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CALCIUM-INDUCED POTASSIUM PATHWAY IN SIDED ERYTHROCYTE MEMBRANE VESICLES

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Summary

We have characterized the asymmetric effect of Ca^{2+} on passive K^+ permeability in erythrocyte membranes, using inside out and right-side out vesicles. Ca^{2+}, but not Mg^{2+}, can induce an increase in K^+ uptake in inside out vesicles. The half-maximal concentration of Ca^{2+} required to induce the K^+ uptake is 0.2 mM, and the permeability increase is not specific for K^+. Thus, the Ca^{2+}-induced permeation process in inside out vesicles is changed from that in the energy-depleted intact cell which requires only micromolar concentrations of Ca^{2+} and is specific for K^+. Removal of spectrin had no effect on the vesicle permeability increase due to Ca^{2+}. Studies with *N*-ethylmaleimide show that the vesicle channel opening is mediated by a protein and passage is controlled by sulfhydryl groups; furthermore, the Ca^{2+}-induced vesicle pathway is distinct from the normal channel for passive K^+ leak in the absence of Ca^{2+}. The protein is sensitive to its phospholipid environment since removal of easily accessible phospholipid head groups on the cytoplasmic face of the vesicles inhibits the Ca^{2+}-stimulated channel opening.

Introduction

Divalent cations, especially Ca^{2+}, are key regulatory factors in many biological processes at the membrane level. In human erythrocytes, Ca^{2+} not only binds tightly to the inside surface of the red cell membrane [1] but will also stimulate permeability to cations under certain conditions [2,3]. Though these processes involve the interaction of Ca^{2+} with the cytoplasmic side of the red cell membrane, the nature of the interaction is not understood. Previously, we have shown that both inside out and right-side out vesicles are about as impermeable to monovalent cations as unwashed ghosts and can, therefore, be

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used effectively to study transport properties of the red cell membrane [4]. We have now used these preparations to characterize the asymmetric effect of Ca^{2+} on passive K^+ uptake in vesicles and have also selectively modified membrane proteins and removed phospholipid head groups to determine the role of these components.

Materials and Methods

Preparation of vesicles. Sealed inside out vesicles and right-side out vesicles from fresh human red blood cells of healthy donors were prepared following the procedure of Steck and Kant [5] with the modifications previously described [4]. The orientation and impermeability to macromolecules of each vesicle preparation were determined by acetylcholinesterase (EC 3.1.1.7) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) activities. As previously reported, inside out vesicles were found to be 79 ± 5.7% (S.D.) inaccessible to acetylcholinesterase and right-side out vesicles were 94 ± 5.1% inaccessible to glyceraldehyde-3-phosphate dehydrogenase activity. Acetylcholinesterase activity was also used as a measure of the content of vesicles in most uptake and binding studies, since the protein composition of right-side out and inside out vesicles are different [6]. By definition, one unit of acetylcholinesterase hydrolyzes 1 μmol/min of acetylcholine, at 23° C, pH 8.0.

Vesicles washed in 2.5 mM Tris-HCl, pH 7.4, were preincubated overnight at 4° C in the solution containing the salt at the concentration that would be used for the experiment. This pretreatment was necessary to prevent drastic volume changes in the vesicles when the reaction medium of higher ionic strength was added [4]. The binding and uptake experiments were, therefore, mostly performed under exchange diffusion conditions which minimize volume and potential difference changes.

Materials. All chemicals were reagent grade. A23187 was a gift from Dr. J. Hosley of Eli Lilly and Company. Valinomycin, *N*-ethylmaleimide, and phospholipase C (*Bacillus cereus*) were purchased from Sigma Chemical Company, St. Louis, MO. All the radioactive isotopes were obtained from New England Nuclear, Boston, MA. Filters were purchased from Millipore Corporation, Bedford, MA.

Modification of vesicles. Extrinsic membrane proteins (bands 1, 2 and 5) were removed by incubating washed ghost pellets in 20 vols. of 0.1 mM ethylenediaminetetraacetic acid, adjusted to pH 8.5 with sodium hydroxide (EDTA/NaOH) [7]. As spectrin depletion from inside out vesicles depended on the time of incubation, ghosts were treated in EDTA/NaOH, pH 8.5, at 37° C for 40 min or overnight. Ovomucoid (5 μg/ml), a trypsin inhibitor, and NaN_3 (0.1 mM) were added to vesicle preparations to prevent proteolysis and bacterial growth during overnight incubations at 37° C.

To remove phospholipid head groups, vesicles (about 2 mg protein/ml) were treated with various concentrations of phospholipase C from *B. cereus* in 10 mM KCl and 2.5 mM Tris-HCl, pH 7.4. Ca^{2+} which is usually added to stimulate enzyme activity [8] was intentionally excluded from the reaction. After an incubation period of 30 min at 37° C, hydrolysis was stopped by

washing the vesicles twice in 5—10 vols. of 10 mM KCl and 2.5 mM Tris-HCl, pH 7.4, at 4°C.

Protein sulfhydryl groups were modified by treating vesicles (3—5 mg protein/ml) with 2 mM *N*-ethylmaleimide for 30 min at 37°C. The vesicles were then washed in 60 vols. of a 4°C buffer solution containing 10 mM KCl and 2.5 mM Tris-HCl, pH 7.4.

All vesicle preparations that had been modified with enzymes or chemical reagents were subsequently checked for their orientation and impermeability to macromolecules. Unless inside out vesicles were at least 75% inaccessible to acetylcholinesterase and right-side out vesicles were 85% inaccessible to glyceraldehyde-3-phosphate dehydrogenase, they were discarded.

Method for measuring ion uptake and binding. Ion uptake into the vesicles was determined by a Millipore filtration method. Reactions were started by addition of vesicles (about 0.2—0.5 mg protein/ml) to a reaction medium containing the radioactive substrate. The reaction media were incubated at 37°C unless otherwise stated. At specified time intervals, 50-μl aliquots were withdrawn and immediately mixed with 5.0 ml of a 4°C non-radioactive wash medium. The mixture was filtered through a 25 mm Millipore filter (pore size 0.45 μm) and rinsed with 5.0 ml wash medium. The entire process takes less than 30 s. The filter paper was then counted in a liquid scintillation counter (Nuclear-Chicago model 6801 S), after addition of 10 ml of scintillation fluid [9]. The reaction medium for measuring Ca^{2+}-stimulated K^+ uptake into inside out vesicles contained: 2.5 mM Tris-HCl, pH 7.4, 10 mM KCl (containing trace $^{86}Rb^+$ at approx. 1.0 μCi/ml) and 0—1.0 mM $MgCl_2$ or $CaCl_2$. The reaction media for measuring uptake of other substances is essentially the same except for the substrate and its radioactive isotope. The wash media consisted of 2.5 mM Tris-HCl, pH 7.4, with 10 mM KCl. Ca^{2+} binding to vesicles was measured using the same Millipore filtration procedure. After various periods of incubation of vesicles in ^{45}Ca (approx. 0.5 μCi/ml) an aliquot was filtered and washed to determine the amount of Ca^{2+} associated with the vesicles. The wash media solution contained 2.5 mM Tris-HCl, pH 7.4, and 10 mM KCl. To determine the amount of tracer that would be inside the vesicles at equilibrium as well as to differentiate between binding and uptake, ionophores were added to the vesicle reaction medium. After 10 min incubation at 37°C with an ionophore an aliquot was diluted and filtered. Usually, since the ionophore is diluted 100 fold before filtration, loss of tracer from vesicles by efflux is minimal during washing. However, as a precaution when K^+ uptake was measured, 0.5% bovine serum albumin which binds valinomycin [10] was included in the wash medium.

Protein and phospholipid determinations. The total amount of membrane protein was determined according to the method of Lowry et al. [11] after solubilization of the membrane proteins with 0.2% sodium dodecyl sulfate. The presence of an equivalent amount of sodium dodecyl sulfate in standard bovine serum albumin had no effect on the measurements. The protein components of the membrane were separated for analysis by gel electrophoresis according to the procedure of Fairbanks et al. [7] with minor modifications [1].

The amount of phospholipid still left on vesicles that had been treated with phospholipase C was determined by measuring organic phosphorus according

to the procedure of Gomori [12]. Phospholipids were extracted from vesicles by a modified method of Bligh and Dyer [13]. 1 vol. of vesicles (3—5 mg protein/ml) was extracted with 10 vols. of methanol. After 10 min at room temperature, 10 vols. of chloroform were added and mixed. This procedure was repeated and the extracts were pooled. The total lipid extract was separated into a neutral lipid phase and a phospholipid phase by adding water and chloroform to obtain a ratio of 8 vols. chloroform : 4 vols. methanol : 3 vols. water. The lower phase which contains the phospholipids was removed for organic phosphorus determinations.

Results and Discussion

Although the Millipore filtration technique has been used for measuring solute uptake into red cell vesicles [14,15], it was necessary to determine the validity and limitations of the filtration technique in measuring solute uptake into inside out and right-side out vesicles of red cell membranes under our conditions. In particular, we wanted to differentiate tracer association with the vesicles, as a measure of uptake into sealed vesicles, from binding to membrane surfaces.

The effect of filtration on monovalent cation association with vesicles was examined first. A direct relationship with a correlation coefficient of 0.97 was found between the amount of vesicle protein filtered and the quantity of tracer associated with the vesicles after 1 h incubation. Approximately 90% of the filtered vesicles are recovered on the Millipore filter as determined by phospholipid measurements. Fig. 1 shows a gradual uptake of $^{86}Rb^+$ by sealed inside out vesicles whereas there is very little tracer associated with unsealed vesicles. $^{86}Rb^+$ entry into sealed vesicles reached an apparently constant rate by about 1 h; addition of 0.5 μM valinomycin in one experiment caused the tracer to come to the 120 min level (within 5%) in 4 min, as shown by the point marked 'val'. The rate of increase in vesicle-associated $^{86}Rb^+$ at 120 min in sealed

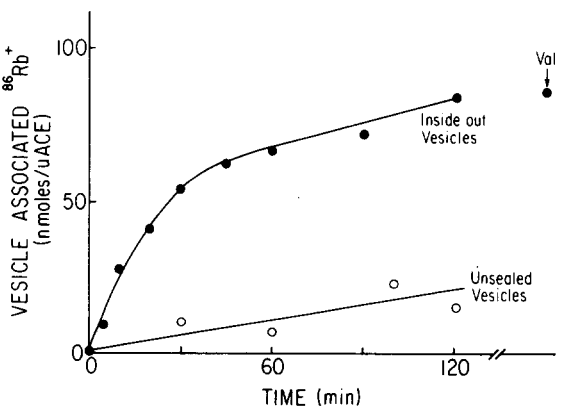

Fig. 1. Time course of K^+ ($^{86}Rb^+$) uptake by inside out and unsealed vesicles in one experiment typical of three. The curves are drawn by eye in this and subsequent figures. Vesicles were incubated at 37° C in 2.5 mM Tris-HCL, pH 7.4, and 10 mM KCl ($^{86}Rb^+$). Valinomycin (0.5 μM) was added to the inside out vesicles at the end of the experiment as indicated by the arrow.

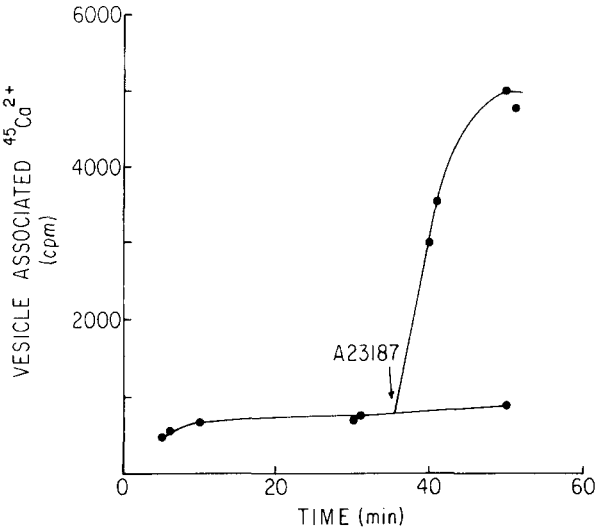

Fig. 2. Effect of the Ca^{2+} ionophore A23187 (4 μg/ml) on Ca^{2+} association with right-side out vesicles in one experiment typical of two. Vesicles were incubated in 2.5 mM Tris-HCl, pH 7.4, 10 mM KCl, and 0.2 mM Ca^{2+} ($^{45}Ca^{2+}$) at 37° C.

vesicles is probably attributable to some inhomogeneities in the population of inside out vesicles. Unsealed vesicles are those that sedimented through the dextran cushion (1.03 g/ml) during preparation, and their uptake probably represents passive processes in vesicles that have subsequently spontaneously resealed. These results indicate that the initial rate of $^{86}Rb^{+}$ uptake by inside out vesicles is a reasonable measure of the uptake process as long as comparisons are confined to a single preparation which may be assumed to be characterized by a constant fraction of inhomogeneous vesicles.

Binding of ions, such as Ca^{2+}, to membrane vesicles can also be measured using the same filtration technique, provided the membrane is relatively impermeable to the ion. Fig. 2 shows that association of $^{45}Ca^{2+}$ with right-side out vesicles reaches a constant rate in about 10—20 min. Subsequent addition of the Ca^{2+} ionophore A23187 (4 μg/ml) causes a further increase of Ca^{2+} associated with the vesicles which reaches a higher stable value in about 10 min. These observations indicate that the vesicle membrane is relatively impermeable to Ca^{2+} in the absence of the ionophore and that the initial uptake level reached in 10—30 min can be taken as a measure of Ca^{2+} binding to the membrane surface. We have used the amount of Ca^{2+} associated with vesicles after 30 min incubation at 37° C as the measure of this binding.

Effect of Ca^{2+} on K^{+} flux

Fig. 3 shows that 1 mM Ca^{2+} causes about a five fold increase in K^{+} uptake by inside out vesicles, though K^{+} flux into right-side out vesicles is not changed. This stimulation appears to be specific for Ca^{2+} as Mg^{2+} has a negligible effect and EGTA inhibits the stimulation as shown in Table I. These results indicate that Ca^{2+} can interact with the inner surface of the membrane and cause an increase in K^{+} permeability and that the effect of Ca^{2+} is specific to the cyto-

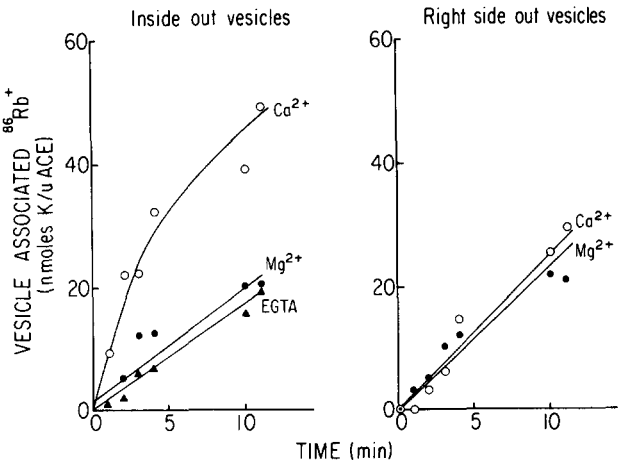

Fig. 3. Effect of Ca^{2+} on initial K^+ uptake into inside out and right-side out vesicles in one experiment typical of three. Vesicles were incubated at 37°C in 2.5 mM Tris-HCl, pH 7.4, and 10 mM KCl ($^{86}Rb^+$) and either 1 mM $MgCl_2$, $CaCl_2$ or EGTA.

plasmic face of the membrane. Furthermore the ineffectiveness of Mg^{2+} shows that the effect is not merely due to a change in ionic strength or other mechanisms related to charge density. We have also studied the effects of other cations and in a single exploratory experiment have found that 0.2 mM of La^{3+}, Cd^{2+}, Ba^{2+}, and Mn^{2+} cause increases in K^+ uptake by factors of two to five, measured in the first 5 min after addition of the divalent cation. The increase in permeability is not specific to K^+, since 1 mM Ca^{2+} also increases the permeability of inside out vesicles to Na^+ from 2.9 to 12.8 nmol/unit acetylcholinesterase per min (one experiment, typical of three).

To obtain the K^+ uptake data in Table I, points were taken at 0, 1, 2, 3, 4, 10 and 11 min, as shown in Fig. 3, and the uptake was determined from the initial linear segment of the time course. This procedure makes no allowance for the backflux which is probably negligible, since the calculated maximal backflux in the first 5 min of a typical experiment is 20% of the uptake,

TABLE I

EFFECT OF EGTA ON Ca^{2+}-INDUCED K^+ UPTAKE IN INSIDE OUT VESICLES

$^{86}Rb^+$ uptake into control vesicles made up in 2.5 mM Tris-HCl, pH 7.4, and 10 mM KCl was measured immediately in 0.2 mM Ca^{2+} or 0.2 mM Ca^{2+} plus 0.5 mM EGTA at 37°C as described in the text. EGTA-treated vesicles were either incubated in 0.2 mM Ca^{2+} for 2 min at 37°C alone or followed by a 3 min incubation in 0.5 mM EGTA before tracer uptake was measured. One experiment typical of two.

Vesicle pretreatment	nmol/unit acetylcholinesterase per min	
	K^+ uptake	Ca^{2+}-induced K^+ uptake
Ca^{2+} (0 min)	11.9	
Ca^{2+} + EGTA (0 min)	1.5	10.4
Ca^{2+} (2 min)	10.3	
Ca^{2+} (2 min) + EGTA (3 min)	2.6	7.7

though in extreme cases it reaches 33%. Table I shows that Ca^{2+} increases the permeability over that observed with EGTA by a factor of 3—7; the increase is reversible since removal of Ca^{2+} by EGTA restores the permeability of the vesicles to K^+ to the original level. Sometimes, EGTA caused the vesicle membrane to become even less permeable to K^+ than in its absence. These observations suggest that Ca^{2+} interacts with the membrane surface and that vesicle preparations sometimes may contain endogenously bound divalent cations. As Fig. 4 shows, Ca^{2+}-stimulated K^+ uptake is concentration dependent; there is a steady increase up to 0.5 mM Ca^{2+} and the effect saturates at about 1.0 mM Ca^{2+}. The lowest Ca^{2+} concentration at which an increase in uptake is detectable is around 0.05—0.1 mM and the half-maximal concentration is about 0.2 mM.

Our results are in disagreement with those of Grinstein and Rothstein [15] who did not find that 0.1 mM Ca^{2+} increased the efflux of Rb^+ from either inside out or right-side out vesicles. Their results on inside out vesicles are not directly comparable to ours since Grinstein and Rothstein studied the efflux from inside out vesicles when Ca^{2+} was added to the cytoplasmic face, whereas we studied the influx into inside out vesicles in the presence of cytoplasmic face Ca^{2+}, similar to the conditions in the intact cell. However, Grinstein and Rothstein's observations on efflux from right-side out vesicles when the ionophore A23187 was added would be expected to be similar to our results. At 0.1 mM cytoplasmic Ca^{2+}, we found that the K^+ (Rb^+) uptake increased from an average of 8.0 (two experiments; 8.5, 7.5) nmol/unit acetylcholinesterase per min to 11.7 (two experiments; 12.8, 10.5) nmol/unit acetylcholinesterase per min at 37°C. This difference is not large and may have escaped detection by their method. In some instances Grinstein and Rothstein also added Mg^{2+} to the reaction medium and we have found that Mg^{2+} decreases Ca^{2+} stimulation *.

In order to compare the Ca^{2+}-stimulated K^+ uptake that we have observed with results obtained in intact red cells it is necessary to summarize the literature data in terms of both cation specificity and Ca^{2+} concentration dependence. Ca^{2+} stimulation has been shown to be specific for K^+ in energy-depleted red cells [3] and ghosts [17], although Romero and Whittam [18] have found Ca^{2+} also to stimulate Na^+ flux in outdated bank blood. These results suggest that the specificity of the induced channel is dependent upon the state of the red cell.

A similar conclusion appears to apply to the concentration dependence. Micromolar concentration of Ca^{2+} were sufficient to stimulate K^+ efflux in adenosine triphosphate-depleted fresh red cells [3,19] and resealed ghosts [17]. However, Romero and Whittam used millimolar concentrations of Ca^{2+} to stimulate K^+ efflux from outdated bank blood [18]. Furthermore, Lew and Ferreira [16] observed a half-maximal Ca^{2+} concentration of about 0.5 mM for stimulation of K^+ influx in intact red cells in which the Ca^{2+} concentration

* Mg^{2+} decreases Ca^{2+}-stimulated K^+ uptake. The Ca^{2+}-stimulated K^+ uptake is 0.66 ± 0.5 nmol/unit acetylcholinesterase per min. (S.D., four experiments) at an alkali cation concentration of 0.05 mM Ca^{2+} plus 0.95 mM Mg^{2+}. This is to be compared with a figure of about 1.5—2 nmol/unit acetylcholinesterase per min as interpolated from Fig. 4.

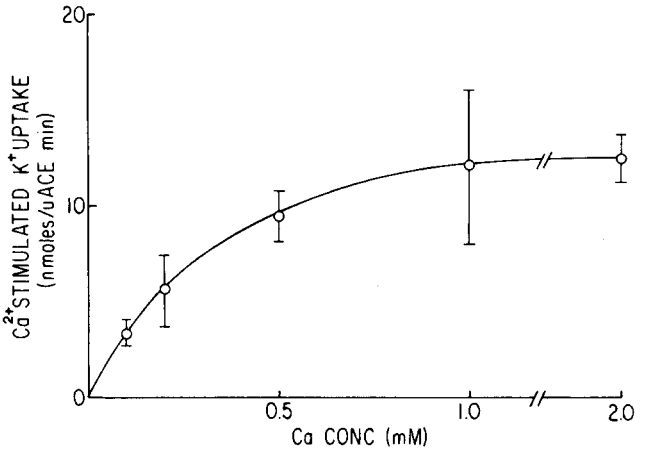

Fig. 4. Effect of Ca^{2+} concentration on K^+ uptake by inside out vesicles in four experiments. Vesicles were incubated at 37°C in 2.5 mM Tris-HCl, pH 7.4, and 10 mM KCl ($^{86}Rb^+$) plus various concentrations of $CaCl_2$. The vertical bars show the standard deviation in two or three duplicate experiments.

could be regulated by treatment with low concentrations of the ionophore A23187. They consider these red cells to be 'minimally disturbed' and suggest that this low affinity Ca^{2+} binding is typical of the normal cell. It is not clear whether the low affinity process observed by Lew and Ferreira maintains the sharp discrimination against Na^+ characteristic of the high Ca^{2+} affinity process in adenosine triphosphate-depleted red cells.

In our experiments, the half-value for Ca^{2+} stimulation is 0.2 mM, similar to that found by Lew and Ferreira [16] to be effective for 'minimally disturbed' red cells but greater by about three orders of magnitude than the effective concentration in adenosine triphosphate-depleted red cells. The Ca^{2+} stimulation of Na^+ uptake that we observe is also different from what is found in energy-depleted red cells. However, both the Ca^{2+} concentration range and the stimulation of Na^+ flux agree with Romero and Whittam's findings in outdated bank blood. As expected, ouabain which Lew [19] has shown to be without effect on the Ca^{2+}-stimulated K^+ efflux in intact red cells at adenosine triphosphate concentrations below 10^{-6} M had no effect on the Ca^{2+}-stimulated permeability increase of inside out vesicles.

It is disappointing that inside out vesicles do not exhibit the same specific Ca^{2+}-induced K^+ leak that characterizes adenosine triphosphate-depleted intact cells. It may well be that the differences reflect relatively small changes in the system since under some conditions, the intact red cell system is characterized by a Ca^{2+} stimulation effect similar to ours. Though many of the characteristics of the intact cell are preserved in inside out vesicles including adenosine triphosphate-dependent Na^+ transport [4,20], our experiments show that inside out vesicles differ significantly from adenosine triphosphate-depleted intact red cells with respect to Ca^{2+}-induced K^+ flux. This clearly indicates that the conformation of one class of Ca^{2+}-binding sites depends upon specific environmental conditions.

Concentration dependence of Ca^{2+} binding to vesicles

Ca^{2+} acts quickly to increase K^+ flux; the time course shown in Fig. 3 gives no evidence of a point of inflection so that the Ca^{2+} binding responsible for the stimulation must be effective in less than a minute or so. The kinetics of the Ca^{2+} binding in Fig. 2 show that there is at least one component that requires some 5—10 min for saturation of at least 30% of the apparent binding sites *. Hence there must be a diffusion barrier which inhibits this Ca^{2+} binding, whereas the Ca^{2+} directly implicated in stimulating K^+ flux must bind to sites which are immediately accessible, and probably peripheral. This leads to the conclusion that the effective sites must be included in the 70% of the sites filled by 5 min, the time of our first point in Fig. 2.

Studies on Ca^{2+} binding in inside out vesicles have shown that there are three Ca^{2+}-binding sites [1] and we were anxious to see if one of them was responsible for the Ca^{2+} stimulation effect. Ca^{2+} binding to inside out vesicles is decreased by the presence of 10 mM KCl (an essential component of our K^+ uptake medium). Cohen and Solomon [1] provided data and equations from which we can compute the binding constant in the presence of 10 mM K^+. These binding constants are (dissociation constant in parenthesis): site 1, $3 \cdot 10^6\ M^{-1}$ ($3 \cdot 10^{-7}$ M); site 2, $8 \cdot 10^3\ M^{-1}$ ($1 \cdot 10^{-4}$ M); site 3, $5 \cdot 10^2\ M^{-1}$ ($2 \cdot 10^{-3}$ M). Since the half-value for the Ca^{2+} stimulation effect in inside out vesicles, is $2 \cdot 10^{-4}$ M, stimulation cannot be attributed to site 1 whose association constant is $3 \cdot 10^{-7}$ M. Our half-value agrees well with the $1 \cdot 10^{-4}$ M association constant for site 2. Ca^{2+} is bound less tightly to site 3 whose association constant of $2 \cdot 10^{-3}$ M is one order of magnitude greater than our half-value. If the population of site 3 is homogeneous, as Cohen and Solomon's Scatchard plot shows [1], binding to that site will not be responsible for the stimulation effect. Site 2 comprises some 25% of the total number of sites found by Cohen and Solomon, consistent with the conclusion from Fig. 2 that no more than 70% of the Ca^{2+} sites can be involved in the K^+ stimulation effect. Thus, attribution of Ca^{2+} stimulation of K^+ uptake in inside out vesicles to Ca^{2+} binding to site 2 is consistent with these data as well.

The effect of temperature on Ca^{2+} binding to inside out vesicles and Ca^{2+}-stimulated K^+ transport was next investigated. Table II shows that, though binding of Ca^{2+} to inside out vesicles after 30 min was decreased by about 50% at 4°C, there is no observable temperature dependence of the Ca^{2+}-stimulated portion of K^+ uptake at 0.2 mM Ca^{2+}, the half-maximal concentration. Since site 3 comprises some 75% of the Ca^{2+}-binding sites, the temperature dependence of the binding could be ascribed to site 3, thus making the temperature dependence results consistent with our tentative assignment of the Ca^{2+} stimulation effect to binding at site 2. Table II also shows that K^+ uptake in the absence of Ca^{2+} is decreased at low temperatures, indicating that the Ca^{2+}-induced permeability pathway is separate from the K^+ pathway in the absence of Ca^{2+}.

* A preliminary experiment using the temperature jump technique (Verkman, A.S. personal communication) shows a Ca^{2+}-binding component with a half-time in the range of 0.1 s.

TABLE II

EFFECT OF TEMPERATURE ON Ca^{2+} BINDING AND Ca^{2+}-INDUCED K^+ UPTAKE IN INSIDE OUT VESICLES

The reaction medium contained inside out vesicles in 2.5 mM Tris-HCl, pH 7.4, 10 mM KCl and various concentrations of Ca^{2+}. The Ca^{2+} binding was measured at 30 min. The control K^+ uptake indicates K^+ influx in the absence of Ca^{2+}. The number of experiments is given in parenthesis. Errors are S.D.

Ca^{2+} conc. (mM)	Temperature (°C)	Ca^{2+} binding (nmol/unit acetylcholinesterase)	nmol/unit acetylcholinesterase per min	
			Control K^+ uptake	Ca^{2+}-induced K^+ uptake
0.1	4	1.7 ± 0.2 (2)	2.0 ± 0.2	1.9 ± 0.3 (2)
	37	3.6 ± 0.4 (4)	8.0 ± 0.7	3.6 ± 0.9 (2)
0.2	4	4.5 ± 1.9 (2)	2.0 ± 0.2	5.8 ± 1.5 (2)
	37	8.4 ± 1.3 (4)	8.0 ± 0.7	6.3 ± 2.8 (2)
0.5	4	7.8 (1)	1.8	15.9 (1)
	37	16.5 (1)	7.5	10.9 (1)

Nature of the Ca^{2+} site

We next investigated the effect of removing spectrin and other extrinsic membrane proteins on Ca^{2+}-induced K^+ uptake in inside out vesicles. About 50% of the extrinsic proteins, such as bands 1, 2, and 5 can be selectively

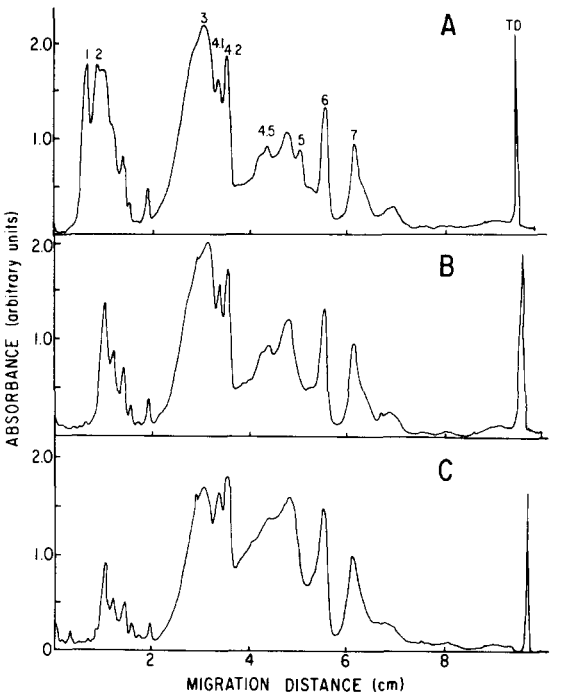

Fig. 5. SDS-polyacrylamide gel electrophoresis of inside out vesicles depleted of specific proteins. (A) Control inside out vesicles. (B) Inside out vesicles after 40 min treatment with 0.1 mM EDTA, pH 8.5. (C) Inside out vesicles after 18 h treatment in 0.1 mM EDTA, pH 8.5, in the presence of ovomucoid (5 μg/ml) and NaN_3 (0.1 mM).

TABLE III

EFFECT OF Ca^{2+} ON K^+ UPTAKE INTO VESICLES DEPLETED OF EXTRINSIC PROTEINS

Vesicles were incubated in 0.1 mM EDTA (pH 8.5) at 37°C. The reaction medium used to measure K^+ uptake contained 2.5 mM Tris-HCl, pH 7.4, 10 mM KCl and 1.0 mM Ca^{2+} or 1 mM Mg^{2+}.

Vesicle preparation	nmol/unit acetylcholinesterase per min		Ca^{2+}-induced K^+ uptake
	K^+ uptake		
	Mg^{2+}	Ca^{2+}	
Normal inside out vesicles	4.1 ± 2.0	15.5 ± 7.4	11.4 ± 5.9 (6)
Spectrin depleted, 40 min	3.8 ± 1.8	14.0 ± 4.5	10.3 ± 3.3 (5)
Spectrin depleted, 18 h	3.7 ± 2.1	14.3 ± 7.3	10.5 ± 5.5 (5)

removed by treatment of the vesicles with 0.1 mM EDTA, pH 8.5, for 40 min [4]. Fig. 5 shows that further incubation for 18 h resulted not only in further loss of bands 1, 2, and 5 but also partial degradation of band 3 even in the presence of ovomucoid and azide. However, even these drastic changes in membrane protein composition had no effect on the gross orientation of the vesicles since the acetylcholinesterase inaccessibility remained in the range of 85 ± 2%. Table III shows that 18 h depletion did not alter the Ca^{2+}-induced K^+ uptake, which means that bands 1, 2, and 5 are not the major sites of Ca^{2+} interaction and that intact band 3 is not necessary. Table IV shows that Ca^{2+} binding to these vesicles at concentrations of 0.1—0.2 mM did not differ significantly from normal though, at higher Ca^{2+} concentrations, vesicles extensively depleted of extrinsic proteins showed a decrease in Ca^{2+} binding.

Many interactions are known to exist between Ca^{2+} and membrane proteins. Ca^{2+} is thought to bind to spectrin (see Kirkpatrick [21]) and Ca^{2+} interactions affect the shape and elasticity of red cell membranes [22—24], the distribution of intramembrane particles [25,26], and sialoglycoproteins [27]. Furthermore, isolated spectrin aggregates in the presence of approx. 1 mM divalent cations [28]. Thus, it is surprising that removal of most of the spectrin has so slight an effect on Ca^{2+} binding.

To determine whether other membrane proteins were involved in the increase in permeability due to Ca^{2+}, the effect of sulfhydryl reagents on Ca^{2+}-stimulated K^+ uptake was investigated. Certain sulfhydryl groups in the membrane have been implicated in maintaining a low membrane permeability to

TABLE IV

EFFECT OF REMOVING EXTRINSIC PROTEINS ON Ca^{2+} BINDING TO INSIDE OUT VESICLES

Ca^{2+} conc. (mM)	Ca^{2+} binding (nmol/unit acetylcholinesterase at 30 min)		
	Control	40 min depleted	18 h depleted
0.1	3.6 ± 0.4 (4)	4.9 ± 0.1 (2)	3 ± 1 (2)
0.2	8 ± 1 (4)	10.8 (1)	7.4 (1)
0.4	17 ± 1 (2)	19 ± 1 (2)	12.1 ± 0.2 (2)
1.0	31.2 ± 0.1 (2)	35 (1)	23 ± 4 (2)

TABLE V

EFFECT OF *N*-ETHYLMALEIMIDE ON Ca^{2+}-INDUCED K^+ UPTAKE INTO INSIDE OUT VESICLES

K^+ uptake was measured in a reaction medium containing 2.5 mM Tris-HCl, pH 7.4, 10 mM KCl, and 0.2 mM Ca^{2+}. Vesicle proteins were modified with 2 mM *N*-ethylmaleimide (NEM) for 30 min at 37°C and washed as described in the text.

Vesicle preparation	K^+ uptake (nmol/unit acetylcholinesterase per min)		(Total K^+ uptake (+ Ca^{2+}))/ (Control (— Ca^{2+}))
	Control	Ca^{2+}	
Control inside out vesicles	2.9 ± 0.5	8 ± 4	2.7 ± 0.8 (4)
NEM-treated inside out vesicles	5 ± 2	5 ± 1	1.2 ± 0.1 (3)
NEM-treated right-side out vesicles	1.9	1.8	1 (1)

cations [29,30]. Table V shows that *N*-ethylmaleimide, a sulfhydryl reagent that readily permeates the membrane, inhibits the Ca^{2+} stimulation of K^+ uptake. The decrease in the ratio (total K^+ uptake (+Ca^{2+})/control (—Ca^{2+})) from 2.7 for the untreated vesicles to 1.2 in the *N*-ethylmaleimide-treated ones is significant at a P value of <0.025. This indicates that sulfhydryl groups can control the Ca^{2+}-stimulated influx of these vesicles and directly implicates membrane proteins in the process. The table shows that *N*-ethylmaleimide treatment opens up the normal K^+ leak channel ($P < 0.02$). The evidence that it

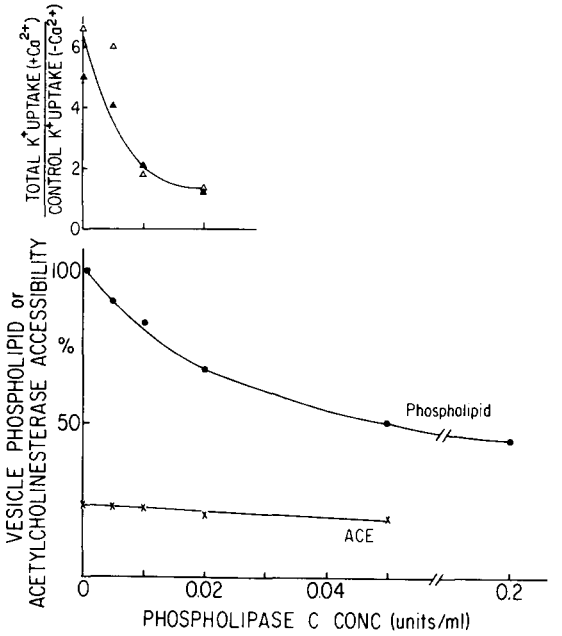

Fig. 6. Effect of phospholipase C concentration on Ca^{2+}-stimulated K^+ uptake, phospholipid content and acetylcholinesterase accessibility. Vesicles were treated with various concentrations of phospholipase C (*B. cereus*) and washed in 2.5 mM Tris-HCl, pH 7.4, and 10 mM KCl. One unit of phospholipase C, according to Sigma Chemical Co., will liberate 1.0 μmol/min of organic phosphorus from phosphatidylcholine at 37°C.

TABLE VI

EFFECT OF PHOSPHOLIPASE C ON Ca^{2+}-INDUCED K^+ UPTAKE INTO INSIDE OUT VESICLES

The reaction medium to measure K^+ uptake included 2.5 mM Tris-HCl, pH 7.4, 10 mM KCl and 0.2 mM Ca^{2+}. K^+ uptake indicates K^+ influx in the absence of Ca^{2+}. The data are the means of two experiments and the error is the excursion from the mean.

Phospholipase C conc. (units/ml)	K^+ uptake (nmol/unit acetylcholinesterase per min)		Ca^{2+}-induced K^+ uptake (nmol/unit acetylcholinesterase per min)
	Control	+ Ca^{2+}	
0	2 ± 1	13 ± 2	11
0.005	3 ± 2	13 ± 5	10
0.01	3 ± 1	7 ± 3	4
0.02	5 ± 1	6 ± 1	1

suppresses the Ca^{2+}-induced K^+ leak comes from the fact that the ratio (total K^+ uptake (+Ca^{2+})/control (—Ca^{2+})) is 1.2 ± 0.1, and strengthens the conclusion drawn from the temperature dependence studies that the pathway for control K^+ influx is different from that of the Ca^{2+}-induced uptake.

We next investigated the effect of removing charged groups of phospholipids from the inner membrane surface. As the cytoplasmic phospholipids of the red cell membrane are primarily composed of negatively charged phospholipids such as phosphatidylserine and neutral phospholipids such as phosphatidylethanolamine [31], it seemed possible for Ca^{2+} to interact with the negative groups to cause the permeability increase. Fig. 6 shows that partial removal of the head group of phospholipids from vesicles by phospholipase C (*B. cereus*) did not affect the gross orientation and vesicle impermeability as judged by acetylcholinesterase accessibility. Since some 50% of the phospholipids are on the cytoplasmic face of the vesicle (Fig. 6; Ref. 32), Fig. 6 shows that virtually all the head groups of phospholipids on this face can be removed without vesicle breakdown. However, at higher phospholipase C concentrations, above 0.02 unit/ml, the vesicles became increasingly leaky to K^+, so measurements of K^+ flux are no longer reliable.

The top section of Fig. 6 shows that the ratio (total K^+ uptake (+Ca^{2+})/ Ca^{2+} control (—Ca^{2+})) falls from an average value of 5.5 in the absence of phospholipase C to just about unity at a phospholipase C concentration of 0.02 unit/ml. The ratio decreases sharply at the lower enzyme concentrations and has already fallen to a value of 1.3 at a phospholipase C concentration of 0.01 unit/ml which has only removed 20% of the phospholipids. Thus that fraction of the phospholipid which is most sensitive to phospholipase C attack is just that fraction most effective in controlling the Ca^{2+}-induced K^+ uptake. These results clearly implicate the negatively charged head groups in the permeation process and suggest that the most superficial or easily accessible groups are the most important.

We did not detect any change in Ca^{2+} binding to inside out vesicles treated with phospholipase C. Vesicles treated with 0.005 and 0.01 unit/ml phospholipase C showed 6.9 and 7.6 nmol bound Ca^{2+}/unit acetylcholinesterase at 20 min, compared to a control of 7.4 nmol/unit acetylcholinesterase at 20 min

in one experiment. This is not inconsistent with our previous suggestion that site 2, which contains some 25% of the bound Ca^{2+}, may be responsible for Ca^{2+}-mediated K^+ uptake.

Our data show that the preparation of inside out vesicles causes the properties of the site for the Ca^{2+}-induced increased permeation of K^+ to change in important ways. The studies of Romero and Whittam [18] and Lew and Ferreira [16] had indicated that the properties of the site were labile, in that micromolar concentrations of Ca^{2+} did not always cause a specific increase in K^+ permeability. The present data demonstrates that Ca^{2+} induces the formation of a cation channel in inside out vesicles, whose selectivity is also different from that in intact cells and ghosts [16,17]. The sensitivity to micromolar concentrations of Ca^{2+} is lost when the vesicles are prepared, an observation which confirms Grinstein and Rothstein's [15] findings in this respect. Thus, it seems likely that the Ca^{2+}-induced cation channel in inside out vesicles is a modification of the one found in intact cells but we cannot exclude the possibility that the original channel is sealed in the process of vesicle formation and a new one opened.

The studies with *N*-ethylmaleimide show that the vesicle membrane channel is mediated by a protein and the passage is controlled by sulfhydryl groups. Furthermore, these studies confirm the conclusion from the temperature dependence results that the Ca^{2+}-induced vesicle pathway is distinct from the normal channel for passive K^+ leak. Since removal of most of the spectrin does not affect the properties of the vesicle channel, it seems most unlikely that it is controlled by Ca^{2+} binding to spectrin. Rather the channel protein is sensitive to its phospholipid environment since removal of easily accessible phospholipid head groups on the cytoplasmic face of the vesicles inhibits the Ca^{2+}-stimulated channel opening. Though the site which opens the channel cannot discriminate between Ca^{2+} and some other divalent ions such as La^{3+} and Mn^{2+}, it can discriminate very well between Ca^{2+} and Mg^{2+}; indeed, the presence of Mg^{2+} has an inhibitory effect on the Ca^{2+} activation. These several properties of the Ca^{2+} channel in vesicles may well possess a broader significance in view of the importance of Ca^{2+}-controlled cation channels in other biological systems.

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