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# THE MODULATION OF THE CALCIUM PUMP OF HUMAN RED CELLS BY $Na^+$ AND $K^+$

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**1. The sidedness of $Ca^{2+}$-pump activation by $Na^+$ and $K^+$ was studied by atomic absorption spectrophotometry in human erythrocyte ghosts, which had been prepared in dextran solutions and resealed to alkali cations. 2. When ghosts were incubated in an all-choline medium, the increase in $Na_i^+$ elicited an inhibitory-stimulatory effect on $Ca^{2+}$ extrusion. By contrast, only a stimulatory action was induced when choline was replaced by $Na_o^+$. 3. A dual effect on active $Ca^{2+}$ efflux was also produced by increasing $K_i^+$ or $K_o^+$. The biphasic response to the latter, however, was absent from high-$K^+$ ghosts. Furthermore, the stimulation obtained at high $K_o^+$ was additive to that elicited by $K_i^+$. 4. The results suggest that $Na^+$ and $K^+$ stimulate the $Ca^{2+}$ pump of human red cells through two different mechanisms. The first one appears to be an electric coupling between $Ca^{2+}$ efflux and the external activating cation. The other seems associated with the molecular reactions of the $Ca^{2+}$-pump protein.**

## Introduction

The biconcave-disk shape of human erythrocytes is critical for efficient gas transport and normal cell survival. Maintenance of this shape seems to rely upon proper activity of two ionic pumps. On one hand, by extruding $Na^+$ in exchange for $K^+$, the $Na^+$ pump prevents cell swelling and subsequent haemolysis. The $Ca^{2+}$ pump, on the other hand, by expelling this ion impedes opening of the $K^+$ channel and concomitant cell shrinkage [1–3].

The above pumps seem functionally related, as activity of one pump appears affected by action of the other. Thus, the $Na^+$ pump becomes inhibited by raising $Ca_i^{2+}$ [1,4,5], whereas the $Ca^{2+}$ pump is stimulated by increasing $Na_o^+$ or $K_o^+$ [6–8]. Although some asymmetrical activating effects on $Ca^{2+}$ extrusion have been reported for these alkali cations [7–10], their sidedness of action is not clear.

The present work is a systematic study of the $Na^+$ and $K^+$ action on the $Ca^{2+}$ pump of human erythrocyte ghosts, which have been resealed to alkali cations in dextran solutions. A biphasic activating effect of both $Na^+$ from inside and $K^+$ from either side of the membrane was found. By contrast, only a stimulatory action was elicited by either $Na_o^+$ in high-choline ghosts or $K_o^+$ in high-$K^+$ ghosts.

## Methods

Analytical-quality reagents were used whenever possible. EGTA, dextran ($M_r$ 80000), adenine, inosine and ATP were purchased from Sigma. The

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latter was freed from $Na^+$ by ion-exchange chromatography, on an amberlite IR-120 column previously loaded with $Mg^{2+}$.

Human blood (mainly O(+) group) was collected in citrate-phosphate dextrose solution and used within 1–2 days after collection.

Red cell ghosts were prepared in dextran solutions and resealed to alkali cations in the presence of an excess of $Ca^{2+}$ over EGTA [7]. Accordingly, 1 vol. of packed cells was lysed at room temperature in 20 vol. of a medium usually containing 4 mM Mg-ATP/2 mM $MgCl_2$/7.5 mM $CaCl_2$/5 mM EGTA/30 mM imidazole-HCl (pH 7.0), with the addition of 3% dextran (w/v).

After vigorous stirring for 30 s, isotonicity was restored with $K^+$, $Na^+$, choline chloride or mixtures of these salts. Thereafter, ghosts were resealed to alkali cations by incubating for 30–40 min at 37 °C, in the presence of inorganic phosphate (4 mM), adenine (5 mM) and inosine (10 mM). Under these conditions, ghosts pumped out the excess $Ca^{2+}$ while resealing and at the same time ATP is regenerated from the substrates provided.

Ghost total $Ca^{2+}$ was generally 4–5 μmol $Ca^{2+}$/ml ghosts at the end of resealing. By assuming that no leakage of EGTA occurred during this period, ghost free $Ca^{2+}$ was calculated from the above values and the $Ca^{2+}$/EGTA ratio originally present in the haemolytic medium. The ghost free $Ca^{2+}$ was usually about 20–40 μM, which was sufficient to saturate the $Ca^{2+}$ pump during subsequent incubation.

After resealing, the ghosts were washed thrice with a high-choline medium and finally incubated (5–10% haematocrit) for up to 6 min at 37 °C, in a medium containing 160 mM choline chloride/5 mM $CaCl_2$/5 mM adenine/10 mM inosine/20 mM imidazole-HCl (pH 6.8 at 37 °C). Where appropriate, choline was replaced by an osmotically equivalent amount of $K^+$ or $Na^+$. Ghosts were further treated as described under method b by Romero [7].

In some experiments, ghosts were prepared with different $Ca^{2+}$/EGTA ratios, set by trial and error in order to obtain free $Ca^{2+}$ concentrations between 0.2–10 μM by the end of resealing. $Ca^{2+}$-EGTA buffers were calculated as detailed elsewhere [11].

The $Na^+$, $K^+$ and $Ca^{2+}$ concentrations of ghosts were determined by atomic absorption flame photometry essentially as described earlier [7], using a Varian Techtron 1000A spectrophotometer. Initial rates of $Ca^{2+}$ extrusion were calculated from regression analyses of the linear decrease with time of ghost $Ca^{2+}$.

## Results

*Action of $Na_i^+$*

The activation of $Ca^{2+}$ pumping by $Na_o^+$ is well substantiated in human erythrocytes [6–9]. The effect of $Na_i^+$, however, appears contradictory. Thus, on one hand, the rate of $Ca^{2+}$ efflux attained by high-$Na^+$ ghosts when incubated in an all-$Na^+$ medium remains practically unaltered when choline or $Mg^{2+}$ replaces $Na^+$ on both sides [7]. On the other hand, after resealing to alkali cations, substitution of $Na_i^+$ by choline markedly diminishes $Ca^{2+}$ extrusion into a high-choline medium [8].

As disodium ATP was used in the above experiments, high-choline ghosts were not essentially $Na^+$-free. It was thus of interest to re-assess the effect of $Na_i^+$. Accordingly, ghosts were loaded with both $Mg^{2+}$-ATP and variable $Na^+$ concentrations. Thereafter, initial rates of $Ca^{2+}$ extrusion were determined by incubating in an $Na^+$-free choline medium.

A peculiar effect on $Ca^{2+}$ extrusion was elicited by altering $Na_i^+$. Thus, $Ca^{2+}$ efflux became drastically reduced when ghost $Na^+$ was raised from 0.5 up to 10 mmol $Na^+$/litre ghosts. Under the latter condition, $Ca^{2+}$ extrusion was diminished by 77%, reaching a rate of nearly 0.04 μmol $Ca^{2+}$/ml ghosts per min (Fig. 1). Half-maximal inhibition was obtained with about 2 mmol $Na^+$/litre ghosts. Further raising $Na_i^+$ from 10 to 60 mmoles $Na^+$/litre-ghosts increased the efflux rate up to approx. 0.1 μmol $Ca^{2+}$/ml ghosts per min, without showing signs of saturation.

These results demonstrate a biphasic effect of $Na_i^+$, being inhibitory at low concentrations whilst activating at relatively high levels.

*Pump stimulation by low $Na_o^+$*

As the latter behaviour of the $Ca^{2+}$ pump may arise from an external action of $Na^+$ due to leakage

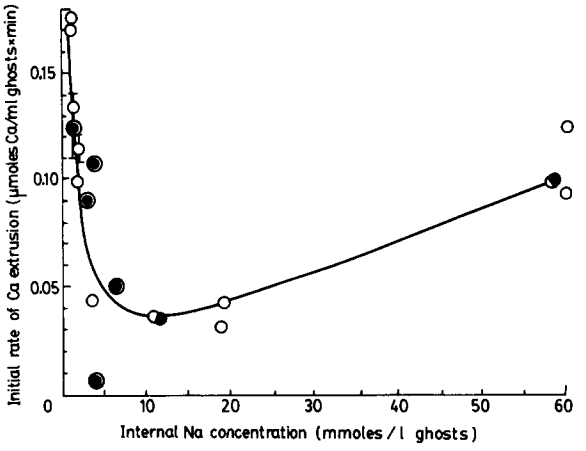


Fig. 1. Biphasic activation of $Ca^{2+}$ pump by $Na_i^+$. Washed human erythrocytes (1 vol.) were lysed in 20 vol. of a medium containing 4 mM Mg-ATP/2 mM $MgCl_2$/7.5 mM $CaCl_2$/5 mM EGTA/30 mM imidazole-HCl (pH 7.0), with the addition of 3% dextran (w/v). Isotonicity was restored with choline chloride, NaCl or mixtures of these salts, in order to obtain the $Na_i^+$ concentrations indicated above. Thereafter, ghosts were resealed to alkali cations as described in Methods and finally incubated at a 5% haematocrit, in a high-choline medium, containing 5 mM $CaCl_2$, 5 mM adenine and 10 mM inosine. The figure shows collected results from different experiments. A bar indicating $\pm 1$ S.D. is given in some cases.

from ghosts, it was important to determine whether $Ca^{2+}$ extrusion can be activated by low $Na_o^+$ concentrations. For this purpose, high-choline ghosts were prepared containing approx. 1 mmol $Na^+$/litre-ghosts and were then incubated in the presence of different $Na^+$ concentrations.

As suspected, $Ca^{2+}$ extrusion was increased from about 0.07 to 0.11 μmol $Ca^{2+}$/ml ghosts per min by raising $Na_o^+$ from 0 up to 10 mM (Fig. 2). A further rise up to 160 mM $Na^+$ only brought about a slight increment in extrusion rate, thus indicating that it had nearly reached its maximum. The $Na_o^+$ concentration required for half-maximal stimulation was approx. 1 mM.

These findings demonstrate that the $Ca^{2+}$ pump is activated by low $Na_o^+$ concentrations.

*Response to $K_i^+$*

In view of the preceding results, it was of interest to investigate the effect on pump activity of varying $K_i^+$. Ghosts were loaded with $K^+$ concentrations ranging from 0.7 up to 80 mmol $K^+$/litre ghosts and then incubated to assess $Ca^{2+}$ extrusion.

Active transport was markedly affected by $K_i^+$. Thus as ghost $K^+$ was increased from 0.7 to nearly 2 mmol $K^+$/litre ghosts, the $Ca^{2+}$ extrusion rate was reduced from 0.18 to about 0.01 μmol $Ca^{2+}$/ml ghosts per min (Fig. 3). Further increasing $K_i^+$ to 5 mmol $K^+$/litre ghosts stimulated $Ca^{2+}$ extrusion 10-fold. The maximal rate was about 0.17 μmol $Ca^{2+}$/ml ghosts per min when the $K^+$ concentra-

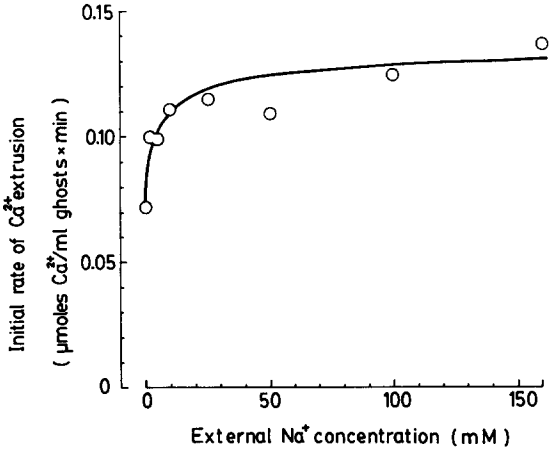


Fig. 2. Activation of $Ca^{2+}$ extrusion by low $Na_o^+$ levels. High-choline ghosts were prepared and incubated as indicated in the text to Fig. 1, in the presence of the various $Na_o^+$ concentrations shown above. The figure presents average values from two experiments.

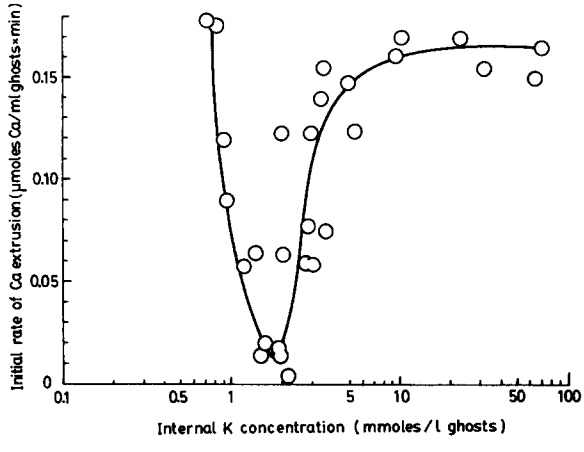


Fig. 3. Dual effect of $K_i^+$. Ghosts were prepared and treated in a manner identical to that indicated in the text to Fig. 1, except for the substitution of $Na^+$ by $K^+$ during restoration of isotonicity. Collected results from at least three experiments are presented.

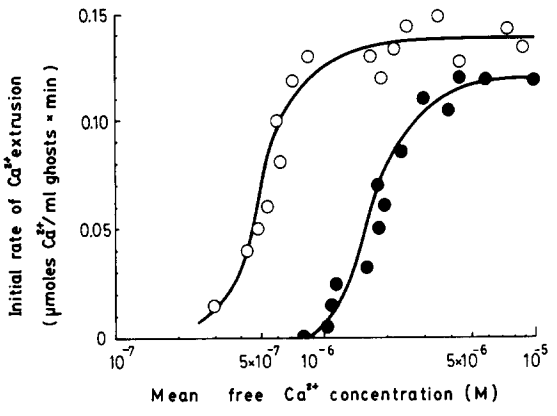


Fig. 4. Increased pump affinity for $Ca^{2+}$ by $K_i^+$. High-choline (●) or -$K^+$ ghosts (○) containing 0.7–1.0 or 70–80 mmol $K^+$/litre ghosts, respectively, and otherwise treated as mentioned in the text to Fig. 1, were loaded with different $Ca^{2+}$ solutions, in order to obtain by the end of the resealing period the range of free $Ca^{2+}$ concentrations shown in the figure. Initial rates of extrusion were calculated over the first minute of incubation in an all-choline medium and then related to the mean free $Ca^{2+}$ concentration attained during this time interval. Collected results from various experiments are given.

tion was at or above 10 mmol $K^+$/litre ghosts. Half-maximal effect was obtained with a ghost $K^+$ content of 2.7 mmol $K^+$/litre ghosts for activation, whilst the corresponding value for inhibition was nearly 1.

The results show that $K_i^+$ acts like $Na_i^+$, eliciting a biphasic response when increased from low levels up to physiological concentrations.

*Enhanced $Ca^{2+}$ affinity at high $K_i^+$*

In order to ascertain to what extent $K_i^+$ affects some kinetic parameters of the pump, high-$K^+$ or -choline ghosts, containing free $Ca^{2+}$ levels ranging from $1 \cdot 10^{-5}$ to $2 \cdot 10^{-7}$ M, were incubated in an all-choline medium.

$Ca^{2+}$ extrusion from high-choline ghosts was maximal at or above 5 $\mu$M $Ca^{2+}$, reaching a value of about 0.12 $\mu$mol $Ca^{2+}$/ml ghosts per min (Fig. 4). The $Ca^{2+}$ concentration for half-maximal effect was 1.6 $\mu$M. A Hill analysis of the results showed a straight line ($r^2 = 0.93$), with a slope of $3.7 \pm 0.31$ (S.E.), highly different from a slope of 1 ($\hat{t} = 8.79$; $P < 0.005$).

In high-$K^+$ ghosts (containing about 70–80 mmol $K^+$/litre ghosts), $Ca^{2+}$ pumping saturated at or above 1 $\mu$M $Ca^{2+}$, reaching a maximal value of 0.14 $\mu$mol $Ca^{2+}$/ml ghosts per min. Half-maximal activation was obtained with 520 nM $Ca^{2+}$. A Hill plot revealed a straight line ($r^2 = 0.65$), with a slope of $1.3 \pm 0.28$ (S.E.), not statistically different from a slope of 1 ($\hat{t} = 0.91$; $P > 0.1$).

These results demonstrate that at physiological levels, $K_i^+$ increases the pump affinity for $Ca^{2+}$ whilst leaving practically unaltered the maximal extrusion rate.

*The action of $K_o^+$*

The preceding experiments have shown an inhibitory-activating transition of the $Ca^{2+}$ pump upon varying $K_i^+$. Since such a behaviour may arise from an action of $K_o^+$ due to leakage from ghosts, it was important to assess $Ca^{2+}$ extrusion over a wide $K_o^+$ range. Accordingly, ghosts were loaded with 160 mM choline and then incubated in various choline media, containing different $K^+$ concentrations.

Active $Ca^{2+}$ efflux was highly dependent on $K_o^+$. Thus, the $Ca^{2+}$ extrusion rate was diminished from nearly 0.13 to 0.02 $\mu$mol $Ca^{2+}$/ml ghosts per min by increasing $K_o^+$ from 0 up to 5 mM (Fig. 5), the $K_o^+$ concentration for half-maximal inhibition being about 1.5 mM.

Increasing $K_o^+$ beyond 5 mM stimulated $Ca^{2+}$ extrusion in a saturable fashion, reaching a maxi-

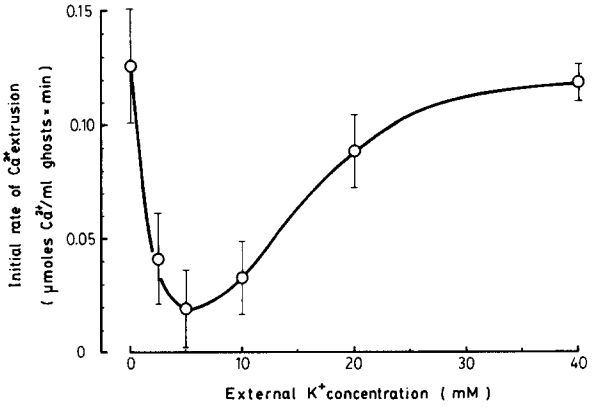


Fig. 5. Pump activation by $K_o^+$. High-choline ghosts, prepared as indicated in the text to Fig. 1, were incubated at a 10% haematocrit, in various choline media containing the $K^+$ concentrations shown above. Choline replaced an osmotically equivalent amount of $K^+$ where appropriate. Results are given as mean values of at least three experiments. Vertical bars denote $\pm 1$ S.D.

TABLE I

THE LEAKAGE OF $K^+$ INTO HIGH-CHOLINE GHOSTS

The increase in $K^+$ content of high-choline ghosts was assessed after 6 min incubation in choline media containing the various $K^+$ concentrations shown below. Ghost $K^+$ content was $0.8 \pm 0.17$ (7) mmol $K^+$/litre ghosts before incubation. All results are expressed as mean values $\pm 1$ S.D. of the number of experiments given within parenthesis.

| $[K^+]$ in external medium (mM) | Ghost $K^+$ content (nmol $K^+$/litre ghosts) after 6 min incubation |
|---|---|
| 0 | $0.73 \pm 0.09$ (7) |
| 2.5 | $0.77 \pm 0.06$ (3) |
| 5 | $0.98 \pm 0.02$ (3) |
| 10 | $1.15 \pm 0.17$ (7) |
| 20 | $1.45 \pm 0.29$ (4) |
| 40 | $2.45 \pm 0.39$ (4) |

mal rate of 0.12 μmol $Ca^{2+}$/ml ghosts per min at 40 mM $K_o^+$ (Fig. 5) and remaining constant by further raising $K_o^+$ up to 160 mM (results not shown). Activation was half-maximal at 14.2 mM $K_o^+$.

These results make clear that $K_o^+$ affects $Ca^{2+}$ extrusion in a biphasic manner, being markedly inhibitory at physiological levels. As $K_o^+$ concentrations between zero and 5 mM are also inhibitory, it is highly unlikely that the activating portion of the biphasic response to $K_i^+$ arises from an external action due to leakage from ghosts.

*Pump activation by $K_o^+$ in high-$K^+$ ghosts*

When choline-loaded ghosts are incubated in the presence of low $K_o^+$ concentrations, practically no $K^+$ entry is detected after 6 min (Table I). However, as $K_o^+$ is raised from 5 to 40 mM, ghost $K^+$ increases from roughly 1 to 3 mmol $K^+$/litre ghosts. In view of the fact that $Ca^{2+}$ extrusion is highly sensitive to $K_i^+$ within this range, the question arises as to whether the biphasic response to $K^+$ previously described is caused by an increased ghost $K^+$. If this were the case, $K_o^+$ would either by without effect or inhibit the pump activity both at low and high concentrations.

To test the above possibility, $Ca^{2+}$ extrusion from high-$K^+$ ghosts was assessed in the presence of various $K_o^+$ concentrations, ranging from 0 to 160 mM.

In the absence of $K_o^+$, $Ca^{2+}$ was expelled at a

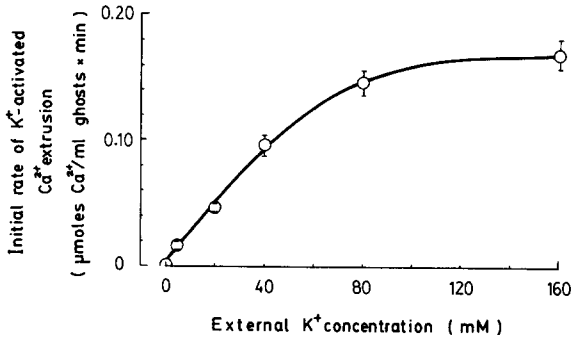


Fig. 6. Stimulation of $Ca^{2+}$ extrusion by $K_o^+$ in $K^+$-loaded ghosts. Ghosts, loaded with about 110 mmol $K^+$/litre ghosts and treated otherwise as explained in the text to Fig. 5, were incubated in the presence of the various $K^+$ concentrations shown in the figure. The initial rate of $K_o^+$-stimulated $Ca^{2+}$ efflux was calculated by subtracting the basal rate obtained in an all-choline medium from that found at each $K_o^+$ concentration. Results are shown as mean values $\pm 1$ S.D. of at least three experiments.

rate of nearly $0.17 \pm 0.008$ μmol $Ca^{2+}$/ml ghosts per min (mean value of five experiments $\pm$ 1 S.D.). Additions of increasing amounts of $K_o^+$ progressively enhanced $Ca^{2+}$ extrusion, which reached a maximal rate of about $0.35 \pm 0.01$ μmol $Ca^{2+}$/ml ghosts per min in a 160 mM $K^+$ medium.

After subtracting the activity found in the absence of $K_o^+$ from that obtained in its presence, typical Michaelis-Menten kinetics are revealed (Fig. 6), as inferred from linear regression analyses of reciprocal plots ($r^2 = 0.98$). The fraction of $Ca^{2+}$ efflux sensitive to $K_o^+$ reached a maximal value of about 0.17 μmol $Ca^{2+}$/ml ghosts per min at or above 80 mM $K_o^+$, the $K_m$ being nearly 36 mM $K_o^+$.

The results clearly show that $K_o^+$ exerts a monotonous activating effect on $Ca^{2+}$ extrusion from high-$K^+$ ghosts. In addition, they also show that the action of $K_o^+$ is additive to that elicited by this ion from inside.

## Discussion

The use of ghosts resealed in dextran solutions has allowed a systematic study of the action of $Na^+$ and $K^+$ on the $Ca^{2+}$ pump of human red cells.

Some peculiarities regarding this type of ghost and its present utilization are worth considering

before discussing the results.

Active transport was generally assessed at free $Ca^{2+}$ concentrations (at or above 20 $\mu$M) which were sufficient to keep saturated the $Ca^{2+}$ pump and, at the same time, adequate to maintain inhibition of the $Na^+$ pump [4,5].

The latter situation would minimize the possibility of rate-limiting $Ca^{2+}$ extrusion, otherwise resulting from a reduced ATP availability by $Na^+$ pump consumption. Moreover, ghosts conserved intact their metabolic system and, in addition to direct ATP loading, they were further incubated in the presence of substrates that give rise to ATP. This circumstance would ensure adequate ATP levels within ghosts, capable of maintaining the $K^+$ channel in a low $Ca^{2+}$-affinity state [12] and presumably closed under the experimental conditions.

In support of the latter point of view, high-$Na^+$ or -$K^+$ ghosts showed, after resealing to alkali cations, almost identical leakage of either ion (about 3 mmol/litre ghosts after 6 min) when measured under comparable conditions. Such a finding is not expected from an open $K^+$ channel.

On the other hand, a large proportion (70–80%) of the cation loss referred to above occurs within the first few minutes of incubation, as shown earlier [7], thus suggesting that a considerable fraction of the ghost population did recover a low permeability to alkali cations.

*Influence of $Na_i^+$*

Internal $Na^+$ exhibited a biphasic action, being inhibitory at low concentrations whilst stimulatory at higher levels. It is unlikely that the latter behaviour arises from an $Na_o^+$ action due to leakage from ghosts, as may be inferred from the following considerations. Taking into account a 5% haematocrit, the leak from high-$Na^+$ ghosts mentioned before would increase in 0.1 mM the $Na_o^+$ concentration by the end of incubation. As activation of $Ca^{2+}$ efflux is half-maximal at 1 mM $Na_o^+$ (see Fig. 2), 0.1 mM will have a negligible effect on $Ca^{2+}$ pumping.

The dual $Na_i^+$ behaviour may explain early findings which are apparently in conflict [7,8]. In such experiments, isotonic choline replaced $Na_i^+$ in two different ghost preparations, namely, leaky [7] and resealed to alkali cations [8]. Since both types were loaded with 4 mM $Na_2^+$-ATP, a retention of $Na^+$ at inhibitory concentrations may occur within resealed ghosts. In fact, ghost $Na^+$ (in mmol $Na^+$/litre ghosts) was about 6 after resealing, while it was less than 1 in the leaky preparation. It is thus evident that substitution of $Na_i^+$ by choline in both types of ghosts is bound to affect differently the $Ca^{2+}$ pump activity.

The biphasic nature of the $Na_i^+$ effect seems to indicate the presence of two classes of regulatory binding sites, accessible from the inner membrane surface. One class, possessing high affinity, leads to inhibition of $Ca^{2+}$ extrusion. Another class of lower affinity is responsible for an $Na^+$ effect leading to reversal of the inhibition.

*Role of $Na^+$*

The results presented above clearly show that $Na_i^+$, though not being essential for $Ca^{2+}$ extrusion, activates the pump at relatively high concentrations. This effect does not appear to be additive to that elicited by $Na_o^+$ in leaky ghosts, as may be inferred from the low efflux rate attained after incubating high-$Na^+$ ghosts in an all-$Na^+$ medium [7]. By contrast, in high-$K^+$ ghosts which have been resealed to alkali cationis, $Na_o^+$ further stimulates $Ca^{2+}$ efflux [8], thus showing a synergistic action with $K_i^+$.

Since at least part of the alkali cation-stimulated $Ca^{2+}$ efflux is coupled to influx of the activating cation [8], $Na_o^+$ may enhance $Ca^{2+}$ extrusion by dissipating an unfavourable electric membrane potential, associated with $Ca^{2+}$ pump activity [13,14]. In this context, pump electrogenicity has been questioned on the basis of an obligatory $Ca^{2+}$-$H^+$ exchange, shown in liposomes reconstituted with purified red cell $Ca^{2+}$-ATPase [15]. Although a similar exchange was also demonstrated in inside-out vesicles from human erythrocytes, the stoichiometry still remains to be established [16].

Theoretically, under an electroneutral exchange, a diffusion potential may originate from the electrochemical $H^+$ gradient created during $Ca^{2+}$ extrusion, thus driving $Na^+$ influx. As $Na^+$ entry cannot collapse the $H^+$ diffusion potential, a stimulation of $Ca^{2+}$ extrusion by $Na_o^+$ is not expected under these conditions. This, obviously, was not the case. Moreover, the ghosts used in the

present experiments were both loaded and incubated in the presence of a pH-buffering capacity (20–30 mM imidazole) sufficient to prevent development of an $H^+$ gradient.

It thus appears that stimulation of the $Ca^{2+}$ pump by $Na_o^+$ is not related to the set up to an $H^+$ gradient but seems consistent with an electrogenic $Ca^{2+}$ transport. Such a stimulation would be absent from leaky ghosts, thus explaining the lack of additivity of the $Na^+$ effect mentioned before.

The above considerations seem to point out two different mechanisms of pump activation by $Na^+$. The effect from outside appears related to an electric coupling. Stimulation by high $Na_i^+$, by contrast, may be associated with the molecular mechanism involved in $Ca^{2+}$ extrusion, as suggested earlier [7].

Our findings are at variance with those of Kratje et al. [10], who reported activation by $Na^+$ or $K^+$ only when these ions were present inside ghosts. The resealing conditions employed by these authors, however, are likely to result in a poor resealing to alkali cations. This circumstance would obscure an effect of these ions when tested from outside, as inhibitory levels may gradually build up within ghosts.

*Dual action of $K_i^+$*

Unexpectedly, $K_i^+$ was found to behave like $Na_i^+$, eliciting a biphasic action on $Ca^{2+}$ extrusion. The effect appears genuine, since, by reasoning in an analogous way to the case of $Na_i^+$, the rise in $K_o^+$ due to leakage from ghosts (much less than 1 mM by the end of incubation) is unlikely to explain an activation from outside. Moreover, an inhibition is more probable, as will be outlined below.

There appear to exist two classes of $K^+$-binding sites at the inner membrane surface, leading to inhibition and activation of $Ca^{2+}$ extrusion. Unlike the case of $Na_i^+$, however, the difference in affinities betwen the sites is very small, both showing high $K_i^+$-affinity.

The maximal stimulation elicited by $K_i^+$ at physiological levels (about 0.17 $\mu$mol $Ca^{2+}$/ml ghosts per min) is not much different from that obtained with $Na_i^+$ at comparable concentrations, thus demonstrating that $Na^+$ and $K^+$ are equally effective from inside and suggesting that they may act through the same mechanism.

*Increased $Ca^{2+}$ affinity at high $K_i^+$*

An interesting finding was that high $K_i^+$ increases the pump affinity for $Ca^{2+}$ without altering the maximal extrusion rate (see Fig. 3). The effect seems associated with a reduction in the degree of cooperativity required for $Ca^{2+}$ transport, as indicated by a diminution in the Hill coefficient from about 4 (found with choline) to 1 (obtained in the presence of $K_i^+$). Such a facilitation of $Ca^{2+}$ binding to pump sites would place $K_i^+$ as an important effector in regulating $Ca^{2+}$ pump activity under physiological conditions.

*Response to $K_o^+$*

In the virtual absence of internally added alkali cations, $K_o^+$ elicits a dual effect on active $Ca^{2+}$ efflux, being markedly inhibitory at very low levels. When ghosts are loaded with physiological $K_i^+$ concentrations, by contrast, the biphasic response is lost and there remains only a stimulatory action which is additive to that of $K_i^+$. Under these conditions, both pump affinity for $Ca^{2+}$ [6] and maximal extrusion rate are increased.

The results suggest that $K^+$ acts from outside through different mechanisms, depending on the presence or absence of $K_i^+$. One of these mechanisms may be related to the electric coupling discussed before. The other may be associated with the molecular reactions of the $Ca^{2+}$-pump protein, as suggested also for activation by high $Na_i^+$.

Early work with fragmented membranes from human erythrocytes has shown that, in the presence of $Ca^{2+}$ and ATP, both the phosphorylation and dephosphorylation rates and the phosphoenzyme level are increased by $K^+$ [17]. This action would certainly lead to an increased pumping rate, as presently found with $K^+$ on both sides of the ghost membrane.

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