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# CALCIUM-DEPENDENT POTASSIUM EXCHANGE IN HUMAN RED CELL GHOSTS

BY T. J. B. SIMONS*

*From the Physiological Laboratory, University of Cambridge, Cambridge CB2 3EG*

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SUMMARY

1. The properties of the Ca-dependent K transport system of human red cell ghosts have been examined under equilibrium exchange conditions.

2. K transport is stimulated half-maximally by about 0·4 $\mu$M-$Ca_i^{2+}$ or 5 $\mu$M-$Sr_i^{2+}$, but much higher concentrations of $Ba_i^{2+}$ give only slight stimulation. Mg is a weak antagonist to Ca.

3. The free $Ca^{2+}$ concentration in human red cells is estimated to be below 0·25 $\mu$M.

4. The curve relating the rate of K transport to the intracellular $Ca^{2+}$ concentration is complicated and suggests that internal Ca acts at three or more sites.

5. K, Rb and possibly Cs ions are transported by the Ca-dependent system. Under comparable conditions the relative rates are 1(K):1·5(Rb): < 0·05(Cs).

6. No Ca-dependent transport of Na, Li or choline could be detected. If Na is transported, it must be at less than 1/40 the rate of K.

7. The rate of K transport is almost linearly related to the K concentration in the 0–200 mM range, but the curve is sigmoid close to the origin.

8. Intracellular, but not extracellular Na inhibits K transport, in a way that suggests competition with K at more than one site.

9. These results suggest that the transport system has a complex mechanism.

## INTRODUCTION

Rises in intracellular Ca increase the K permeability of a variety of cells. In some cases this effect may play a physiological role, for example in nerve cells (Krnjević & Lisiewicz, 1972; Meech, 1974) and in cardiac Purkinje fibres (Isenberg, 1975). A Ca-sensitive mechanism exists in human red cells, but

* Present address: Department of Physiology, University of London, King's College, Strand, London WC2R 2LS.

it cannot be observed under normal physiological conditions. It is seen after metabolic depletion in a Ca-containing medium, which results in a large loss of K (Gárdos, 1956; Romero & Whittam, 1971; Lew, 1971). The Ca-dependent K permeability has no obvious function in red cells (although it might be involved in the destruction of aged cells). Nevertheless, it may represent a permeability mechanism of general physiological importance, which can be studied in isolation in the red cell.

The aim of the experiments in this paper was to investigate the properties of the Ca-dependent permeability mechanism in resealed ghosts, prepared from human red cells. Intracellular Ca was controlled with Ca buffers (the ghosts were free of ATP), and K efflux was measured with all permeant ions at equilibrium, with the same concentrations on either side of the membrane. Under these conditions there should be little or no membrane potential or volume changes. The experiments were of two kinds: in one, the K concentration was held constant, while internal divalent cations were varied, and in the other, internal Ca was held at optimal levels while monovalent cations were varied. The results are interpreted on the basis of two separate pathways for cation transport, under the experimental conditions. The first is Ca-dependent, with specific characteristics, and the second is a small non-specific leak, with properties similar to ionic diffusion in solution. The evidence for this separation is presented in the previous paper (Simons, 1976). A brief summary of some of the present results has already been published (Simons, 1975).

## METHODS

These were the same as in the preceding paper (Simons, 1976), with the following additions.

*Divalent cations.* Mg was included in the haemolysing solution, when required. Sr and Ba buffers were incorporated into ghosts in the same way as Ca buffers, except that HEPES (*N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) replaced phosphate as the hydrogen ion buffer, both in the haemolysing solution and the washing medium. EDTA (ethylenediamine-*NNN'N'*-tetra-acetic acid) and HEDTA (*N'*-(2-hydroxyethyl)ethylenediamine-*NNN'*-triacetic acid) were used to buffer Sr and Ba. For EDTA, the apparent stability constants at 37° C, pH 7·1 and ionic strength 0·1 M, were calculated to be $10^{5\cdot68}$ $M^{-1}$ (for Sr) and $10^{4\cdot60}$ $M^{-1}$ (for Ba), using the results of Bohigian & Martell (1960) (quoted by Sillén & Martell, 1964). The nearest values obtainable for HEDTA were at 20° C, rather than 37° C. They were $10^{4\cdot29}$ $M^{-1}$ (for Sr) and $10^{2\cdot91}$ $M^{-1}$ (for Ba), calculated from the data of Kroll (1959), as quoted by Sillén & Martell (1964).

*Monovalent cation substitutions* were carried out by partial or complete replacement of internal K by other cations. Normally the cations in the external medium were altered in parallel to prevent the setting up of concentration gradients which might affect the membrane potential. Cation concentrations were measured by flame photometry: an EEL instrument was used for K, and a Zeiss PMQ II spectrophotometer with flame attachment for Li, Na, Rb and Cs.

*Tracer fluxes* were measured by the method described previously for K efflux. The standard external medium contained 98 mM-KCl, 2 mM phosphate and 0·4 mM-EGTA (1,2-di(2-aminoethoxy)ethane-*NNN'N'*-tetra-acetic acid) and its pH was adjusted to 7·1 at 37° C with a mixture of KOH and Tris base. $^{86}$Rb and $^{134}$Cs were counted by $\gamma$-spectrometry, and [$^{14}$C]choline by liquid scintillation, using B.D.H. dioxan scintillator, and allowing $^{42}$K to decay first.

## RESULTS

### *Effect of Ca*

Experiments in the previous paper (Simons, 1976) showed there was a complicated relationship between the rate of K exchange and the intracellular $Ca^{2+}$ concentration. The results are included in Fig. 1 of this paper. As little as 0·1 $\mu$M-$Ca^{2+}$ suffices to raise the K flux above its basal level, and the curve rises steeply up to 1 $\mu$M-$Ca^{2+}$, then less steeply to a peak at around 5 $\mu$M-$Ca^{2+}$. Further increases in Ca depress K movements, at first rapidly, but then more slowly. These effects are foreshortened by the logarithmic scale of Fig. 1. They can be seen more clearly with a linear Ca scale: the upper end of the range is covered in Fig. 2, and the lower end by the upper curve in Fig. 3, which also brings out the highly sigmoid shape of the foot of the Ca-activation curve.

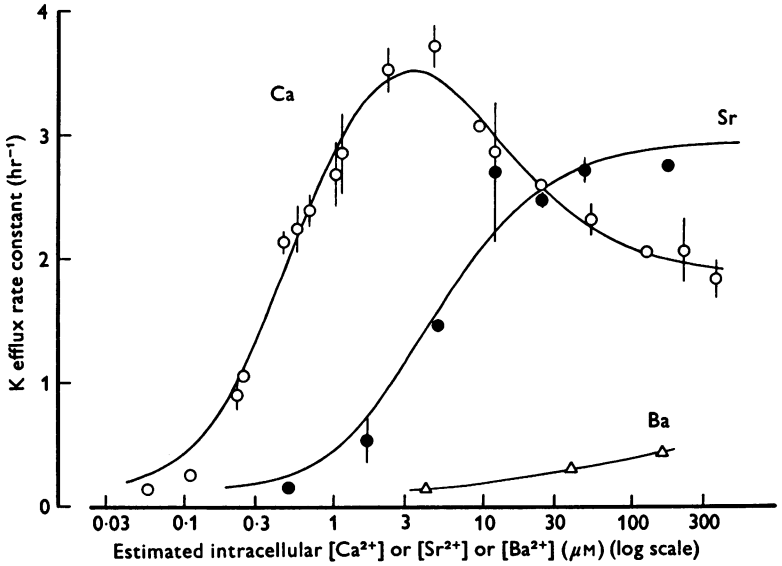


Fig. 1. Comparative effects of intracellular $Ca^{2+}$ (○), $Sr^{2+}$ (●) and $Ba^{2+}$ (△) on K efflux from 100 mM-K ghosts in a 100 mM-K medium. For Ca the points are from Text-fig. 3 of Simons (1976), and for Sr and Ba they are the average of the results in Table 2 of this paper. Vertical lines indicate ±1 S.E. of mean (for Ca) and range (for Sr). The Ca and Sr curves are drawn to fit polynomial expressions, explained in the text, and the Ba curve is drawn by eye.

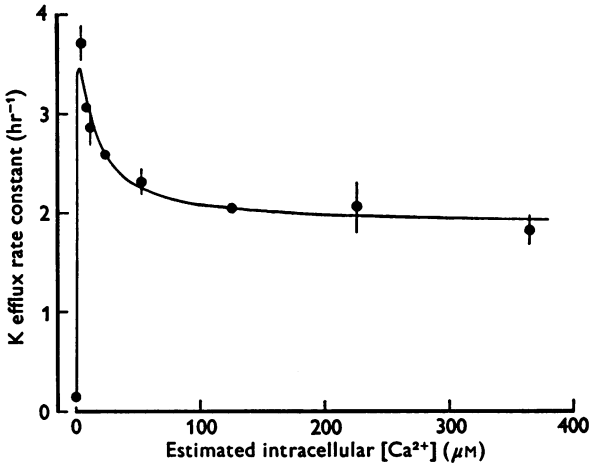


Fig. 2. Relation between K efflux and $Ca^{2+}$ concentration, on a linear scale (upper range). The results in Text-fig. 3 of Simons (1976) are here replotted, with a linear Ca scale. All points between 0 and 4 $\mu$M-$Ca^{2+}$ are omitted, the vertical lines indicate $\pm 1$ S.E. of mean, and the curve is explained in the text.

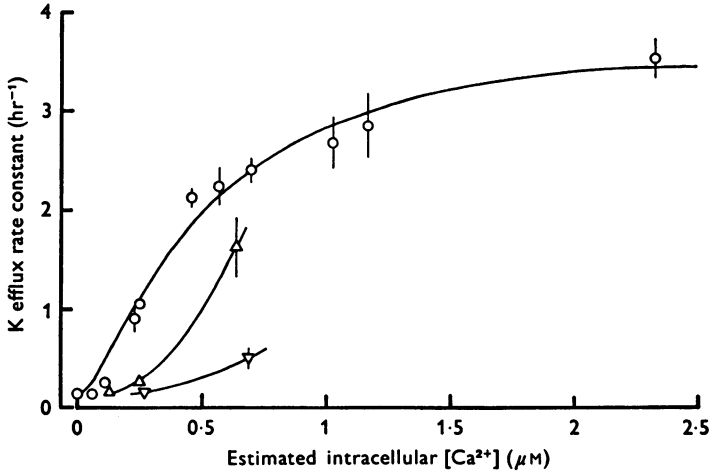


Fig. 3. Effect of Mg on the foot of the Ca activation curve. The ghosts were prepared with no (○), 1 mM (△) or 2 mM (▽) added Mg, and the estimated intracellular $Mg^{2+}$ concentrations are 0·1 mM (○), 0·9–1·0 mM (△) and 1·7–1·8 mM (▽). The 0-Mg points are replotted from Fig. 1, and the others are taken from Table 1. The K concentration was 100 mM in the ghosts and in the external medium, and the vertical lines indicate $\pm 1$ S.E. of mean. The 0-Mg curve is taken from Fig. 1, and the others are drawn by eye.

In Figs. 1–3, the curve drawn to the Ca results obeys the equation

$$\text{rate constant} = 0{\cdot}136\ \text{hr}^{-1} + \frac{a[\text{Ca}]^2}{(b+[\text{Ca}])^2} \times \frac{c+d[\text{Ca}]}{c+[\text{Ca}]}.$$

The parameters, $a = 5{\cdot}05\ \text{hr}^{-1}$, $b = 0{\cdot}316\ \mu\text{M}$, $c = 7{\cdot}67\ \mu\text{M}$ and $d = 0{\cdot}342$, were obtained by a least-squares method. It corresponds to a model in which two Ca ions have to occupy equivalent high-affinity sites before K transport can occur, while a single Ca ion acts at a third site to reduce the maximum rate of K transport. (The $0{\cdot}136\ \text{hr}^{-1}$ is to allow for transport by the Ca-insensitive route.)

The Sr results in Fig. 1 could obviously be fitted by a simpler expression. The one chosen was

$$\text{rate constant} = 0{\cdot}136\ \text{hr}^{-1} + \frac{a[\text{Sr}]^2}{(b+[\text{Sr}])^2},$$

with $a = 2{\cdot}83\ \text{hr}^{-1}$ and $b = 2{\cdot}02\ \mu\text{M}$, and assumes that two Sr ions have to occupy sites of equal affinity before K transport can occur. The corresponding one-site model gives a substantially worse fit.

### *Effect of Mg*

Red cells contain about 2 mM-Mg, and part of this is free. They have a low K permeability, so internal Mg is unlikely to act in the same way as Ca. However, it might be an antagonist to Ca. This hypothesis was tested by incorporating Mg into ghosts, together with Ca and EGTA. EGTA binds Mg only weakly, so it can buffer $Ca^{2+}$ at a low concentration, in the presence of a much higher concentration of $Mg^{2+}$. The experiments are described in Table 1, and in Fig. 3 the results are compared with those obtained without added Mg. They show that Mg progressively depresses the Ca-stimulated K efflux towards the basal (Ca-insensitive) level. About 0·9 mM-$Mg^{2+}$ is required to counteract the effect of 0·3 $\mu$M-$Ca^{2+}$, which suggests that the sites regulating K permeability have a much higher

TABLE 1. The effect of intracellular Mg on Ca-dependent K exchange

| Concn. in haemolysing solution (mM) | | | Estimated concn. in ghosts | | |
|---|---|---|---|---|---|
| Ca | EGTA | Mg | $Ca^{2+}$ ($\mu$M) | $Mg^{2+}$ (mM) | $^{42}$K efflux rate constant ($\text{hr}^{-1}$) |
| 1·5 | 3 | 1 | 0·13 | 0·9 | 0·145, 0·174 |
| 2 | 3 | 1 | 0·25 | 0·9 | 0·245, 0·288 |
| 2·5 | 3 | 1 | 0·64 | 1·0 | 1·32, 1·93 |
| 0 | 3 | 2 | — | 1·5 | 0·141 |
| 1 | 3 | 2 | 0·07 | 1·6 | 0·125*(a) |
| 2 | 3 | 2 | 0·27 | 1·7 | 0·132*(b), 0·164 |
| 2·5 | 3 | 2 | 0·69 | 1·8 | 0·403*(c), 0·602 |

* $^{22}$Na efflux rate constants, measured simultaneously, were $0{\cdot}128\ \text{hr}^{-1}$ (*a*), $0{\cdot}129\ \text{hr}^{-1}$ (*b*) and $0{\cdot}099\ \text{hr}^{-1}$ (*c*).

The estimates of intracellular $Mg^{2+}$ allow for Mg contributed by the red cells, and for binding by EGTA and phosphate (present at 2 mM in the ghosts). Internal and external K were 100 mM, internal Na 1 mM.

affinity for $Ca^{2+}$ than $Mg^{2+}$. Recently, Porzig (1975) has published some results in qualitative agreement with these.

*Effects of Sr and Ba*

Like Ca, these ions form buffer solutions when mixed with chelating anions. Table 2 gives details of several experiments to measure cation

TABLE 2. Na and K efflux from resealed ghosts containing Sr and Ba buffers

| Haemolysing solutions contents | | Estimated intracellular concn. ($\mu$M) | Efflux rate constants ($hr^{-1}$) | |
|---|---|---|---|---|
| Anion (concn. = 3 mM) | Sr (mM) | $Sr^{2+}$ | $^{42}K$ | $^{22}Na$ |
| EDTA | 0·5 | 0·5 | 0·116, 0·182 | 0·181, 0·115 |
| EDTA | 1·2 | 1·7 | 0·349, 0·706 | — |
| EDTA | 2 | 5·0 | 1·42, 1·50 | 0·168, 0·119 |
| EDTA | 2·5 | 12 | 2·12, 3·25 | — |
| HEDTA | 1 | 25 | 2·42, 2·53 | 0·089 |
| HEDTA | 1·5 | 48 | 2·61, 2·81 | 0·084, 0·093 |
| HEDTA | 2·5 | 180 | 2·71, 2·79 | 0·068 |
| | Ba (mM) | $Ba^{2+}$ | | |
| EDTA | 0·5 | 4·1 | 0·125, 0·164 | 0·110, 0·161 |
| EDTA | 2 | 39 | 0·281, 0·327 | 0·104, 0·172 |
| HEDTA | 0·5 | 160 | 0·427 | 0·088 |

The fluxes were measured at 37° C and pH 7·1, with about 100 mM-K inside the ghosts and in the suspending medium, and about 1 mM-Na in the ghosts.

fluxes in resealed ghosts containing these buffer solutions. It shows that Sr, and to a lesser extent Ba, can stimulate K movements. Parallel measurements of Na efflux show that neither Sr nor Ba has an undue deleterious effect on the ghosts. The average Na efflux rate constant ($\pm$ S.E. of mean) is $0{\cdot}115 \pm 0{\cdot}014\ hr^{-1}$ for ghosts containing Sr, and $0{\cdot}127 \pm 0{\cdot}017\ hr^{-1}$ for those with Ba. These are not significantly different from $0{\cdot}119 \pm 0{\cdot}007\ hr^{-1}$, observed previously with Ca-containing ghosts (Simons, 1976).

The stimulatory effects of Sr and Ba on K transport are compared with that of Ca in Fig. 1. Increasing concentrations of $Sr^{2+}$ seem to produce a different response from $Ca^{2+}$: they increase the rate of K efflux at low concentrations, but do not decrease it again, within the concentration range studied. The maximum velocity with $Sr^{2+}$ is slightly less than with $Ca^{2+}$, and the affinity is lower: the rising part of the activation curve is shifted to the right by about one log unit. $Ba^{2+}$ only activates K efflux slightly in the concentration range studied, which suggests that the transport system has a much lower affinity for it than for $Ca^{2+}$ or $Sr^{2+}$.

*Transport of monovalent cations other than K*

Ions other than K might interact with the Ca-dependent transport system in several ways: they might be transported, and they might affect K transport, either directly (by competition with K, for example), or indirectly (by competition with Ca). An ion will be considered to be transported by the system if its movements are accelerated by intracellular Ca in the absence of appreciable concentration gradients. The evidence so far is that K is transported, and Na is not (for Na, see Table 3 of the previous paper (Simons, 1976)).

The first ion to be investigated was choline. Ghosts were made with a variety of compositions: 100 mM-K, 50 mM-K and 50 mM choline, or 100 mM choline, both with and without Ca. They were suspended in Ca-free solutions of similar K and choline contents, so there would be no K concentration gradients. K and choline effluxes were measured with tracers, and Fig. 4 gives the result. It shows the usual large stimulation of

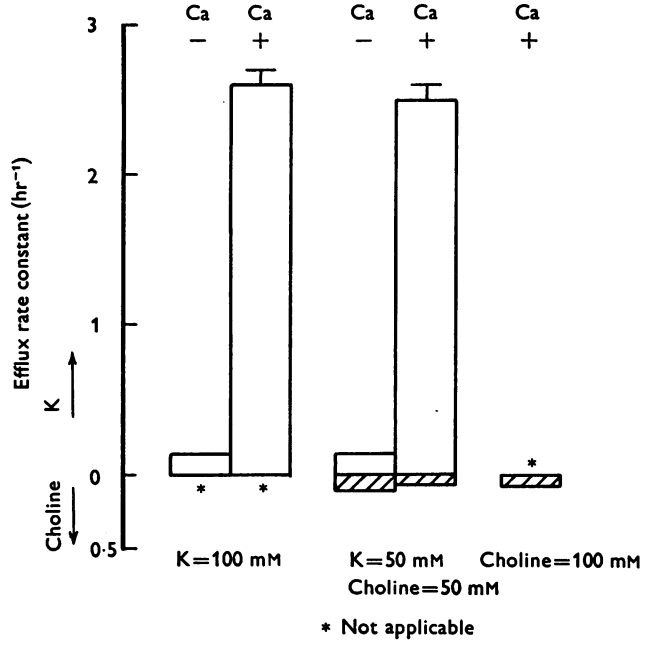


Fig. 4. Effects of Ca on choline and K transport. The Figure gives the efflux rate constants, +1 S.D., and the cation compositions of the ghosts and the medium. Ghosts without Ca (Ca −) had 3 mM-EGTA and 2 mM-Mg incorporated at haemolysis; those with (Ca +) had 3 mM-EGTA, 3 mM-Ca and 2 mM-Mg. (This is not a buffered mixture: the $Ca^{2+}$ concentration was probably at least 20 $\mu$M.)

K efflux by Ca, but the rate constant for choline efflux was always lower than the K efflux rate constant in the absence of Ca, and was, if anything, slightly diminished by Ca. The conclusion is that choline ions are not transported by the Ca-dependent route.

The next experiment was designed to test the effects of a variety of cations. Ghosts were prepared containing optimal levels of $Ca^{2+}$ (i.e. about 5 $\mu$M), 9 mM-K and 84 mM of another monovalent cation. They were suspended in a medium which contained 9 mM-K and 91 mM choline. K efflux was measured with tracers, and flame photometry was used to measure the net effluxes of La, Na, Rb and Cs. In this experiment there were concentration gradients of monovalent cations, so some of the results presented in Fig. 5 were probably influenced by shrinkage and membrane potential effects. Nevertheless, some tentative conclusions can be drawn.

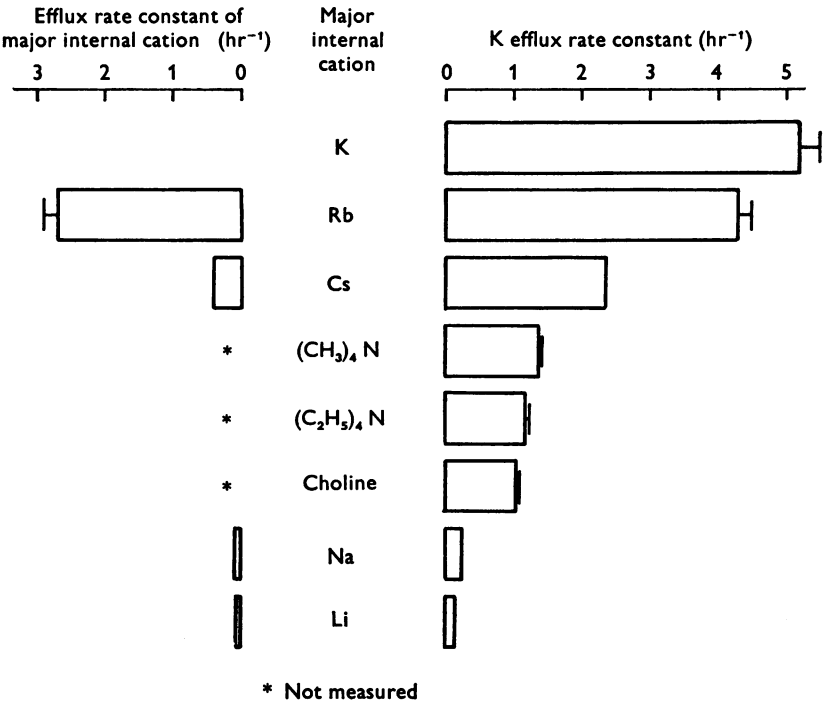


Fig. 5. Cation substitution experiment with Ca-rich ghosts. The ghosts contained 2 mM-Ca, 3 mM-HEDTA, 9 mM-K and 84 mM of the cation shown (plus Tris to 100 mM). They were washed and suspended in a 9 mM-K, 91 mM choline medium, and the figure gives the effluxes of $^{42}K$ (tracer) and the major cation (net), +1 S.D. (When K was the major cation its internal concentration was initially 93 mM.) Another experiment, not included, gave similar results, both for net cation efflux, and the effect of cations on K efflux.

Comparison of the net efflux rates of Rb, Cs and Li with that of Na suggests that Rb and possibly Cs are transported by the Ca-dependent system, while Li is probably not. Fig. 5 also shows that the K efflux rate constant is reduced when most of the internal K is replaced by another cation. The order of increasing inhibitory effect is Rb < Cs < $(CH_3)_4N$, $(C_2H_5)_4N$, choline < Na, Li. It is unlikely that this apparent inhibition could be caused by membrane hyperpolarization, except in the case of Rb, because a concentration gradient of an impermeant ion cannot influence the membrane potential.

Table 3 gives details of three further experiments, which attempted to confirm that Rb and Cs are transported by the Ca-dependent system.

TABLE 3. Comparative effects of Ca on K, Rb and Cs exchange

| Cation used | Ca | Experiment (1) | Experiment (2) | Experiment (3) |
|---|---|---|---|---|
| K | + | 4·6 ±0·4 | 3·2 ±0·1 | — |
| Rb | + | 5·5 ±0·5 | 5·6 ±0·3 | 5·5 ±0·3 |
| | − | 0·18 ±0·02 | 0·54 ±0·06 | 0·36 ±0·02 |
| Cs | + | — | 0·67 ±0·05 | 0·19 ±0·01 |
| | − | — | 0·46 ±0·05 | 0·13 ±0·03 |

The Table gives efflux rate constants ($hr^{-1}$, ±1 S.D.), for $^{42}K$, $^{86}Rb$ and $^{134}Cs$, each measured under exchange conditions, with 100 mM of the relevant cation inside the ghosts and in the external medium. 'Ca +' ghosts contained 2 mM-Ca and 3 mM-HEDTA, 'Ca −' had 3 mM-EGTA.

In these experiments there were no concentration gradients, and each batch of ghosts contained just one monovalent cation. Ca gave a clear stimulation of Rb transport, making it about 50% larger than the rate of K transport, under comparable conditions. On the other hand, the stimulation of Cs transport by Ca was barely significant.

Two problems arose with the experiments in Table 3. The Rb fluxes in Ca-rich ghosts were so fast that over 90% equilibration of tracer was reached during the period of measurement. This led to an underestimate of the rate constants, because the plots of log (1-(counts in medium/counts in suspension)), against time, ceased to be linear, and bent upwards systematically above 90% tracer equilibration. Secondly, some of the rate constants for Rb and Cs efflux from Ca-poor ghosts were surprisingly high. These were associated with abnormally high rates of ghost lysis, as demonstrated by haemoglobin release during the efflux period.

*Effects of cation substitution*

The results in Fig. 5 imply that certain monovalent cations may affect K movements, without being transported by the Ca-dependent system. This phenomenon was studied in detail for two substituent cations, Na and choline. In a series of experiments, Ca-stimulated K efflux was

studied as a function of K concentration, from 0 to 100 mM. K was replaced by either Na or choline, keeping the internal and external concentrations equal. The result, given in Fig. 6, was that the rate constants for K efflux increased with K concentration in the 3–30 mM range, but seemed to be constant in the 30–100 mM range. At low K concentrations, choline-rich ghosts had higher K efflux rate constants than Na-rich

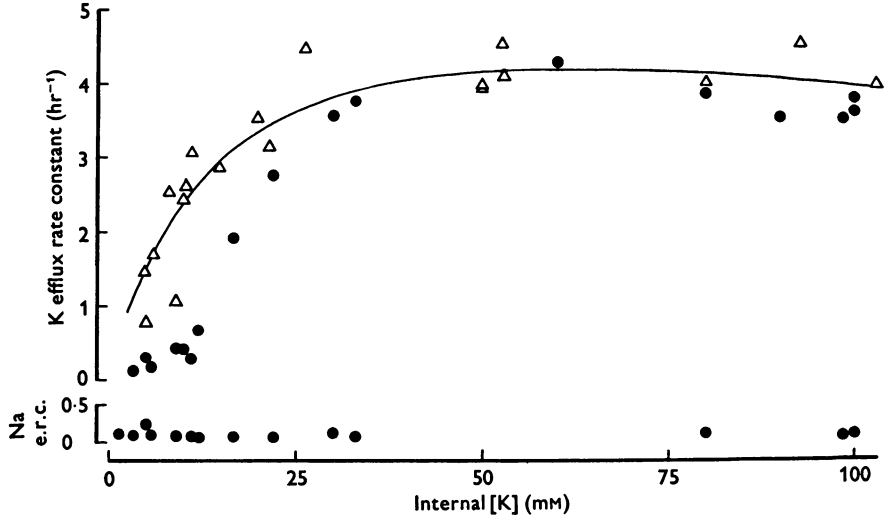


Fig. 6. Na and K exchange as a function of K concentration. K was replaced by Na (●) or choline (△) in ghosts which contained 2 mM-Ca and 3 mM-HEDTA. The external medium contained the same concentrations of K and the counter-cation as the ghosts, to within 10 %. Each point represents a single measurement. The curve is drawn to fit the K efflux results in choline-containing ghosts, and is explained in the text.

ghosts, although this difference disappeared above 30 mM-K. The obvious conclusions is that Na ions inhibit K movements, but it is not possible to say whether the decrease in K efflux rate constant at low K concentrations in choline-rich ghosts is a result of inhibition by choline, or is just a consequence of the interaction of K with the transport mechanism.

Riordan & Passow (1973) have also investigated the effects of monovalent cation substitutions on K movements in resealed ghosts. They used extracellular Ca to induce K movements, so the intracellular Ca levels were probably increasing during the course of their experiments. With equal concentrations of K and choline in the cells and medium, they found a maximum in the K efflux rate constant at 5–10 mM-K. This was not seen in the present experiments, in which the K movements were 3 or 4 times faster than those seen by Riordan & Passow (1973). They

also observed an inhibition of K efflux by intracellular Na, but only when there was an outward-directed K gradient, not in the absence of K gradients. In those experiments the internal K concentration was 70 mM: Fig. 6 shows that Na ions do inhibit K movements in the absence of K gradients, provided the K concentration is low enough.

Parallel measurements of Na efflux, also included in Fig. 6, show that the efflux rate constant had its usual value of about 0·12 $hr^{-1}$, irrespective of the Na concentration. Previous observations of the lack of effect of internal Ca on Na efflux were all made with a high K:Na concentration ratio (100:1), and it might have been argued that K inhibition prevented a Ca-dependent Na efflux from being seen. The independence of Na efflux rate constant from Na concentration in Ca-rich ghosts enables this hypothesis to be rejected.

A considerable effort was made to fit empirical curves to the results in Fig. 6. A Ca-insensitive component of 0·136 $hr^{-1}$ was assumed throughout. All attempts to fit the results to a one-site model, of the form R.C. = $0{\cdot}136\ hr^{-1}+a/(b+[K])$, failed. It should be noted that this expression would apply whether or not choline is a competitive inhibitor of K transport. A two-site model, of the form R.C. = $0{\cdot}136\ hr^{-1}+a[K]/(b+[K])(c+[K])$, fits the K efflux results in choline-containing ghosts reasonably well, and is drawn on Fig. 6. The parameters, $a$ = 1375 mM $hr^{-1}$, $b$ = 193 mM and $c$ = 20·2 mM, were obtained by a least-squares method. A three-site model gives a slightly, but not appreciably better fit. The general form of the K efflux results in Na-containing ghosts suggests that Na competes with K at the high affinity site(s), but not the low-affinity ones. However, attempts to fit the two-site model gave very poor results, and the same was found for a three-site model, of the form R.C. = $0{\cdot}136\ hr^{-1}+a[K]^2/(b+[K])(c+[K])^2$.

(Note that the formulae given above contain one less power of concentration in the numerator than is usual, because the rate constant, rather than the flux, is being fitted.)

It is often more useful to have graphs of flux against concentration, rather than rate constant against concentration. Fig. 7*A* shows how the Ca-dependent K efflux varies with K concentration when choline is the K substitute. The relationship appears to be linear above 5 mM-K, but sigmoid close to the origin. As there was no sign of saturation at K concentrations up to 100 mM, the range of observations was extended to 200 mM, using ghosts resealed at double the usual osmotic pressure (Fig. 7*B*). Again the ghosts contained optimal levels of Ca, and choline was the K substitute. In one experiment the results lay on a straight line, and in the other along a slight curve. The only conclusion that can be drawn is that if saturation does occur, it must be at K concentrations above 200 mM.

*Site of cation action*

Fig. 6 clearly demonstrates that Na ions inhibit K movements, but does not indicate on which side of the membrane they act, although the experiment in Fig. 5 would lead one to suspect it was the inside. This was

confirmed in two further experiments, one of which is described in Table 4. The K concentration was 10 mM on either side of the membrane, and the predominant cations were Na and choline. These could be interchanged freely without disturbing the membrane potential, as neither is transported by the Ca-dependent system. Table 4 shows that replacing external choline by Na has no effect on K efflux, whereas replacing internal choline by Na does inhibit it. It also shows that the rates of change of the ghost K

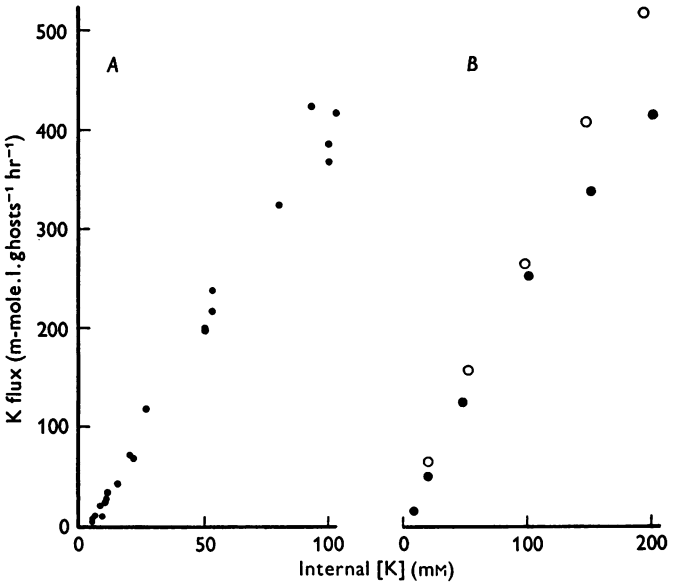


Fig. 7. K efflux as a function of K concentration, under equilibrium exchange conditions. The sum of the K and choline concentrations was about 100 mM in Fig. 7*A* and 200 mM in Fig. 7*B* (note the different scales on the abscissa). All the ghosts contained 2 mM-Ca and 3 mM-HEDTA. The points in Fig. 7*A* were calculated from Fig. 6; those in Fig. 7*B* are from two additional experiments.

TABLE 4. An experiment to show the site of inhibition by Na

| Cation composition (to 100 mM) | | $^{42}$K efflux rate constant ($hr^{-1}$) | Rate constant for change in internal [K] ($hr^{-1}$) |
|---|---|---|---|
| Internal | External | | |
| 10 mM-K + choline | 10 mM-K + choline | 2·44 ± 0·07 | + 0·03 ± 0·07 |
| 10 mM-K + choline | 10 mM-K + Na | 2·23 ± 0·13 | + 0·01 ± 0·10 |
| 10 mM-K + Na | 10 mM-K + choline | 0·46 ± 0·01 | − 0·02 ± 0·05 |
| 10 mM-K + Na | 10 mM-K + Na | 0·42 ± 0·01 | 0·00 ± 0·05 |

The ghosts contained 2 mM-Ca and 3 mM-HEDTA. The rate constants for change in internal [K] were calculated from the slopes of graphs in which the K content of the ghosts was plotted against the incubation time.

content are not significantly different from zero. In other words, the internal K concentration was constant, or the K influx equalled the K efflux.

*Interactions between K and Ca*

Until now the effects of divalent cations on K movements have been considered separately from the actions of monovalent cations, yet various interactions might occur. For example, K might compete with Ca at sites which activate K efflux, or Ca might compete with K for transport sites. Such interactions might be detected from shifts in the curve relating

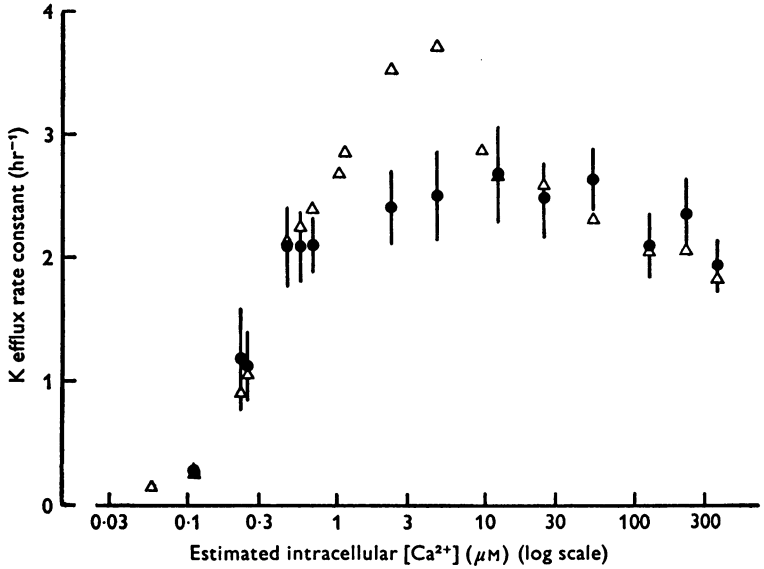


Fig. 8. The effect of Ca on K exchange in 10 mM-K ghosts. Each point (●) gives the average result from three or more measurements at that $Ca^{2+}$ concentration, ±1 S.E. of mean. (The Ca buffer solutions incorporated into the ghosts are described in Table 3 of Simons, 1976.) In theory, the ghosts and the medium both contained 10 mM-K and 90 mM choline, and measurements showed that intracellular K was in the 9–11 mM range. The values obtained with 100 mM-K ghosts (△) are taken from Fig. 1, and are included for comparative purposes.

TABLE 5. Effect of ouabain on Ca-dependent K exchange

| | Ouabain − | Ouabain + |
|---|---|---|
| Expt. 1 | 3·91 ± 0·08 | 3·59 ± 0·13 |
| Expt. 2 | 3·47 ± 0·12 | 3·39 ± 0·08 |

The Table gives rate constants for K efflux ($hr^{-1}$, ±1 S.D.), under equilibrium exchange conditions, from ghosts which contained 2 mM-Ca and 3 mM-HEDTA. The ouabain concentration was 100 μM.

K efflux to $Ca^{2+}$ concentration (Fig. 1), if it were determined again at a different K concentration. Fig. 8 presents the results of a series of experiments in which this was done, using 10 mM-K, 90 mM choline ghosts, suspended in a 10 mM-K, 90 mM choline medium. The results obtained previously with 100 mM-K ghosts are also included in Fig. 8, to facilitate the comparison. In fact, the two sets of results are not significantly different over most of the $Ca^{2+}$ concentration range. The only noticeable difference is the peak at about 5 $\mu$M-$Ca^{2+}$, present with the 100 mM-K ghosts, but not at 10 mM-K. This is hard to understand, and suggests there are no simple interactions between K and Ca.

*Effect of ouabain*

It has been suggested that the Na pump is responsible for the Ca-dependent K movements in human red cells (Blum & Hoffman, 1971). The main evidence for this was the inhibitory effect of ouabain, but Lew (1974) has now shown that this effect disappears when the cells are completely depleted of ATP, which suggests an indirect role for ouabain. Two experiments with resealed ghosts tended to confirm this (Table 5), but the errors were too large to exclude a small inhibitory effect. A comparison of the other properties of the Ca-dependent K permeability mechanism with those of the Na pump would reveal some similarities and some differences. The balance of the evidence suggests that a common mechanism is not involved, but convincing evidence must await studies with purified membrane components.

## DISCUSSION

*The mechanism of Ca-dependent K transport*

These experiments provide a reasonably detailed kinetic description of the Ca-dependent K transport system in human red cell ghosts. They are confined to one particular set of conditions – equilibrium exchange – but they should still enable one to put forward a tentative model of the mechanism responsible. Because of the complexity of the results, it is difficult to do this and keep the model physically plausible. Nevertheless, a number of hypotheses can be rejected.

All the available evidence suggests that Ca-dependent K transport is passive. Exchange and net downhill movements of K are possible, but there are no reports of net uphill movements of K against an electrochemical gradient. The assumption is often made that a single mechanism is responsible for all Ca-dependent K movements. This may or may not be true: multiple mechanisms make an easy explanation for complicated results. Even a single mechanism can give complex transport patterns if

the cells are heterogeneous. Resealed ghosts are more heterogeneous than intact red cells, because of a minority of ghosts that fail to reseal. However, ghost suspensions behave like a homogeneous two-compartment system, under the conditions used here, and it is hard to see how heterogeneity could be invoked to explain the results.

If we accept the single-mechanism hypothesis, the next assumption is that the system exists in two states: 'on', in which K movements are possible, and 'off', in which they are not. The system would be switched on when Ca ions bind to a site (or sites) on the inner surface of the membrane. This model predicts that K movements should be proportional to $\left(\frac{[Ca]}{a+[Ca]}\right)^n$, where $n$ equivalent Ca-binding sites have to be filled. It can explain the rising parts of the curves in Fig. 1, but not the apparent inhibition by high $Ca^{2+}$ concentrations. The minimum value of $n$ compatible with the Ca and Sr results is 2, although a higher value would be needed to give a good fit at low $Ca^{2+}$ concentrations. It is harder to explain the inhibitory effect of $Ca^{2+}$. This appears to be large at 100 mM-K, but absent at 10 mM-K (Fig. 8). The 10 mM-K results are the averages of widely scattered results from different experiments, but when each experiment is considered individually there is a small though significant inhibition of K movements in high-Ca ghosts, relative to those with intermediate levels of $Ca^{2+}$. The inhibitory effects of high $Ca^{2+}$ might be explained as an indirect consequence of Ca-binding to membrane components not involved in K transport. This does not explain the lack of effect on Na or Cl transport (Simons, 1976), or the different effects in high-K and in low-K ghosts. Competition of Ca with K can also be rejected. This predicts there should be greater inhibition at low K concentrations than at high, yet the reverse is true. Competition with K also fails to explain why the inhibitory effect saturates at moderately high $Ca^{2+}$ levels (Fig. 2). The limited inhibitory effect of $Ca^{2+}$ also enables one to reject a model in which K transport is blocked by the binding of Ca to a low-affinity site. A more complicated model is needed, for example one in which Ca binds to a regulatory site, which modulates the rate of K transport (as assumed for the curve drawn in Figs. 1–3). The modulation has to depend on K, as well as Ca, to explain the differential effect seen in Fig. 8. Another way of looking at such a model is to say that the system has two (or more) 'on' states, whose relative occupation is a function of K and $Ca^{2+}$ concentrations.

This suggestion seems highly speculative. Let us ignore it for the moment, assume there is a single 'on' state, and try to explain the effects of monovalent cations on K movements. The relationship between K flux and K concentration is almost linear over the 0–200 mM range, except for a

sigmoid region close to the origin (Fig. 7). The behaviour at low K concentrations is not compatible with simple models in which K ions act at only one site. The simple pore and simple carrier models predict either a linear or a rectangular hyperbolic relation between flux and concentration under equilibrium exchange conditions (Lieb & Stein, 1974*a*, *b*). Sigmoid behaviour implies multiple sites – either more than one K ion is carried at a time, or K ions have to be present at a regulatory site before transport can occur. Fig. 6 shows that the results can be fitted by a two-site model when choline is the K-substitute, but the inhibitory effect of internal Na requires at least four sites, if it is to be explained on the basis of competition with K. Four sites for monovalent cations seem hardly plausible, particularly when one also has to postulate three or four sites for divalent cations. Perhaps the system consists of a number of subunits, each of which can bind a single Ca ion, and transport a single K ion. The results, as a whole, would have to be explained by the interactions between the subunits. This seems slightly more plausible than a single-unit model, but is still in the realms of speculation. It is not worth advancing a formal model in the absence of structural evidence, or of detailed studies of K movements in the presence of concentration gradients.

*Nature of the Ca-binding sites*

The results enable one to make some suggestions about the chemical nature of the Ca-binding sites. The response to Ca implies that the highest-affinity site has a log stability constant in the 6–7 range, at pH 7·1. This is only possible with carboxylic acids, of the chemical groupings likely to be present, and cannot be reached by hydroxyl, phosphate or polyphosphate groups. Consideration of simple model compounds suggests that at least three carboxylic acid residues would be needed at each site, but a combination of other groups with two carboxylic acid residues might be possible.

*Comparison with snail neurones*

It is possible to compare the results in this paper with some recent studies of Ca-dependent K currents in snail neurones. The two systems seem to be similar in some respects, but different in others. For example, Meech (1974) has shown that the K permeability of snail neurones is increased by the injection of Ca buffers containing 0·9 $\mu$M-$Ca^{2+}$, which suggests a similar sensitivity to $Ca^{2+}$. On the other hand, an appreciable fraction of the Ca-dependent ionic current in snail neurones seems to be carried by ions other than K (Meech & Standen, 1975). It is not certain whether Na is the other ion involved, but if it is, the results of Meech & Standen (1975) imply that the K:Na selectivity of the Ca-sensitive channel

in snail neurones is much less than 40:1, which is the lower limit for the K:Na selectivity of the Ca-dependent permeability mechanism in human red cells. Another difference is that tetraethylammonium ions (TEA) inhibit both the delayed rectifier and the Ca-dependent K current in snail neurones (Meech & Standen, 1975), whereas in red cell ghosts TEA behaves like choline, and does not produce marked inhibitory effects, like those seen with Na and Li (Fig. 5).

*Ionized Ca in human red cells*

This may be estimated in the following way. Ouabain-insensitive K efflux from red cells has an average value of about 1·5 m-mole/l. cells hr (Glynn, Lew & Lüthi, 1970). This corresponds to a rate constant of 0·015 $hr^{-1}$, assuming 100 mM-K in the cells. The free $Ca^{2+}$ in the cells must be less than the concentration which activates the Ca-dependent system to that extent. In order to proceed further, the free $Mg^{2+}$ concentration must be known. For oxygenated red cells, the best estimate available is 0·67 mM (Gerber, Berger, Jänig & Rapoport, 1973), which takes into account binding by ATP, DPG and haemoglobin. Resealed ghosts have a higher basal K permeability than red cells. Fig. 3 shows that the K efflux rate constant from ghosts containing 0·25 $\mu$M-$Ca^{2+}$ and 0·9 mM-$Mg^{2+}$ is 0·1 $hr^{-1}$ above the basal level. The increment is six times the passive K permeability of red cells, so red cells probably contain appreciably less than 0·25 $\mu$M-$Ca^{2+}$. This estimate is lower than Schatzmann's (1973) upper limit of 1 $\mu$M-$Ca^{2+}$, estimated from the kinetics of the Ca pump. It depends on two untested assumptions (apart from those mentioned previously): (i) the sensitivity of ghosts to Ca is no greater than red cells, and (ii) Ca-dependent K transport is unaffected by substances normally present in red cells, but absent from the ghosts (for example, ATP).

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