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Role of Membrane-Bound Ca in Ghost Permeability to Na and K

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Summary. The permeability of red cell ghosts to K is determined by the amount of membrane-bound Mg which, in turn, depends on internal Mg. Contrasting with such effect, an increase in cellular Ca raises K permeability. To test whether this action is due to a competitive displacement of membrane Mg, the free Ca content of human red cell ghosts was altered by means of Ca-EGTA buffers. Net Na and K movements as well as Ca and Mg bindings were assessed after incubation in a Na-medium at 37 °C. Raising Ca from 3×10^{-7} to 1×10^{-2} M caused a large K efflux with very little Na gain. Under similar conditions, Ca binding was increased without affecting membrane-bound Mg. Both Ca binding and K loss were markedly diminished by either adding ATP to the hemolytic medium or increasing internal Mg at a fixed Ca concentration. A Scatchard analysis showed three Ca binding sites, two of them having high affinity. It is concluded that Ca action does not arise from a displacement of membrane-bound Mg but from binding to different sites in the membrane. Presumably, high affinity sites are involved in the control of K permeability.

The low K permeability of red cells to Na and K is maintained under physiological conditions by activity of the calcium pump. Whenever this pump is inactivated and Ca is present in the incubation medium, there is a net Ca entry and a concomitant increase in K permeability (Whittam, 1968; Lew, 1970; Romero & Whittam, 1971).

On the other hand, the restoration of a low K permeability of erythrocyte membranes after an osmotic shock, markedly depends on the presence of Mg in the hemolytic medium (Romero, 1973). Furthermore, a strict relationship between the extent of K retention by ghosts and the level of membrane Mg was found, suggesting that K permeability is determined by the amount of Mg bound to the ghost membrane (Romero, 1974).

Since Ca and Mg are antagonists in their effects on membrane permeability, it is possible that the Ca action arises from a competition

with Mg for binding sites in the membrane. This possibility was investigated in human red cell ghosts containing Ca-EGTA buffers.

The results showed that at the concentrations tested, Ca does not displace Mg from its binding sites. They further demonstrated a tight correspondence between Ca-induced K loss and the amount of membrane-bound Ca, suggesting that interaction of Ca with specific sites in the membrane is a requirement for the permeability change.

Materials and Methods

Analytical grade reagents were used whenever possible and were purchased from BDH Chemicals, England. EGTA (ethyleneglycol-bis(β-amino-ethylether) N,N′-tetraacetic acid) was obtained from Sigma Chemical Co., USA. The pH of all solutions was adjusted at room temperature within a range of ± 0.02 units, using a Radiometer TTT-lc pH-meter with scale expander.

Preparation of Ghosts

Red cells were obtained from human blood which had been stored for 4–5 weeks at 4 °C in acid-citrate-dextrose solution. Ghosts were prepared as described previously (Romero, 1974), except that the hemolytic medium contained in addition 5 mM EGTA and various amounts of $CaCl_2$.

Following resealing, the ghosts were washed thrice and then incubated by duplicate for 30 min at 37 °C, in 40 vol of a medium containing (mM): NaCl, 160; Tris-HCl buffer, 20 (pH 7.6). After which, they were washed thrice either in fresh Na-solution or 170 mM choline chloride + 20 mM Tris-HCl medium (pH 7.6). Ghosts were finally lysed in 100 vol distilled water (original lysate).

Preparation of Fragmented Membranes

A known volume of original lysate was centrifuged at $40{,}000 \times g$ for 15 min at 4 °C. The membranes were resuspended in 3 ml water and the suspension was transferred into thick-walled test tubes. They were immersed in a mixture of CO_2-ethanol and thawed under tap water. This procedure was repeated once.

The fragmented membranes were washed twice by resuspending in a volume of water equal to that of the original lysate from which they were obtained and centrifuging under conditions stated above. In this way, weakly bound Ca is washed out from the membranes. These were finally resuspended in 3 ml of water and kept at −4 °C for chemical analysis.

Estimations of Alkaline Cations

Na and K were determined in dilutions of the original lysate by flame emission at 588.8 and 766.3 nm, respectively, using a Varian Techtron 1000 spectrophotometer.

Due to unbalanced Na and K movements, ghosts shrank after incubation. In order to correct for these changes, the cation content of ghosts was referred to the original

volume using ghost hemoglobin as a volume index (Whittam & Ager, 1964). The error associated with these determinations was always below 10%.

Determination of Hemoglobin

Oxyhemoglobin was measured at 540 nm in suitable dilutions of the original lysate in 0.01 N NH_4OH (Wooton, 1964).

Measurements of Ca and Mg Bound to Membranes

A known volume (1.5 ml) of membrane suspension was centrifuged at $40{,}000 \times g$ for 20 min at 4 °C and the sediment was solubilized with 0.5 ml of 10% Triton X-100 in 8 M urea (Romero, 1974).

Ca and Mg were determined in the solubilized material by atomic absorption at 422.3 and 285.1 nm, respectively, using the above mentioned instrument. K (5 mg/ml) was present in both samples and standards to overcome ionization and La (2 mg/ml) was added as a releasing agent (Ramakrishna, West & Robinson, 1968).

Ca and Mg concentrations were referred to lipid P content of the membranes. Duplicate samples usually agreed within 10%.

Pilot experiments have shown that the amount of bound cations is practically unaffected when fragmented membranes are prepared in distilled water either at pH 5.5 or 7.

Determination of Lipid P

Phospholipids were extracted from an aliquot of membrane suspension (Rose & Oklander, 1965) and digested with 60% perchloric acid for 3 hr at 200 °C (King, 1932). Thereafter, the acid was precipitated as $KClO_4$ and the supernatant solution was kept for inorganic phosphate assays (Fiske & Subbarow, 1925).

Ca-EGTA Buffers

The buffers were prepared from 1 M stock solutions of EGTA (K salt) and $CaCl_2$. The final concentration of EGTA in the hemolytic medium was 5 mM. Variable amounts of Ca were added in order to obtain ionic contents from 3×10^{-9} to 1×10^{-2} M.

Free Ca concentrations above 10^{-4} M were checked by a modification of the murexide method (Schatzmann, 1973). Results agreed with theoretical calculations within less than 10%.

Upon warming the incubation medium at 37 °C, there was a pH shift of 0.28 ± 0.05 units (SD of 5 experiments) towards the acid side. Therefore, the apparent association constant for Ca-EGTA was calculated at pH 7.3, using true association constants of $10^{11.00}$ and $10^{5.33}$ for K_{MeL} and K_{MeHL}, respectively (Portzhel, Caldwell & Rüegg, 1964). The value obtained for $K'_{MeL_{total}}$ was $10^{7.28}$.

The amount of Ca required for a desired ionic concentration in the buffer, was calculated employing the following equation obtained from simple mass action:

$$[Ca]_T = ([Ca^{2+}]/\alpha)\ ([EGTA]_T + \alpha)$$

where $\alpha = [Ca^{2+}] + (1/K'_{MeL_{total}})$; $[Ca]_T$ and $[EGTA]_T$ are total concentrations of Ca and EGTA, respectively and $[Ca^{2+}]$ denotes the free Ca concentration in the buffer.

Results

Selective Rise of K Permeability

When energy-depleted cells are incubated with Ca, they develop an increased permeability to Na and K and hemolyze to an appreciable extent. The effect is more marked on K permeability and clearly depends on internal Ca (Romero & Whittam, 1971). Accordingly, ghosts become leaky to K when Ca is present in the hemolytic medium, unless a chelating agent such as ATP or EDTA is also added (Hoffman, 1962; Riordan & Passow, 1971).

These observations suggest that ionic Ca may be responsible for the permeability change, as pointed out earlier by Lew (1970). To investigate this possibility, the ghost-free Ca was varied and both net Na and K movements and degree of lysis were assessed after incubation.

A 100-fold increase in Ca concentration from 3×10^{-9} M has no effect on membrane permeability (Fig. 1). However, raising Ca above 3×10^{-7} M caused a large K loss without additional Na gain. Thus, in 30 min, the Ca-induced K loss was 110 μEquiv K/ml ghosts while the total Na gained was only 10 μEquiv Na/ml ghosts.

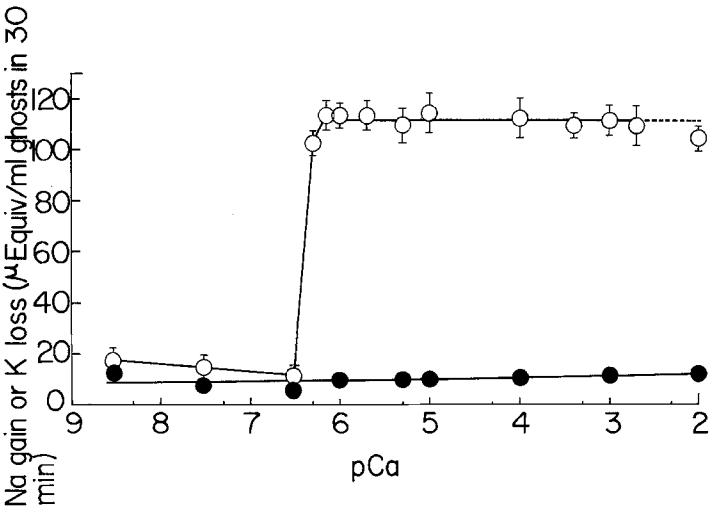

Fig. 1. Selective increase in K permeability of red cell ghosts. Human erythrocytes from cold-stored blood were lysed in 30 vol of a medium containing (mM): $MgCl_2$, 2; Tris-acetate buffer, 10 (pH 6.5); EGTA, 5 and sufficient amount of $CaCl_2$ to give the free Ca concentrations shown above. K loss (open circles) and Na gain (solid circles) were determined after 30 min incubation at 37 °C in a 160 mM NaCl+20 mM Tris-HCl medium, pH 7.6. Results are shown as the average of at least 4 experiments. Vertical bars show ±1 SD of mean and when not drawn, they are enclosed by the circles

Such alteration of K loss occurred abruptly, becoming maximal at about 7×10^{-7} M and remaining constant when Ca is further increased to 10 mM. At this concentration, Na gain was practically similar to that found at lower Ca levels and the degree of lysis was negligible.

These results demonstrate that a rise in free internal Ca induces a selective increase in K permeability.

Internal Ca and Membrane-Bound Mg

The above observation suggests a direct interaction of Ca with the ghost membrane. In view of the role of Mg described earlier, it is probable that the primary site of action might be some membrane ligands normally occupied with Mg. If so, there should be competition between these two ions for common binding sites.

To determine whether membrane-bound Mg is displaced by Ca, ghosts were prepared containing 0 or 2 mM free Ca. Total Mg in the former type of ghosts was slightly increased to account for chelation by EGTA and thus maintain ionic Mg at 2 mM.

As was expected, both K loss and Na gain were relatively small in the absence of Ca, being about 15 and 5 μEquiv/ml ghosts in 30 min incubation (Table 1). Over the same period with Ca, K loss was increased by nearly 100 whereas Na gain showed no statistical variation.

Concomitant with these changes and irrespective of the presence of Ca, membrane Mg remained unaltered, averaging about 2 nmoles/μg

Table 1. Parallel increase between K permeability and Ca binding to red cell membranes

Free Ca concentration added to hemolytic medium (mM)	Ghost cation content (μEquiv/ml ghosts)		Membrane-bound cations (nmoles/μg lipid P)	
	K	Na	Ca	Mg
0	123±7.6	5±1.5	0.8±0.21	2.2±0.71
after 30 min incubation	108±4.8	10±1.5	0.9±0.26	1.9±0.54
2	124±5.2	5±1.7	2.1±0.56	1.8±0.50
after 30 min incubation	10±4.8	13±2.5	4.3±0.49	2.2±0.76

Cold-stored cells were hemolyzed in the presence of (mM): $MgCl_2$, 2; Tris-HCl buffer, 10 (pH 6.5); EGTA, 5; with and without the addition of sufficient amount of $CaCl_2$ to give the final free Ca concentration shown above. Resealed ghosts were incubated for 30 min at 37 °C in an isotonic medium similar to that described in Fig. 1. The results are mean values (±1 SD) of 6 different experiments.

lipid P. By contrast, membrane Ca (in nmoles/μg lipid P) was increased from about 2 to 4.3 under conditions of a raised K permeability.

These findings show that Ca does not displace Mg from its binding sites but interacts with other sites in the membrane. Since Na permeability was not affected by Ca, subsequent experiments will refer to K alone.

Ca Binding and K Loss

To test if an increase in membrane Ca levels is related to a similar change in K efflux, internal free Ca was varied over a wide range of concentrations and both Ca binding and K loss were determined after incubation.

Membrane Ca altered from 0.6 to 0.9 nmoles/μg lipid P when internal free Ca was raised from 3×10^{-9} to 1×10^{-7} M (Fig. 2). Under these conditions, K loss was approximately 10 μEquiv/ml ghosts in 30 min, which is about 10 times the normal passive movement in intact red cells (Glynn, 1956). Such an increased leakage is mainly due to the presence of a certain fraction of ghosts which did not recover a low permeability after resealing (Bodemann & Passow, 1972).

An increase in membrane Ca (in nmoles/μg lipid P) to about 1.2

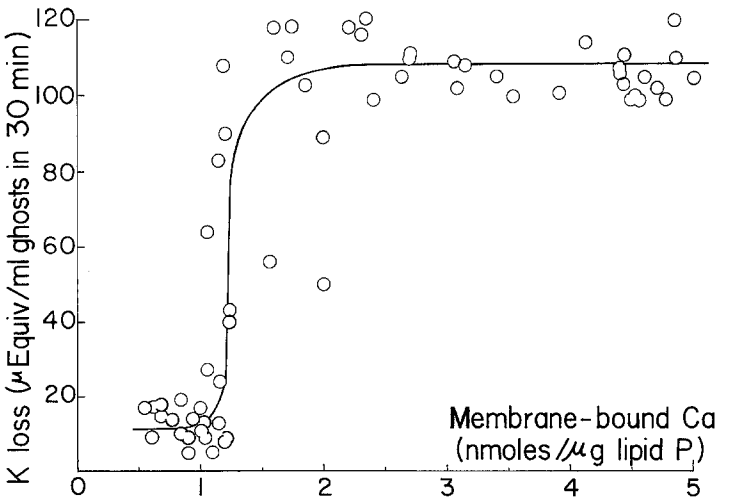

Fig. 2. The relationship between K loss and membrane-bound Ca. Ghosts were prepared containing free Ca concentrations from 3×10^{-9} to 1×10^{-2} M. The loss of K was determined after 30 min incubation in a medium similar to that of Fig. 1 and was related to the respective amount of membrane-bound Ca. The graph shows collected results from different experiments. Each point corresponds to a single experiment

was practically corresponded with the maximal K loss. This was maintained when membrane Ca was further raised to about 5.

Analysis of a Hill plot obtained from the same data, gave a regression coefficient of 0.75, corresponding to a line of equation $Y = -2.32X + 0.42$. A standard error of 0.61 and 0.30 was obtained for the whole regression line and slope, respectively. Although there is not a good fit of the data, the regression analysis indicates that membrane Ca is sigmoidally related to K loss.

These results suggest that K permeability is related to membrane-bound Ca. If there is a strict relationship, conditions that alter Ca binding should modify K permeability and conversely. Two of such conditions were investigated and are described below.

Prevention by ATP of Ca-Induced K Loss

It is known that ATP inside ghosts prevents the Ca-associated K efflux (Hoffman, 1962; Romero & Whittam, 1971). To test whether the amount of membrane-bound Ca is maintained at low levels under this condition, ghosts were prepared to contain both Ca and ATP.

As expected in the absence of ATP, K loss was about 110 μEquiv/ml ghosts in 30 min incubation and membrane Ca was almost doubled (Table 2). In the presence of ATP, by contrast, the loss of K over the same period was only 10 and the level of membrane Ca remained at about 1 nmole/μg lipid P.

The above observations demonstrate that membrane Ca is kept at low levels when the Ca-induced permeability change is prevented.

Table 2. The prevention by ATP of Ca-induced K loss

Additions to hemolytic medium (mM)	Ghost K content (μEquiv/ml ghosts)	Membrane-bound Ca (nmoles/μg lipid P)
2 Ca	120±8.7	2.3±0.36
after 30 min incubation	10±2.3	4.3±0.40
2 Ca+2 ATP	125±7.0	0.9±0.26
after 30 min incubation	115±4.5	0.8±0.31

Red cells were hemolyzed in a medium similar to that described in Table 1, but containing 2 mM free Ca with and without the addition of ATP as indicated above. Results are given as mean values±1 SD of 4 experiments.

Inhibition by Mg of Ca-Induced K Loss

Early work has shown that Ca can be released from erythrocyte membranes by relatively high Mg concentrations (Tolberg & Macey, 1972). It has been further demonstrated that Ca-dependent K efflux is markedly reduced by increasing internal Mg (Simons, 1975; Porzig, 1975).

To determine if the Ca-induced permeability change and the level of membrane Ca are affected similarly by Mg, ghosts containing 5×10^{-5} M Ca and Mg concentrations from 2 to 10 mM were incubated under otherwise identical conditions.

When Mg was increased up to 10 mM, K loss (in μEquiv/ml ghosts in 30 min) was reduced from 120 to nearly 5 (Fig. 3). This effect was paralleled by a reduction in membrane Ca from 2.3 to about 1 nmole/μg lipid P. These results show that inhibition of Ca-dependent K loss is concurrent with a decrease in membrane Ca levels.

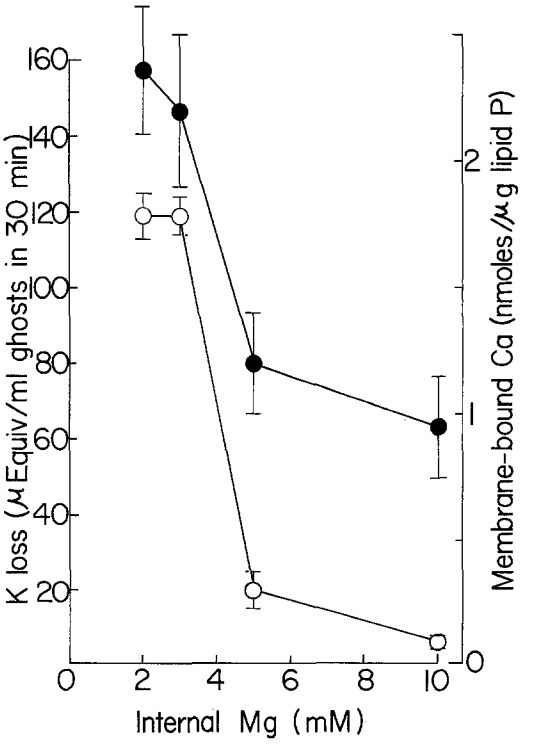

Fig. 3. The inhibition by internal Mg of Ca-induced K loss. Ghosts were prepared containing 5×10^{-5} M free Ca and the amounts of Mg indicated in the graph. The loss of K (open circles) and the amount of membrane-bound Ca (solid circles) was determined after 30 min incubation in a Na-medium. Results shown are average values of 4 experiments. Vertical bars denote ±1 SD of mean

The above findings taken together, indicate that K loss is strictly associated with membrane-bound Ca.

Binding Sites for Ca

As the permeability change occurs abruptly when membrane Ca is slightly increased, the binding of this ion was estimated over a wide range of concentrations to study whether it was preferentially bound. The number of sites were assessed from a graphical analysis originally proposed by Scatchard (1949).

Two points have to be stressed at this stage. First, weakly bound Ca is lost during washing of membranes. Secondly, the amount of membrane Ca after 30 min was not altered by a further 15 min incubation, indicating that binding had reached equilibrium.

Three linear regions can be observed in Fig. 4, which indicate that binding occurs at three different sites. Two sites have a high affinity, with apparent dissociation constants of 3×10^{-8} and 5×10^{-6} M and a maximal binding capacity of about 1.2 and 2.3 nmoles Ca/μg lipid

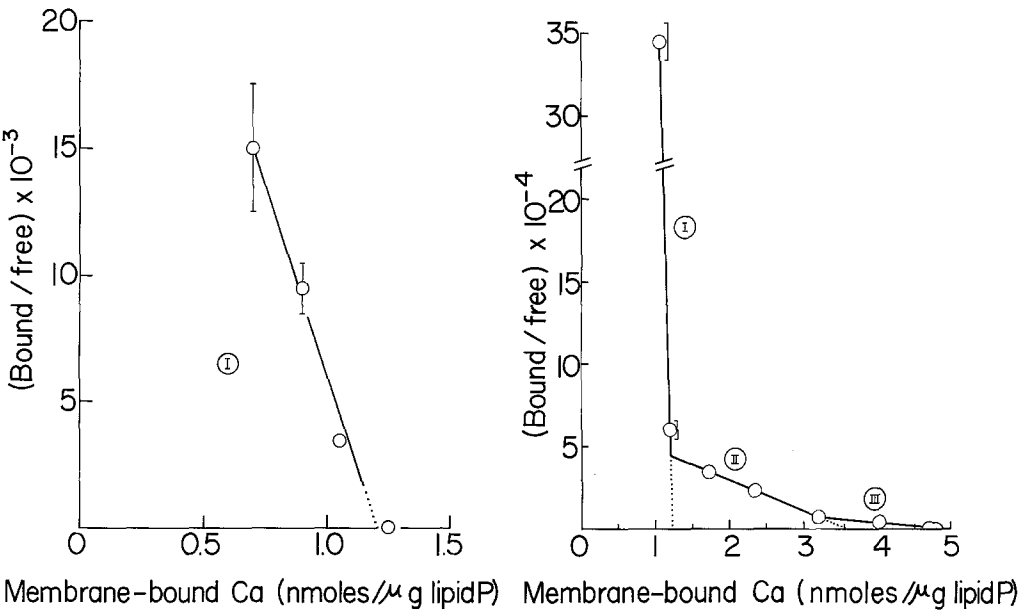

Fig. 4. Scatchard plots of Ca binding to human erythrocyte membranes. (*a*) resolves the highest affinity site. (*b*) shows another site of high affinity and the lowest affinity site. Results are mean values of at least 3 experiments. Vertical bars indicate ±1 SD of mean and when not shown, they are enclosed by the circles

P, respectively. The third site has an apparent dissociation constant of 5×10^{-4} M and a binding capacity of nearly 1.3.

These results show that Ca is preferentially bound to two sites in the membrane.

Discussion

Membrane K permeability seems to be regulated by internal Ca in a wide variety of tissues, including cardiac muscle (Isenberg, 1975), liver (van Rossum, 1970) nerve (Meech, 1972; Gorman & Marmor, 1974), and red cells (Romero & Whittam, 1971).

The use of Ca-EGTA buffers allowed us to confirm that the alteration of K permeability in red cell ghosts is associated with an increase in internal free Ca (Lew, 1970; Porzig, 1975; Simons, 1975).

The Ca-induced K loss occurs abruptly, thus resembling the typical response of excitable membranes, when ionic Ca is raised beyond 3×10^{-7} M.

Selective Increase of K Permeability

Unlike in erythrocytes from cold-stored blood, the permeability change in ghosts is highly specific for K. Such a finding disagrees with Porzig's observation that increasing internal Ca above 3 mM alter ghost permeability to both Na and K (cf. Porzig, 1975).

Another difference with intact cells is that practically no lysis was detected whatever the level of internal Ca studied. No good correlation between degree of lysis and internal Ca has been found (Romero & Whittam, 1971; Long & Mouat, 1973). However, hemolysis is certainly associated with a raised internal Ca since cells with an active calcium pump do not hemolyze in the presence of external Ca. These observations are compatible with the lack of a Ca effect on Na permeability.

The question is raised that the Ca action observed in resealed ghosts may not be the same found in intact cells. It is possible that the ghost membrane may suffer some rearrangement after the osmotic shock and restoration of isotonicity. In this way, some sites controlling Na permeability may be hindered and become inaccessible to Ca. A similar hypothesis was proposed to explain the exposure of certain phospholipids in red cells (Juliano, 1973).

Membrane Ca and K Permeability

A tight relationship between K loss and membrane Ca levels was found in the present work, suggesting that K permeability is regulated by the amount of Ca bound to the ghost membrane.

This idea is in conflict with a recent paper, where the increased K permeability seems to be explained on the sole basis of a rise in ghost free Ca.

In the absence of added Ca, propanolol has a Ca-like effect which is inhibited by raising the internal EGTA concentration (Porzig, 1975). This author suggested that the release of membrane Ca by propanolol, would induce the permeability change by increasing the level of internal free Ca.

The amount of membrane Ca was not measured directly in the paper cited above, but was estimated by determining the minimal amount of EGTA required to suppress the permeability change. This method is not necessarily valid because EGTA may equally well be competing for Ca with membrane binding sites. Removal of this ion from those sites could lead to a decrease in K permeability.

The amount of membrane Ca required to alter K permeability is about 1.2 nmoles/μg lipid P. Assuming that 1 ml of packed red cells contains about 10^{10} cells (Whittam, 1964) and that there are 1.22×10^{-8} μg lipid P per cell (Dodge, Mitchell & Hanahan, 1963), the above value is equivalent to 0.16 μmoles Ca/ml packed cells. Such an estimate is ten times higher than the Ca content of fresh cells (Harrison & Long, 1968) and about a half of that reported for 3–4 weeks stored cells (Romero & Whittam, 1971). However, it must be recalled that weakly bound Ca was not measured in the present work. Therefore, the total Ca content of the membranes must be higher than the estimate given above.

Lew (1971) showed that an increase in cell Ca to about 1 or 2 μmoles/liter of packed cells is sufficient to alter the permeability to K of fresh cells incubated with iodoacetamide or iodoacetamide plus inosine. The present experiment indicates that this change occurs when membrane-bound Ca reaches a concentration roughly equivalent to 100 times that reported by Lew.

It can be argued that such discrepancies arise from an overestimation of membrane-bound Ca, due to further binding which might occur during the preparation of membrane fragments and subsequent washing with water. This is not the case, as indicated by the following reasons. First,

Lew (1974) has shown that freezing and thawing red cell membranes eliminates the difference of ^{45}Ca activity between membrane pellet and supernatant solution, otherwise found when depleted cells containing ^{45}Ca are lysed in water. This finding clearly indicates that Ca, instead of becoming further bound, is released from the membrane pellet when fragmented by freezing and thawing.

Secondly, it is well known that a reduction of ionic strength leads to an increase in Ca binding to erythrocyte membranes (Long & Mouat, 1971). However, at the same time that ionic strength is decreased by lysing the ghosts in 100 vol distilled water, the Ca concentration in the internal bulk solution is consequently diminished, thus preventing further binding.

The discrepancies mentioned above may arise from both substantial differences between fresh and stored cells and the use of a metabolic inhibitor plus a nucleoside. It is known that metabolic poisons like iodoacetate potentiate the effect of Ca on K permeability (Romero & Whittam, 1971). Furthermore, the addition of a nucleoside together with the inhibitor is more effective than the inhibitor alone (Gárdos, 1967). Perhaps, iodoacetamide would act like iodoacetate, and thus a much lower internal Ca concentration may be required to elicit the permeability change.

Lew (1974), studying ^{45}Ca binding to both red cell membrane and fragmented membranes, found that all activity could be removed easily by washing. He suggested that the Ca responsible for the increased K permeability does not become tightly bound to membrane sites.

The results reported in the present work are not in agreement with the above view. They clearly show the existence of a tight relationship between the amount of Ca strongly bound and the Ca-induced increase in K permeability. We have no explanations at the present for such discrepancies.

Ca Binding Sites

A Scatchard analysis revealed two sites of high affinity. The highest affinity site becomes saturated with about 1.2 nmoles Ca/μg lipid P, which is coincident with the amount of Ca required to trigger the permeability change.

If calculations are made using both the amount of free Ca required to produce half maximal effect on K permeability and the apparent dissociation constants obtained from the Scatchard analysis, it is possible

to evaluate the degree of saturation of each site. Under such conditions, site I is about 93% saturated while site II is only 7%. These observations suggest that high affinity sites are involved in the control of K permeability.

Our experiments cannot rule out the possibility that the interaction of Ca with both site I and II is required for the increased permeability. However, two aspects of this effect may give an insight to the problem. First, a sigmoidal relationship between K loss and membrane-bound Ca was found. Secondly, a Hill plot of K loss and membrane Ca gave a straight line of slope equal to 2.3. These findings seem to indicate involvement of a cooperative effect between high affinity sites, as has been reported for activity of the sodium pump at low external K (Sachs & Welt, 1967).

Site of Ca Action

The permeability of phospholipid bilayers to alkaline ions is extremely low (Bangham, 1968). However, it is increased by Ca when bilayers are made of acidic phospholipids such as phosphatidic acid, phosphatidyl-serine and phosphatidyl-inositol (Papahadjopoulos & Watkins, 1967).

These phospholipids bind most of the lipid Ca (about 80%) in human erythrocyte membranes (Forstner & Manery, 1971). However, the action of Ca on ghost permeability cannot be explained entirely in terms of phospholipid interaction since phospholipid bilayers, unlike red cell ghosts, are nonselective towards alkaline cations (Papahadjopoulos, Nir & Ohki, 1971).

Ca is most probably exerting an effect on membrane proteins, which account for almost 85% of the total binding (Forstner & Manery, 1971; Duffy & Schwarz, 1973). Furthermore, strongly bound Ca is confined to two high molecular weight polypeptides (Duffy & Schwarz, 1973), suggesting a restricted location of bound Ca. On the other hand, Ca induces aggregation and reorientation of some membrane proteins (Bond, 1972; Carraway, Triplett & Anderson, 1975). It is therefore tempting to involve a Ca-induced conformational change of some membrane protein(s) in the mechanism of the raised K permeability of erythrocyte ghosts.

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