ghosts stored at 0 °C

at the Ca^{++}-binding sites at the inner face of the transport system.

A delay of the addition of external KCl to the Ca^{++} buffer-loaded ghosts for 10 min renders the ghosts totally refractory: after this time a challenge

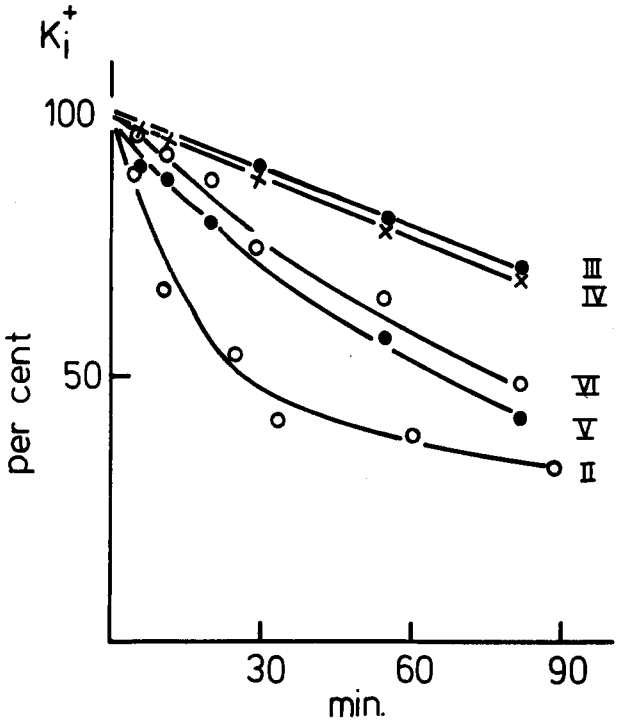

Fig. 11. Partial maintenance by external Ca^{++} of responsiveness during incubation in K^+-free media. In contrast to the experiment represented in Fig. 7, where the responsiveness was induced by Ca_o^{++} plus K_o^+, in this experiment the ghosts contained Ca^{++} buffer and the response was elicited by the addition of K_o^+. Ghosts were resealed in choline medium containing Ca^{++}/Mg^{++}/EDTA buffer with a concentration of free Ca^{++} of 4 μM. The sealed ghosts were washed, incubated for 30 min, washed again (all procedures at 0 °C) and subsequently resuspended at 37 °C. The media contained (in mM):

II		III		IV		V		VI		t (min)
K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	K^+	Ca^{++}	
(−)		0	0	0	0.1	0	1.0	0	10.0	−10
2.5	0	2.5	0	2.5	0.1	2.5	1.0	2.5	10.0	0

$t=0$: concentrations as established at zero time in the figure. −10: start of incubation in absence of K^+ at the Ca^{++} concentrations indicated. (−): packed ghosts stored at 0 °C

by external KCl no longer induces KCl efflux (Fig. 10, Nos. II and III). Thus the Ca^{++} buffer-loaded ghosts are much more sensitive to the absence of KCl than ghosts that are stimulated by a combination of external KCl with external $CaCl_2$.

If the external Ca^{++} is added shortly (30 sec) before the external K^+ to Ca^{++}-buffer-containing ghosts, then no further augmentation of the rate of K efflux beyond that seen in ghosts that are challenged with external K^+ without previous addition of Ca^{++} can be observed (Fig. 10). However, the irreversible loss of responsiveness to a challenge by external Ca^{++} plus K^+ that can be induced in ghosts that do not contain Ca^{++} buffers by exposure to Ca^{++} in K^+-free media (*see* Fig. 7) does not develop in the Ca^{++} buffer-filled ghosts. If anything, the external Ca^{++} prolongs the responsiveness to KCl rather than reducing it (Fig. 11).[3]

So far, all data were presented by plotting the full time course of K^+ release for the various experimental conditions. Except in Fig. 3 we avoided the calculation of rate constants or fluxes. This is explained by the fact that at less than maximally activating concentrations of Ca_i^{++} and K_o^+ the rate of loss changes with time. At these concentrations, there is an initially high rate of loss which tends to level out after 30–60 min and then continues at a very much lower rate. A change of Ca_i^{++} at fixed Ca_o^{++} and of K_o^+ at fixed Ca_i^{++} leads to an increase of the amount of K^+ that is lost during the initial rapid phase of K^+ exit, while the length of time required for the transition from the fast to the slow phase of loss, changes comparatively little (*see*, for example, Figs. 8 and 9).

As has been pointed out previously (Riordan & Passow, 1971, 1974) this time course may reflect the average behavior of all cells of the population and indicate that in each cell the rate of loss changes with time. Alternatively, it could mean that each individual cell responds to a given calcium concentration in an all-or-none fashion either with a high or a low rate of loss where the rate constants for the two processes are time-independent. In this case, increasing the Ca^{++} concentration would increase the number of cells in the population in which the threshold for the all-or-none response is exceeded. Such behavior had previously been demonstrated in lead poisoning of red cells, which produces effects that are essentially similar to those induced by intracellular Ca^{++}. The cooperativity suggested by the small range of Ca^{++} concentrations (1–4 μM) between zero and maximal effect (*see* Fig. 9*a*) could, perhaps, best be accounted for on the assumption of an all-or-none response in individual red cell ghosts with a statistical variation of threshold values rather than by a similar and gradual response of all ghosts in the population.

If one accepts this interpretation, then the total

[3] This result suggests that K^+-free incubation increases the threshold for the effects of Ca^{++} by reducing the affinity of the channel for Ca^{++}. It should be noted, however, that in this experiment the concentration of *external* Ca^{++} was varied. It remains to be established, therefore, whether or not the observations depicted in the figure are indeed simply due to a significant increase of intracellular Ca^{++} within 10 min or if it represents a composite action by combination with an inhibitory site on the outer membrane surface and the K^+ loss promoting site on the inner membrane surface.

amount of K lost during the rapid phase of K^+ efflux should be proportional to the number of ghosts in which the threshold is exceeded. This amount can be approximately equated to that amount that is lost within the first 100 min after exposure to Ca_i^{++} plus K_o^+. Thus, a plot of the percentage loss of K^+ during this length of time would represent a rough measure of the percentage of cells that underwent the permeability change. Fig. 9*b* represents such a plot and provides one way of expressing the results of the experiment by a single curve. If one calculated the efflux from the initial slopes, one would obtain a somewhat similar curve. However, if one agrees that the effect is all-or-none then the initial slope had to be normalized with respect to the numbers of cells that are responsible for the rapid phase of efflux. After such normalization, one finds that the rate of efflux is only slightly dependent on $[Ca_i^{++}]$ (*see also* Riordan & Passow, 1971; for a more detailed discussion).

The comments concerning a graded *vs.* an all-or-none response do not only apply if one considers the measures that elicit the response. They are also pertinent for the interpretation of those experiments in which a pretreatment reduces the response to a challenge. Even though the challenge was always carried out with concentrations of Ca^{++}/K_o^+ or Ca_i^{++}/K_o^+ which produce a maximal response in fully responsive ghosts, the effects in pretreated ghosts are often less then maximal. The time course of loss still remains biphasic, suggesting that perhaps the changes of responsiveness observed reflect changes of threshold values in the individual cells of the population rather than equal graded responses in all cells of the population. An example is represented in Fig. 3*b*. Here, in contrast to Fig. 9*b*, the initial slopes have been used to calculate rate constants. This is based on the assumption that the inhibitory effects or preincubation in K^+-free media affect all cells in the population to approximately the same extent (graded response). It is clear, however, that a shift of threshold values could offer an equally good explanation. Further work will be needed to decide which of the two types of responses prevails under the existing experimental conditions. Up to this time, the representations in Figs. 3*b* and 9*b* may serve as a purely descriptive way of presenting data in a more condensed form, without further implications concerning the underlying kinetics.

Whatever the final explanation will be, it is interesting that this conspicuous kinetic behavior is not only obtained when the Ca^{++} concentration inside the ghosts is varied at a fixed concentration of K^+

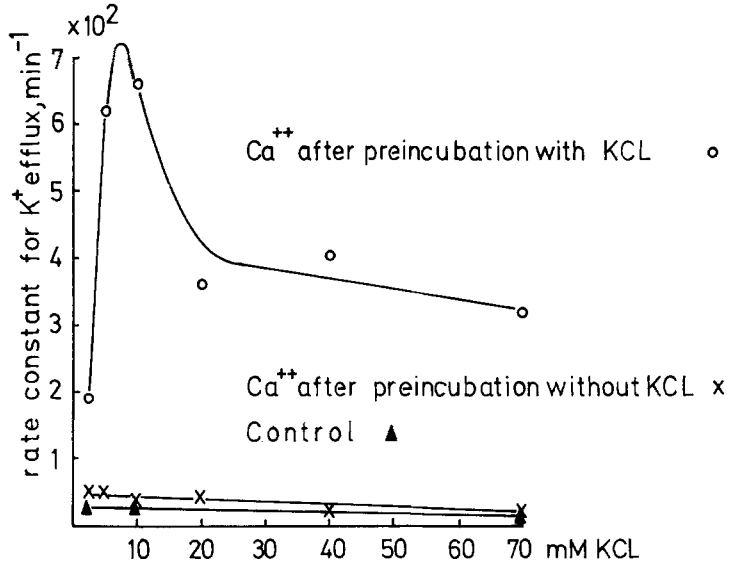

Fig. 12. Effect of K^+-free preincubation on K^+/K^+ equilibrium exchange as measured by means of ^{42}K. Ghosts were resealed in media that contained 20 mM TRIS-Cl, pH 7.6 and choline-Cl+KCl at a fixed concentration of 140 mM. The ratio choline-Cl/KCl was varied to obtain the KCl concentrations indicated on the abscissa. After resealing, each batch of ghosts was subdivided into two. One was washed twice and incubated in the absence of KCl, the other in the presence of 1 mM KCl in media containing 140 mM choline-Cl and 20 mM TRIS-Cl, pH 7.6. Hematocrit 0.25%, temperature 37 °C. After 30 min, the ghosts were washed once again and resuspended in fresh medium of the same composition as before. Incubation was then continued for another 60 min at the hematocrit and temperature indicated above. After another wash, each batch of ghosts was resuspended in a medium that contained KCl at the same concentration as the cell interior, whereby isotonicity was maintained by the addition of choline-Cl. The media contained 4 μM $CaCl_2$ plus 2 μM of the ionophore A 23187. ^{42}K efflux was measured at 37 °C, 0.25% hematocrit, and the rate constants indicated on the ordinate were calculated. Corresponding samples were prepared that received neither ionophore nor Ca^{++} (control)

outside, but also when K^+ outside is increased at constant Ca_i. This raises the question: what elicits the response in intact cells? The intracellular Ca^{++} concentration or the concentration of extracellular K^+? In the light of the present experiments, one would need to conclude that both K_o^+ and Ca_i^{++} are equally important. An action of extracellular K^+ solely as a regulator of Ca^{++} entry, can be definitely excluded.

The Effect of Pre-incubation in K^+-Free Media on Ca^{++}-Stimulated K^+ Equilibrium Exchange

The data presented in the preceding sections were interpreted on the assumption that the observed effects on net KCl efflux reflect modifications of the Ca^{++}-stimulated K^+-selective channel. However, the rate of net KCl efflux depends on both the permeability for K^+ and Cl^-. Hence, in theory at least, there exists the possibility that inhibition of net KCl efflux by K^+-free preincubation is due to an inhibition of the diffusive permeability for Cl^- (P_{Cl}) rather than a reduction of the rate of K^+ efflux. Contributions of changes of P_{Cl} on changes of K^+ permeability can be eliminated when one measures the effects of K^+-free preincubation on K^+/K^+ equilibrium exchange. In these experiments, after incubation in the absence of external K^+, the ghosts were resuspended in media that had the same K^+ concentration as the ghost interior. The permeability change was elicited by the addition of 4 μM Ca^{++} in the presence of 2 μM of the Ca^{++} ionophore A 23187, and the K^+ efflux was measured by means of $^{42}K^+$. Under these conditions, Ca^{++} entry into the ghosts should be independent of K_o^+ and no net KCl movements take place. There exists no coupling of K^+ and Cl^- movements by a common electrical field. Since the measurements of ^{42}K exchange were performed under steady-state conditions at a maximally stimulating Ca^{++} concentration, the time course of release of $^{42}K^+$ from the ghosts follows the kinetics of a two-compartment system. Rate constants could be calculated, therefore, by fitting the data to a single exponential. The results of such an experiment are represented in Fig. 12. They show that the preincubation in the absence of external K^+ for 90 min largely abolishes the capacity of Ca^{++} to stimulate K^+ efflux. This indicates that the effects described in the previous sections reflect indeed the behavior of the K^+-selective channel.

Conclusions

The results of the work described above can be summarized by the tentative scheme in Fig. 13.

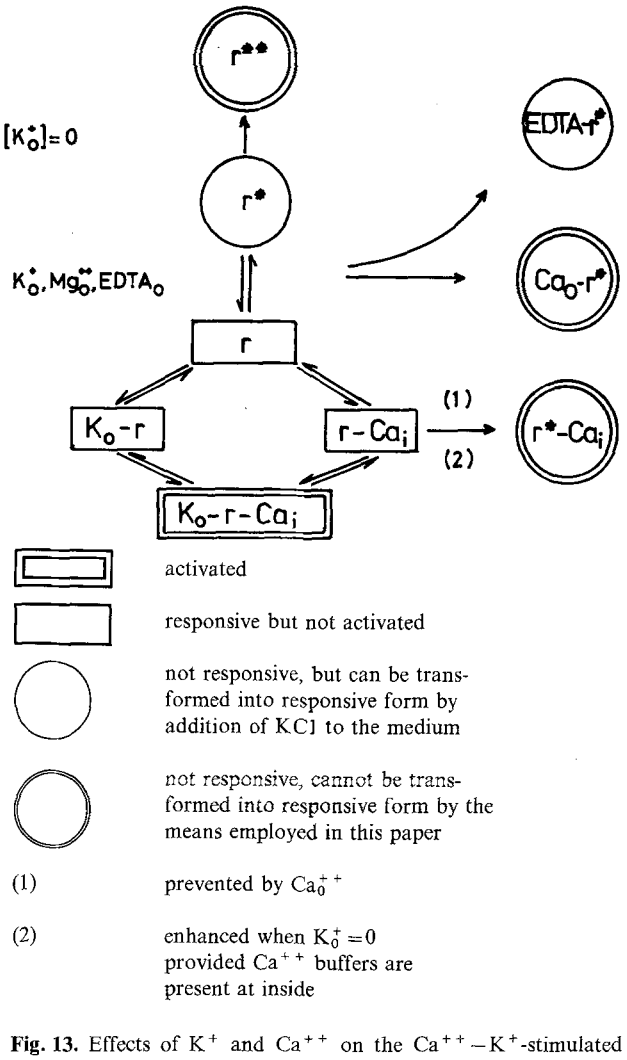

Fig. 13. Effects of K^+ and Ca^{++} on the $Ca^{++}-K^+$-stimulated potassium channel

In the absence of extracellular K^+ and intracellular Ca^{++}, the K^+ channel is responsive but inactivated and thus does not permit net movements of K^+ down the electrochemical potential gradient. This situation is denoted by *r* in a rectangular frame. If only intracellular Ca^{++} or extracellular K^+ are present (K_o-r and $r-Ca_i$, in a single frame), the channel is also responsive but not activated. No enhancement of K^+ efflux is observed. Only when K^+ is present at the outer surface and Ca^{++} at the inner surface is the channel transformed into the activated state where rapid K^+ net movements take place (optimal concentrations 2.5 mM and 4 μM, respectively, at pH 7.6). At very high concentrations of K^+ some inhibition is observed. This suggests additional binding of K^+ to some site on the outward facing portion of the channel (not represented in the figure). In the complete absence of external K^+ the channel undergoes a transition from the responsive but inactive

form r into a nonresponsive form r^*[4]. This transition takes place regardless of whether Ca_i^{++} is present or not. Addition of high concentrations of K^+ to the medium may partially or totally reverse the transition $r^* \rightarrow r$, depending on the time of incubation in the absence of K^+ and on the concentration of subsequently added KCl.

Prolonged incubation in the complete absence of K^+ renders the loss of responsiveness irreversible. The nonresponsive form r^* is transformed into the irreversibly inactivated form r^{**}. Extracellular Ca^{++} enhances this process (provided the ghosts do not contain Ca^{++} buffer), while extracellular K^+, Mg^{++} and EDTA are capable of reducing or preventing it. It is not clear whether or not the conformations of the channels r^{**}, $Ca_o - r^*$ and r^*-EDTA within this scheme are identical. It is also not resolved whether the agents inhibit the irreversible transformation $r^* \rightarrow r^{**}$, or shift an equilibrium $r^* \rightleftharpoons r$ towards the responsive form r.

It is remarkable that under all experimental conditions, for the transitions of the responsive channel r into the nonresponsive forms r^* and r^{**}, the absence of KCl in the external medium is essential. Less than millimolar concentrations of external KCl suffice to prevent those conformational changes that lead to spontaneous irreversible inactivation or render the channel susceptible to the rapidly inactivating effect of Ca_o^{++}. However, it is clear that K_o^+ is not only necessary for the maintenance of the responsiveness of the system. K_o^+ is also required to transform the responsive channel into the activated conformation, although in a concentration range that exceeds that needed for the maintenance of the responsiveness. Finally, at very high concentrations, extracellular K^+ slightly inhibits the K^+ efflux through the activated channel (Knauf et al., 1975; *see also* Figs. 8 and 12). The present work shows that this cannot be related to a reduction of the rate of Ca^{++} entry into the ghosts, as previously proposed by Lew (1974). Thus, there seem to exist at least three K^+-binding sites at the outward facing side of the K^+ channel. The site with the highest affinity maintains the channel in a conformation in which the binding of additional K^+ to another site and the binding of intracellular Ca^{++} become possible. The third site with the lowest affinity either closes the activated channel or interferes with the binding of the activating K^+ (negative cooperativity) or the binding of Ca_i^{++}.

In conclusion of the summary of the effects of K^+ it may be recalled that intracellular Na^+ counteracts the combined effects of K_o^+ and Ca_i^{++}. It is unknown whether this is due to a replacement of Ca_i^{++} or to an allosteric effect that is transmitted across the membrane, leading to a reduction of the affinity of the channel for external K^+, or some other reaction.

[4] The terms channel or pathway are used interchangeably to denote the specific route by which K^+ penetrates across the modified membrane. They bear no implications regarding specific molecular properties.

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References

Blum, R.M., Hoffman, J.F. 1971. The membrane locus of Ca-stimulated K transport in energy depleted human red blood cells. *J. Membrane Biol.* **6**:315

Blum, R.M., Hoffman, J.F. 1972. Ca-induced K transport in human red cells: Localisation of the Ca-sensitive site to the inside of the membrane. *Biochem. Biophys. Res. Commun.* **46**:1146

Bodemann, H., Passow, H. 1972. Factors controlling the resealing of the membrane of human erythrocyte ghosts after hypotonic hemolysis. *J. Membrane Biol.* **8**:1

Dissing, S., Lassen, U.V., Scharff, O. 1979. Magnesium inhibition of hyperpolarization of *Amphiuma* red cell membrane induced by calcium and A 23187. *J. Physiol. (London)* **289**:29P

Ferreira, H.G., Lew, V.L. 1976. Use of ionophore A 23187 to measure cytoplasmic Ca buffering and activation of the Ca pump by internal Ca. *Nature (London)* **259**:47

Gárdos, G. 1958a. The function of calcium in the potassium permeability of human erythrocytes. *Biochim. Biophys. Acta* **30**:653

Gárdos, G. 1958*b*. Effect of ethylenediamine-tetra-acetate on the permeability of human erythrocytes. *Acta Physiol. Acad. Sci. Hung.* **14**:1

Knauf, P.A., Riordan, J.R., Schuhmann, B., Wood-Guth, I., Passow, H. 1975. Calcium-potassium-stimulated net potassium efflux from human erythrocyte ghosts. *J. Membrane Biol.* **25**:1

Kregenow, F.M. 1962. Metabolic control of passive transport and exchange diffusion of Na and K in human red cells. M.D. Thesis. Yale University, New Haven

Kregenow, F.M., Hoffman, J.F. 1962. Metabolic control of passive transport and exchange diffusion of Na and K in human red cells. (*Abstr.*) Biophysical Society, Washington, D.C.

Kregenow, F.M., Hoffmann, J.F. 1972. Some kinetics and metabolic characteristics of calcium-induced potassium transport in human red cells. *J. Gen. Physiol.* **60**:406

Lassen, U.V., Pape, L., Vestergaard-Bogind, B. 1976. Effect of calcium on the membrane potential of *Amphiuma* red cells. *J. Membrane Biol.* **26**:51

Lassen, U.V., Lew, V.L., Pape, L., Simonsen, L.O. 1978. Transient activation of the Ca^{++}-sensitive K^+-channel in red cells by external K^+. *In:* Abstracts of Communications presented at the Meeting of the European Red Cell Club, Sandbjerg. (*Abstr.*)

Lepke, S., Passow, H. 1968. Effects of fluoride on potassium and sodium permeability of the erythrocyte membrane. *J. Gen. Physiol.* **51**:365

Lew, V.L. 1971*a*. On the ATP dependence of the Ca^{2+}-induced increase in K^+ permeability observed in human red cells. *Biochim. Biophys. Acta* **233**:827

Lew, V.L. 1971*b*. Effect of ouabain on the Ca^{2+}-dependent increase in K^+ permeability in depleted guinea-pig red cells. *Biochim. Biophys. Acta* **249**:236

Lew, V.L. 1974. On the mechanism of the Ca-induced increase in K permeability observed in human red cell membranes. *In:* Comparative Biochemistry and Physiology of Transport. L. Bolis, S.E. Luria and R. Lynen, editors. pp. 310–316. North-Holland Publishing Company, Amsterdam

Lew, V.L., Ferreira, H.G. 1976. Variable Ca sensitivity of a K-selective channel in intact red-cell membranes. *Nature (London)* **263**:336

Lew, V.L., Ferreira, H.G. 1978. Calcium transport and the properties of a calcium-activated potassium channel in red cell membranes. *In:* Current Topics in Membranes and Transport, Vol. 10. F. Bronner and A. Kleinzeller, editors. pp. 217–277. Academic Press, New York – San Francisco – London

Lew, V.L., Beaugé, L. 1979. Passive cation fluxes in red cell membranes. *In:* Membrane Transport in Biology. G. Giebisch, D.C. Tosteson, and H.H. Ussing, editors. Vol. II. pp. 81–115. Springer Verlag, Berlin-Heidelberg-New York

Manninen, V. 1970. Movements of sodium and potassium ions and their tracers in propanol-treated red cells and diaphragm muscle. *Acta Physiol. Scand. Suppl.* **355**:1

Øskov, S.L. 1935. Untersuchungen über den Einfluß von Kohlensäure und Blei auf die Permeabilittät der Blutkörperchen für Kalium und Rubidium. *Biochem. Z.* **279**:250

Passow, H. 1961. Zusammenwirken von Membranstruktur und Zellstoffwechsel bei der Regulierung der Ionenpermeabilität roter Blutkörperchen. *In:* Biochemie des aktiven Transports. 12. Colloquium der Gesellschaft für physiologische Chemie in Mosbach, Baden. pp. 54–99. Springer, Berlin – Göttingen – Heidelberg

Passow, H. 1963. Metabolic control of the passive permeability for potassium ions. *In:* Cell Interface Reactions. A.D. McLaren, P. Mitchell and H. Passow, editors. pp. 57–107. Scholar's Library, New York

Passow, H. 1964. Ion and water permeability of the red blood cell. *In:* The Red Blood Cell. C. Bishop and D.M. Surgenor, editors. pp. 71–145. Academic Press, New York

Passow, H. 1969. Ion permeability of erythrocyte ghosts. *In:* Laboratory Techniques in Membrane Biophysics. H. Passow and R. Stämpfli, editors. pp. 22–27. Springer-Verlag, Berlin – Heidelberg – New York

Perrin, O.D., Dempsey, B. 1974. Metall-ion buffers. *In:* Buffers for pH and Metall Ion Control. D.D. Perrin and B. Dempsey, editors. pp. 94–102. Chapman and Hall, London

Porzig, H. 1975. Comparative study of the effects of propanolol and tetracaine on cation movements in resealed human red cell ghosts. *J. Physiol. (London)* **249**:27

Riordan, J.R., Passow, H. 1971. Effects of calcium and lead on potassium permeability of human erythrocyte ghosts. *Biochim. Biophys. Acta* **249**:601

Riordan, J.R., Passow, H. 1974. The effects of calcium and lead on the potassium permeability of human erythrocytes and erythrocyte ghosts. *In:* Comparative Physiology. L. Bolis, K. Schmidt-Nielsen and S.H.P. Madrell, editors. pp. 543–581. North-Holland, Amsterdam – London

Romero, P.J., Whittam, R.R. 1171. The control by internal calcium of membrane permeability to sodium and potassium. *J. Physiol. (London)* **214**:481

Schwoch, G., Passow, H. 1973. Preparation and properties of human erythrocyte ghosts. *Mol. Cell. Biochem.* **2**:197

Simons, T.J.B. 1976*a*. The preparation of human red cell ghosts containing calcium buffers. *J. Physiol. (London)* **256**:209

Simons, T.J.B. 1976*b*. Calcium-dependent potassium exchange in human red cell ghosts. *J. Physiol. (London)* **256**:227

Simons, T.J.B. 1979. Actions of a carbocyanine dye on calcium-dependent potassium transport in human red cell ghosts. *J. Physiol. (London)* **288**:481

Whittam, R. 1968. Control of membrane permeability to potassium in red blood cells. *Nature (London)* **219**:610

Wilbrandt, W. 1937. A relationship between the permeability of the red cell and its metabolism. *Trans. Faraday Soc.* **33**:956

Wilbrandt, W. 1940. Die Abhängigkeit der Ionenpermeabilität der Erythrocyten vom glykolytischen Stoffwechsel. *Pfluegers Arch.* **243**:519

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