*Biochimica et Biophysica Acta*, 644 (1981) 351–362
Elsevier/North-Holland Biomedical Press



BBA 79278

# ACTIVATION AND DEACTIVATION KINETICS OF Ca TRANSPORT IN INSIDE-OUT ERYTHROCYTE MEMBRANE VESICLES

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(Received September 2nd, 1980)
(Revised manuscript received February 20th, 1981)

*Key words: $Ca^{2+}$ transport; Calmodulin; Inside-out vesicle; (Erythrocyte)*

**The kinetics of active $Ca^{2+}$ transport in inside-out vesicles of human erythrocyte membranes has been studied. Hemolysate or purified calmodulin increased the apparent affinity of the $Ca^{2+}$ transport system for $Ca^{2+}$ and increased the apparent maximum velocity of $Ca^{2+}$ transport. However, as $Ca^{2+}$ concentration was increased above 90 $\mu$M in the presence of hemolysate or calmodulin, the extent of activation of $Ca^{2+}$ fluxes decreased. This deactivation was less prominent if the $Mg^{2+}$ concentration was increased. These results may be explained by postulating that calmodulin has a site which binds either $Mg^{2+}$ or $Ca^{2+}$ and if $Mg^{2+}$ occupies this site calmodulin activates $Ca^{2+}$ fluxes, while if $Ca^{2+}$ occupies this site, calmodulin is unable to activate the transport system.**

## Introduction

The human erythrocyte contains a $Mg^{2+}$ plus ATP-dependent $Ca^{2+}$ transport system which maintains intracellular $Ca^{2+}$ concentrations at below $10^{-6}$ M [1,2]. The maintenance of these low $Ca^{2+}$ concentrations is important to protect the cell against rigidity [3,4] impairments in active Na-K transport [5], changes in $K^+$ conductance [6] and inhibition of anion exchange [7,8].

Many studies have evaluated the active $Ca^{2+}$ transport system which regulates free intracellular $Ca^{2+}$ in human red cells [2,5,9]. But technical limitations make kinetic studies of $Ca^{2+}$ fluxes using resealed ghosts or intact cells difficult, while inside-out vesicles have proven useful as a model and avoid many of these limitations. For example, kinetic studies of active $Ca^{2+}$ transport in inside-out vesicles have suggested that the kinetics can be altered by a cytoplasmic protein activator [10–12]. This activator protein appears to increase both the apparent affinity of the $Ca^{2+}$ pump for $Ca^{2+}$ and also the apparent $V$ of $Ca^{2+}$ transport [11]. These effects of activator protein are consistent with similar alterations by activator protein on the kinetics of membrane-bound ($Ca^{2+} + Mg^{2+}$)-dependent ATPase observed in open red cell membranes [13].

The red cell activator protein first described by Bond and Clough [14] has been purified [15] and is believed identical or closely homologous with the $Ca^{2+}$-dependent protein activator of adenylate cyclase and phosphodisterase which has been called modulator protein, calmodulin or calcium-dependent regulatory protein [12,16,18–22].

This report describes experiments which characterize the kinetics of $Ca^{2+}$ transport in inside-out vesicles from human erythrocyte membranes and the effect of activator protein on these kinetics.

## Methods

*Preparation of inside-out vesicles.* Vesicles were prepared from fresh heparinized blood from a single

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Abbreviations: IOV, inside-out vesicles; AChE, acetylcholinesterase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid.

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donor (JDM). The fresh cells were washed three times at 4°C in 150 mM NaCl plus 5 mM $Na_2HPO_4/NaH_2PO_4$, pH 8.0 (at room temperature) by repeated resuspension, centrifugation and aspiration of the supernatant.

White ghosts were made from these washed cells as described by Steck and Kant [23]. 2 ml packed cells were hemolyzed in 36 ml of ice-cold 5 mM $Na_2HPO_4/NaH_2PO_4$, pH 8.0. The suspension was centrifuged at $20\,000 \times g$ for 10 min. The supernatants and any sticky portion of the pellet were aspirated. Three further washes with the same ice-cold solution yielded a pellet of creamy white ghosts.

Inside-out vesicles were made by a slight modification of the method of Steck and Kant [23]. 2 ml homogenized vesicle suspension were layered onto 3 ml of an ice-cold Dextran T-70 barrier (4.46 g/100 ml 0.5 mM $Na_2HPO_4/NaH_2PO_4$) in a 15 ml plastic tube and centrifuged for 40 min at $31\,000 \times g$ at 4°C in an RC-5 Sorvall centrifuge. After centrifugation, about 50% of the added suspension appeared to be sealed and remained on the upper surface of the barrier. Sealed vesicles from the top of two barriers were combined in one 50 ml tube. The vesicles were resuspended to 40 ml with ice-cold 10 mM Tris-HCl/0.5 M EDTA, pH 8.0 (at room temperature) and spun for 20 min at $28\,000 \times g$. Thrice the supernatant fluid was removed thoroughly and the pellet dislodged with vortex mixing and resuspended with 40 ml 10 Tris-HCl, pH 7.5 (at room temperature). The final pellet of vesicles was resuspended to a concentration of 2–4 mg protein/ml and refrigerated until needed. Prior to measurement of the flux an equal volume of ice-cold solution comprising 280 mM choline chloride/20 mM Tris-HCl, pH 7.5 (at room temperature) was added to the suspension and divided into aliquots for use as described below.

*Preparation of hemolysate.* 2 ml packed cells were first washed four times in 150 mM NaCl plus 5 mM $Na_2HPO_4/NaH_2PO_4$, pH 8.0, then hemolysed in 20 vol. ice-cold deionized water and centrifuged for 30 min at $44\,000 \times g$ at 4°C. The supernatant was transferred with a plastic pipette and recentrifuged as above. The supernatant was again removed with a plastic pipette and either directly stored or dialyzed before freezing at −70°C.

*Dialysis.* The crude hemolysate was added to prerinsed tubing and dialyzed against 30 vol ice-cold deionized-distilled $H_2O$ for 4–5 h at 8°C. A second dialysis with fresh water was continued overnight at 8°C. This reduced the $K^+$ content to less than 40 nmol $K^+$/mg of hemolysate hemoglobin. Suspensions of rehydrated, lyophilized calmodulin were dialyzed similarly.

*$Ca^{2+}$ flux determination.* The flux medium containing $^{45}Ca$ tracer was prewarmed to 37°C in a thermostatically controlled chamber containing a temperature probe (model 409 Yellow Springs Instrument Co., Yellow Springs, OH). We initiated the flux at time zero by injecting 150 μl of prewarmed inside-out vesicles suspended in 140 mM choline chloride/10 mM Tris-HCl, pH 7.5, into 15 ml flux medium at 37°C. After about 15 s, we separately filtered three 1-ml aliquots of this suspension under vacuum using Millipore filters (HAWP 02500; average pore size 0.45 μm; Millipore Corp., Bedford, MA) which had been prewashed with 6 ml ice-cold 160 mM KCl. The vesicles on the filter were immediately washed by aspirating 6 ml ice-cold 160 mM KCl through the filter. After 3 min and 6 min, triplicate samples were again removed, filtered and washed as above. The washed filters were placed in liquid scintillation vials to which 10 ml scintillation fluid was added. After 7 min we removed aliquots of the final flux suspension from the chamber with plastic pipettes and stored them in plastic test tubes capped with Parafilm (American Can Co.) in the refrigerator until assayed for specific activity of $^{45}Ca$ or hemoglobin concentration. In some cases the $Mg^{2+}$ concentration was also measured in these aliquots.

The basic composition of the final flux suspension was usually the following: 130 mM NaCl, 2 mM $MgCl_2$, 0.5 mM $Na_2ATP$, 20 mM Mes/Tris buffer, pH 6.5, at 37°C and different levels of $CaCl_2$. The Mes/Tris buffer was prepared by titrating 120 mM Mes (Calbiochem, San Diego, CA) to the desired pH using granular Tris as the titrant. In some cases, dialyzed hemolysate or purified calmodulin were also present in the vesicle suspension. The specific composition of medium used in a particular experiment is given in the legend for that experiment. The small contribution of choline chloride (1.4 mM) common to all experiments was neglected throughout.

*Acetylcholinesterase.* This assay was based on the

method of Ellman et al. [24] as modified by Steck and Kant [18]. Vesicle suspensions were measured for the amount of acetylcholinesterase in the presence and absence of the detergent triton X-100. Vesicle preparations described in this paper ranged from 70 to 83% inside-out based on the accessibility of acetylcholinesterase. This activity in leaky ghosts was also measured to control for the reagents used.

*Atomic absorption spectrophotometry.* All ion measurements are made using a Perkin Elmer Model 460 atomic absorption flame photometer (Perkin-Elmer Inc., Norwalk, CT). Total Ca concentration was determined by measuring Ca concentration in aliquots of the final flux suspension with 1% $La^{3+}$ added to control interference. Using commercial (Fischer Scientific Co., Fair Lawn, NJ) or our own standards prepared from $CaCO_3$, total Ca was determined in an air-acetylene flame. When necessary, the $Mg^{2+}$ concentration was also determined in the presence of 1% $La^{3+}$ using a commercial standard and air-acetylene flame.

*Protein.* Protein concentration was measured by the method of Lowry et al. [25] using bovine serum albumin (Schwartz-Mann, Orangeburg, NY) as a standard.

*Hemoglobin.* Hemoglobin measurement was based on the method of van Kampen and Zijlstra [26]. 1 ml final incubation suspension (when it contained dialyzed hemolysate) was added to 2 ml reagent which contained, per l: 200 mg $K_3Fe(CN)_6$, 50 mg KCN, 140 mg $KH_2PO_4$ and 0.5 ml Acationox (Scientific Products, McGraw Park, IL). The resultant suspension stood 1 h or more at room temperature and then the absorbance was measured at 540 nm in a Gilford Stasar III (Gilford Instruments, Oberlin, OH) spectrophotometer. Blanks contained vesicle suspensions without hemoglobin.

*Purified calmodulin.* Rat testis calmodulin was the generous gift of Dr. Kenneth Seamon of Washington University, St. Louis, MO. The sample was received as a lyosphilized crystal. When needed, the calmodulin was rehydrated with deionized-distilled $H_2O$ and dialyzed as described earlier.

*Chemicals.* Inorganic chemicals were reagent grade from commercial sources, ATP was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) and acetylthiocholine chloride were purchased from Sigma Chemical Co., (St. Louis, MO). EGTA and EDTA were purchased from Eastman-Kodak (Rochester, NY).

*Radioactivity.* $^{45}Ca$ was purchased from ICN (Irvine, CA) and diluted with $CaCl_2$ as needed. The actual specific activity in the final incubation suspension was high enough to give an increase of 1000 cpm or more over a 3 min incubation period when 1 ml of a vesicle suspension containing Mg and ATP was filtered and washed with 160 mM KCl.

*Calculations of $Ca^{2+}$ flux.* The initial rate of tracer influx was measured as the slope of the regression line of the measurements taken at 0.25, 3.25 and 6.35 min. The specific activity of the $^{45}Ca$ was determined from the total Ca concentration and the average radioactivity of $^{45}Ca$ in duplicate 10 $\mu$l aliquots of the final flux suspension. From a measurement of the mg of vesicle protein per unit volume of suspension injected into the prewarmed fluxing medium, the concentration of protein in the final flux medium was calculated. Since the Millipore filters retain vesicles and allow suspending medium to pass, the volume of suspension filtered was proportional to the amount of vesicles remaining on the filter. Since we knew the amount of $^{45}Ca$ which appeared on the filter, the amount of vesicle membranes on the filter and the time interval between filtrations, the $Ca^{2+}$ flux could be calculated as nmol $Ca^{2+}$/mg IOV protein per min.

Fluxes reported in this paper as nmol $Ca^{2+}$/mg IOV protein per min were based on the total vesicle protein in the preparation and were not corrected for the usual 20% of the vesicles which were sealed right-side-out. However the fluxes reported which are normalized to units of acetylcholinesterase were corrected for contamination by right-side-out vesicles.

## Results

Fig. 1 shows the activation kinetics by $Ca^{2+}$ on active calcium flux into inside-out vesicles after different periods of storage at 4°C. The average $K_{1/2}$ value (Ca concentration causing half-maximal flux) was 19 $\mu$M and the average $V$ value (maximum $Ca^{2+}$ influx) was 23.8 nmol $Ca^{2+}$/mg IOV protein per min. When normalized to units of acetylcholinesterase (AChE) the average $V$ value in Fig. 1 was 7.6

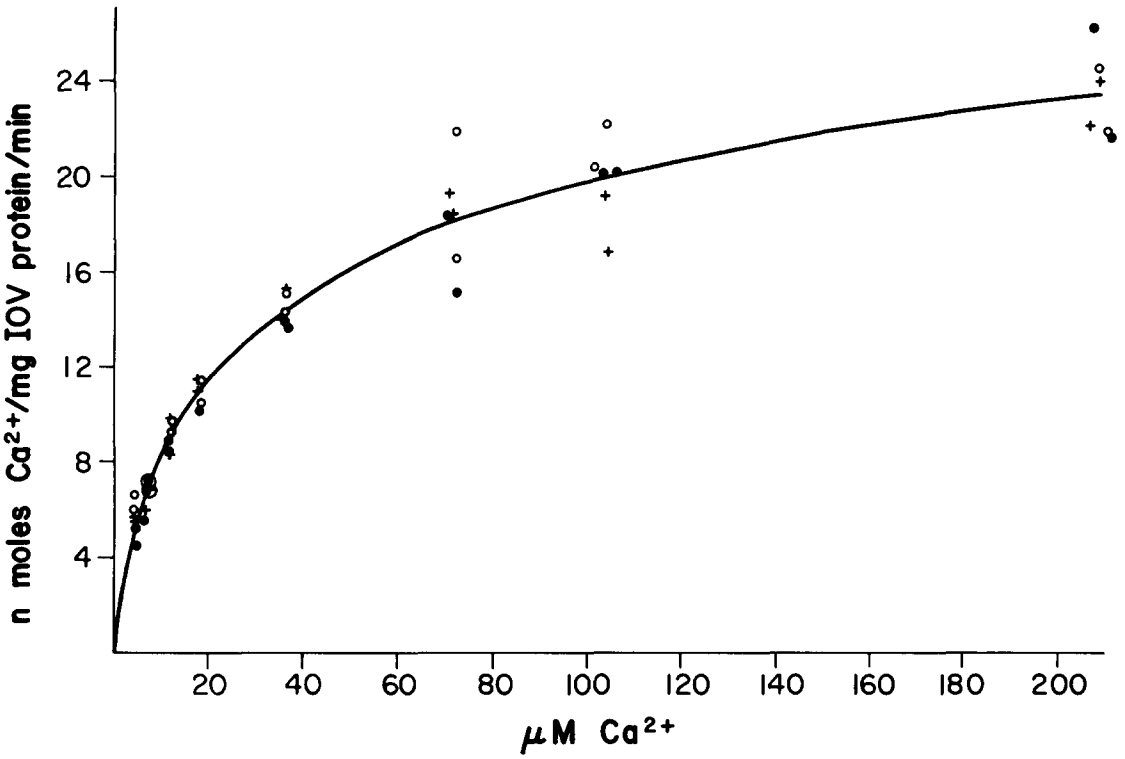


Fig. 1. The kinetics of ATP dependent calcium fluxes after storage at 4°C. $Ca^{2+}$ influx was measured at 37°C in 130 mM KCl/2 mM $MgCl_2$/0.5 mM $Na_2ATP$/20 mM Tris-HCl, pH 7.1 with 18–19 μg vesicle protein/ml. To correct for nonlinearity of $Ca^{2+}$ uptake at $Ca^{2+}$ concentrations over 70 μM, calculations of the initial rates were based on Ca fluxes from 0.25 to 3.25 min. Without this correction $K_{1/2}$ would have been underestimated [27]. The data curves were analyzed by the method of Wilkinson [28].

| | Symbol | $K_{1/2}$ (μM Ca) | $V$ (nmol Ca/mg IOV protein per min) |
|---|---|---|---|
| Day 1 | • | 21.5 ± 2.4 | 23.7 ± 0.9 |
| Day 2 | + | 18.0 ± 2.2 | 23.2 ± 0.9 |
| Day 3 | ○ | 18.1 ± 2.3 | 24.5 ± 1.0 |

nmol $Ca^{2+}$/unit AChE per min. Using 10 units of AChE/ml of packed cells as a standard value [25], then our $V$ value for $Ca^{2+}$ flux in inside-out vesicles may be compared with the $V$ value of 8.5 nmol $Ca^{2+}$/unit AChE per min for active $Ca^{2+}$ efflux from the intact cell [5]. We have found that at pH 7.1 with high $Ca^{2+}$ concentrations the $Ca^{2+}$ uptake over the 6 min is non-linear. This non-linearity was greater at high pH values. For example, at pH 8.0 and 300 μM $Ca^{2+}$, $Ca^{2+}$ influx from 0.25 to 3.25 min was twice the $Ca^{2+}$ influx from 3.25 to 6.25 min. To insure that initial rates of $Ca^{2+}$ flux would be measured if activator was added, the remaining experiments presented in this paper were performed at pH 6.4–6.5. Three experiments were performed as described in Fig. 1 on the same vesicle preparation, the first on the day blood was drawn (day 1) and one each on two successive days thereafter (days 2 and 3). The results indicate no noticeable effect of such storage on basal vesicle $Ca^{2+}$ fluxes.

In Fig. 2a the $Ca^{2+}$ dependency of $Ca^{2+}$ fluxes in four different inside-out vesicle preparations is

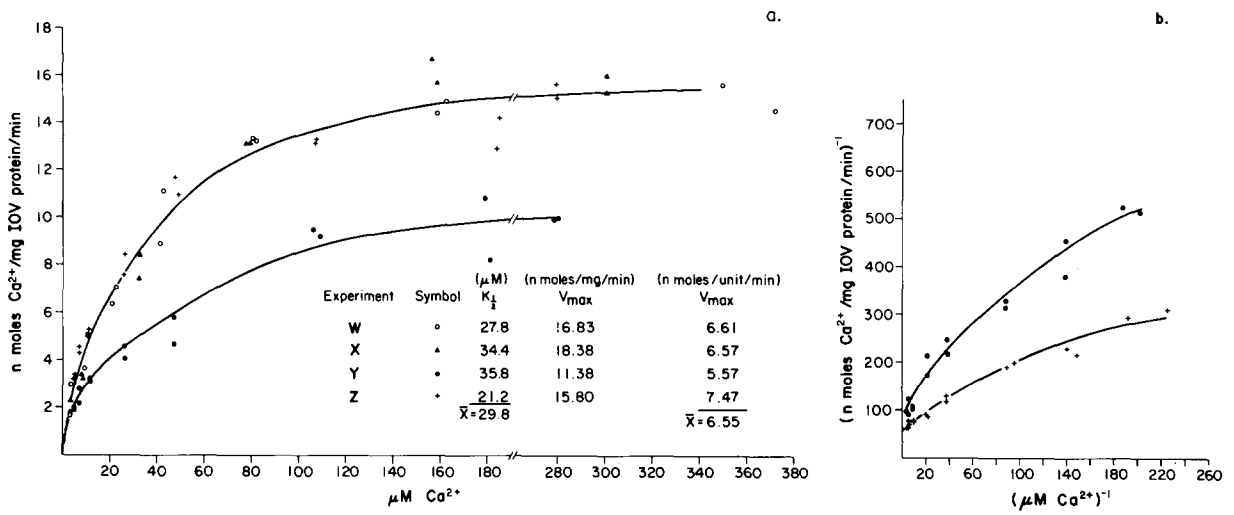


Fig. 2. The reproducibility of $Ca^{2+}$ fluxes among different preparations of inside-out vesicles. All fluxes were measured at 37°C in 130 mM NaCl, 2 mM $MgCl_2$, 0.5 mM $Na_2ATP$, 20 mM Mes/Tris, pH 6.4–6.5, with 10–20 μg vesicles protein/ml. Panel a shows a Michaelis-Menten analysis of $Ca^{2+}$ uptake kinetics in the four different vesicle preparations. The best values for the parameters for the separate hyperbola [28] are given. Only one curve is drawn through three similar experiments for clarity. This curve is used as reference in Figs. 3 and 4. Panel B shows a double-reciprocal plot of the data from experiments Y and Z shown in panel a. The non-linearity of this double reciprocal plot is evidence for two classes of pump in the membranes: one with a low capacity but high velocity and one with a high capacity at a slower velocity.

shown. These fluxes represent baseline values for ATP dependent $Ca^{2+}$ fluxes in our inside-out vesicle preparation at pH 6.4–6.5 in the absence of added activator. The differences in maximal velocity were greatly diminished when they were normalized to the acetylcholinesterase activity of the vesicles, which appeared to be a better measure of functioning pumps than total membrane protein in these vesicle preparations. The average $V$ value of the four experiments was 6.55 nmol $Ca^{2+}$/unit IOV AChE per min and the average $K_{1/2}$ value was 29.8 μM.

Fig. 2b is a Lineweaver-Burk plot of the data from experiments Y and Z in Fig. 2a. Neither reciprocal plot is linear. The same is also true if experiments W or X from Fig. 2a are replotted in double-reciprocal form. These kinetic results may be interpreted to mean that the preparation of vesicles contains $Ca^{2+}$ pumps in two states. As suggested earlier, [13] this could mean that there are many pumps with a low affinity which are deficient in calmodulin while there are a few pumps with a high affinity for calcium which have retained calmodulin. In the calmodulin deficient preparation of vesicles these pump forms would not be interconvertible in the time frame of these experiments otherwise only linear double-reciprocal plots would be obtained. But in vivo or upon adding calmodulin in vitro, low affinity pumps might be convertible to high affinity pumps. This analysis may also suggest that the variation in kinetic constants between experiments (see inset of Fig. 2a) may reflect differences in residual activator or calmodulin bound to vesicles in the different preparations.

In Fig. 3, $Ca^{2+}$ flux into inside-out vesicles was measured as a function of $Ca^{2+}$ concentration in the presence of dialyzed, membrane-free hemolysate. The lowest curve (experiment A) shows the kinetics observed in the absence of hemolysate. Hemolysate significantly enhanced the $Ca^{2+}$ flux at every $Ca^{2+}$ concentration (experiments B and C). Hemolysate elevated the apparent $V$ value and decreased the $K_{1/2}$ for $Ca^{2+}$ relative to the control values. These results were consistent with our earlier report describing a similar effect when lower levels of hemolysate were present [11]. A new finding was that when $Ca^{2+}$ concentration increased beyond 90 μM in the presence of high levels of hemolysate (experiment B), the extent of activation decreased. That is, there

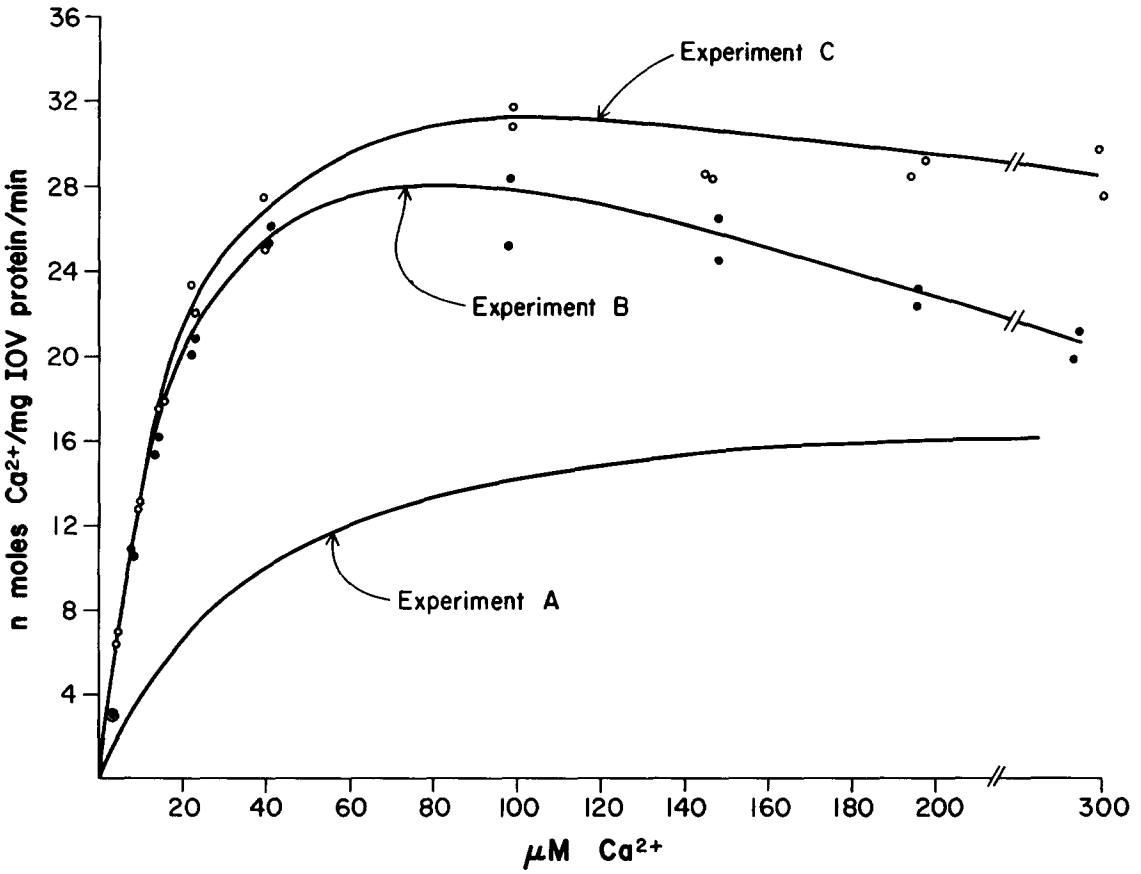


Fig. 3. The influence of dialyzed hemolysate on active $Ca^{2+}$ fluxes into inside-out vesicles. Fluxes were measured at 37°C in a medium of the following final composition: 130 mM NaCl, 2 mM (experiments A and B) or 4 mM (experiment C) $MgCl_2$, 0.5 mM $Na_2ATP$, 20 mM Mes/Tris, pH 6.4, Experiment A is the average replotted from Fig. 2 of experiments W, X and Z to indicate control flux levels. In experiments B and C, there were 15 μg vesicle protein/ml. In experiments B and C the final hemoglobin concentrations were 4.4 mg/ml and 4.1 mg/ml, respectively. This difference in hemolysate concentrations did not significantly affect the qualitative outcome of the experiment.

appeared to be a deactivation of the hemolysate activator. When the $Mg^{2+}$ concentration in the flux suspension was increased from 2 mM (experiment B) to 4 mM (experiment C) in the presence of a comparable amount of hemolysate, the deactivation at high calcium concentrations was less prominent. These results show that increased $Mg^{2+}$ concentration reduced the deactivation at each $Ca^{2+}$ concentration compared to the deactivation observed at lower $Mg^{2+}$ concentrations.

The effect of a single exogenous protein, purified calmodulin, on the kinetics of active $Ca^{2+}$ fluxes (Fig. 4) was essentially the same as the effect of dialyzed hemolysate. The lowest curve (W) shows the $Ca^{2+}$ dependence of the $Ca^{2+}$ flux in the absence of calmodulin. The $V$ was 16 nmol $Ca^{2+}$/mg IOV protein per min and the $K_{1/2}$ value was approx. 30 μM. In curves B and C the $Ca^{2+}$ fluxes were measured in the presence of 0.42 μg calmodulin/ml suspension and 2 mM $Mg^{2+}$ (B) or 4 mM $Mg^{2+}$ (C). In both curves the $K_{1/2}$ for $Ca^{2+}$ was decreased and the apparent $V$ value was increased due to the addition of calmodulin. Above 80–90 μM $Ca^{2+}$ concentration, the extent of activation decreased in a manner similar to that observed using dialyzed hemolysate as the source of activator and $Mg^{2+}$ appeared to protect against the deactivation induced at high $Ca^{2+}$ concentrations. The $Ca^{2+}$ fluxes in

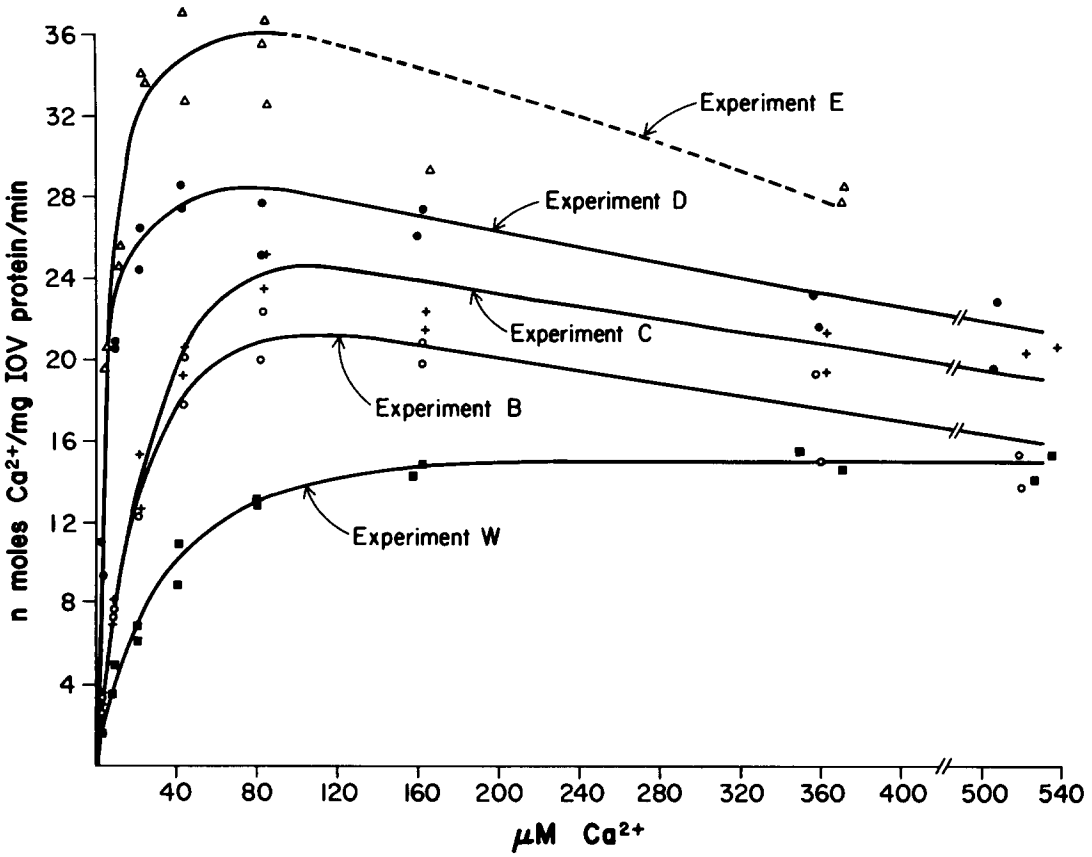


Fig. 4. The influence of purified calmodulin on $Ca^{2+}$ fluxes into inside-out vesicles. ATP dependent $^{45}Ca$ influxes were measured at 37°C in a medium of the following final composition: 130 mM NaCl, 2 mM (experiments W, B, D, E) or 4 mM (experiment C) $MgCl_2$, 0.5 mM $Na_2ATP$, 20 mM Mes/Tris, pH 6.5. Expt. W was performed on a separate vesicle preparation. It is replotted from Fig. 2 to indicate control flux levels. In experiment B, C and D the final vesicle protein concentration was 10 μg/ml. In experiment E, the final vesicle protein concentration was 5 μg/ml. Dialyzed calmodulin was added to the following final concentrations in μg/ml; experiment W, 0; experiments B and C, 0.42; experiment D, 4.2; and experiment E, 34. Calmodulin was responsible for both the enhanced flux and the deactivation kinetics seen with crude hemolysate.

experiments B and C were identical in the $Ca^{2+}$ concentration range 3–40 μM. The apparent $V$ in experiment C (24.5 nmol $Ca^{2+}$/mg IOV protein per min) was higher than the apparent $V$ in experiment B (21.1 nmol $Ca^{2+}$/mg IOV protein per min) and the $K_{1/2}$ (18 μM) was higher than in experiment B (12 μM). These results confirm the interpretation of those shown in Fig. 3 by using purified calmodulin.

Experiments D and E had the same $Mg^{2+}$ concentration as experiment B (2 mM) but greater concentrations of calmodulin; 4.2 μg/ml (10-fold greater) and 34 μg/ml (81-fold greater). The apparent $V$ values were 28.6 and 36 nmol $Ca^{2+}$/mg IOV protein per min at approx. 60 μM $Ca^{2+}$. The apparent $V$ for experiment E was normalized to 14.6 nmol $Ca^{2+}$/unit IOV AChE per min. The $K_{1/2}$ values were approximately 3 and 5 μM in experiments D and E, respectively. In experiment E at 5 μM total $Ca^{2+}$ concentration, $Ca^{2+}$ fluxes were stimulated 7- to 10-fold relative to the control (experiment W). Clearly it was calmodulin which caused both the activation of the active calcium fluxes and permitted the deactivation seen at $Ca^{2+}$ concentrations above 90 μM.

Fig. 5 shows the stimulation of ATP-dependent

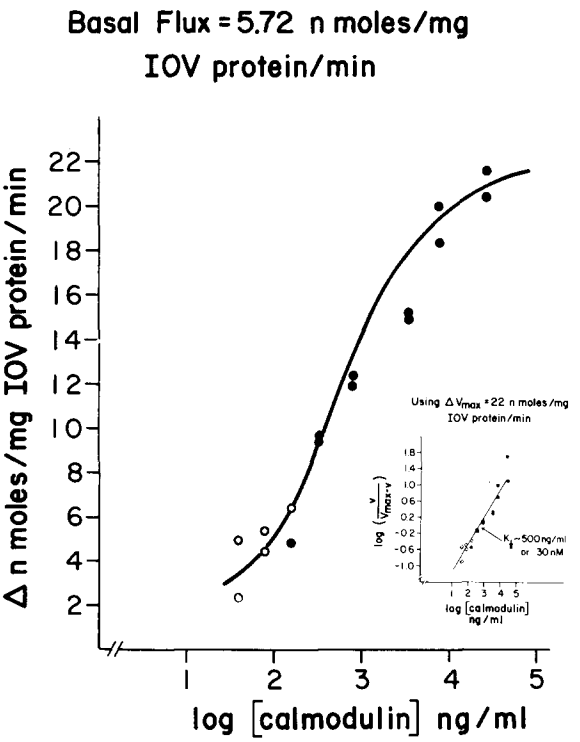


Fig. 5. $Ca^{2+}$ fluxes into inside-out vesicles as a function of calmodulin concentration. All fluxes were measured at 37°C in a medium of the following final composition: 130 mM NaCl, 2 mM $MgCl_2$, 0.5 mM $Na_2ATP$, 20 mM Mes/Tris, 48 μM $CaCl_2$; pH 6.5. Dialyzed calmodulin was present at the final concentration indicated. Vesicles were present at 12 μg vesicle protein/ml. The logarithm of the final calmodulin concentration in ng/ml is indicated on the abscissa. The increase in $Ca^{2+}$ flux beyond the basal flux is given on the ordinate. The inset is a Hill plot of the data given in the main panel. The slope of the plot is approx. 0.7. The $K_{1/2}^{Ca}$ = 30 nM at zero on the ordinate scale. At low calmodulin concentrations where data points are represented by open circles, calmodulin-stimulated $Ca^{2+}$ fluxes were calculated based on $Ca^{2+}$ uptakes from 3.25 to 6.25 min. This is because at these low calmodulin concentrations $Ca^{2+}$ fluxes increased with time.

$Ca^{2+}$ fluxes as a function of purified calmodulin. On the abscissa is the logarithm of the total calmodulin concentration in ng calmodulin/ml suspension and on the ordinate is the increment of the $Ca^{2+}$ flux above the basal flux caused by calmodulin. If 26.7 nmol $Ca^{2+}$/mg IOV protein per min was the $V$ value in this experiment, then the normalized $V$ value based on acetylcholinesterase activity was 11.8 nmol $Ca^{2+}$/unit IOV AChE per min. The inset is a Hill plot of the data. From the Hill plot we determined the concentration of calmodulin required for half-maximum stimulation of the $Ca^{2+}$ flux, $K_{1/2}^{Cal}$, to be 0.5 μg/ml or 30 nM calmodulin (if $M_r$ = 16 836). Our value is higher than reported by Larsen and Vincenzi (0.074 μg/ml) for a similar measurement in inside-out vesicles or the value reported by Jarrett and Kyte (0.24 μg/ml) for the $K_{1/2}$ of calmodulin activation of $(Ca^{2+} + Mg^{2+})$-ATPase in open erythrocyte ghosts [28]. Reasons for these differences are considered in the discussion. Our previous two figures also bear on these differences.

## Discussion

Previous studies of the active $Ca^{2+}$ transport process in human red cells have primarily used resealed ghosts and intact cells as the experimental model system. But the results of these studies often seem in apparent conflict [1,5,9] and the experiments of different laboratories are difficult to compare because substrate concentrations are difficult to control and maintain over the ranges necessary for detailed kinetics studies. The use of inside-out vesicles however enables the accurate measurement of transport kinetics which are relevant to in vivo physiology. In this discussion we will: compare fluxes in inside-out vesicles with those in intact cells; explain the magnitude of the $Mg^{2+}$ effect on $Ca^{2+}$ inactivation of calmodulin using the binding constants measured by Wolff and his associates [17] between $Ca^{2+}$, $Mg^{2+}$ and calmodulin; compare our calculated $K_D$ for calmodulin binding to the pump with those of Larsen and Vincenzi [11] and Jarret and Kyte [29].

The maximum efflux rate from intact human erythrocytes loaded with 1 mM Ca (a level which will saturate the calcium pump mechanism) is 85 nmol $Ca^{2+}$/ml packed cells per min or approx. 8.5 nmol $Ca^{2+}$/unit AChE per min [5,30]. This value is approx. 60% of the maximum $Ca^{2+}$ flux in inside-out vesicles reported here: 14 nmol Ca/unit AChE per min. It is difficult to explain why the maximum $Ca^{2+}$ pump activity is different in the two systems. An important problem is that while the concentration of free $Mg^{2+}$ in the intact cell in vivo has been reported as 0.4 mM [31], the free $Mg^{2+}$ concentration

is unknown for intact cells loaded with 1 mM Ca [32]. It is conceivable that higher levels of free $Mg^{2+}$ within the intact cell would increase the maximum $Ca^{2+}$ pump activity to that observed for the maximum pump activity of these vesicles. More on this point will be considered later in the discussion.

In the absence of added calmodulin the $Ca^{2+}$ activation of the (Mg + ATP)-activated $Ca^{2+}$ fluxes always had two components when analyzed on a double reciprocal graph of $flux^{-1}$ vs. $[Ca]^{-1}$ (Fig. 2b). The high affinity component had a low capacity, while the low affinity component had a higher capacity. Exogenous activator from hemolysate clearly increased the $V$ and decreased the concentration of $Ca^{2+}$ required to give half-maximum fluxes as we reported earlier [11]. These results suggest that the kinetics observed in the absence of added calmodulin may represent $Ca^{2+}$ pumping by primarily calmodulin depleted pumps but also transport by a few pumps which might still retain activator bound to them. This type of kinetics for calmodulin-depleted membranes has previously been seen in studies of $(Ca^{2+} + Mg^{2+})$-ATPase in red cell membranes and our studies of $Ca^{2+}$ fluxes in inside-out vesicles [1,10]. Recently Scharff [33] has found that the dissociation of calmodulin from the holoenzyme is not a simple process and does not follow first-order kinetics when the dissociation is assayed by changes in $(Ca^{2+} + Mg^{2+})$-ATPase activity of the enzyme. Thus while we cannot yet prove that residual activator is the cause of the variation between experiments (Fig. 2), this is a simple suggestion which seems consistent with our data and present knowledge of the calmodulin-pump interactions.

A new finding in this report was that the activation by hemolysate (Fig. 3) was not sustained at $Ca^{2+}$ concentrations above 90 $\mu$M. Since the same pattern of activation and deactivation was observed with purified calmodulin (Fig. 4) these effects cannot be attributed to the components in the hemolysate other than calmodulin. In addition, this loss of sustained activation by calmodulin was reduced by doubling the $Mg^{2+}$ concentration. These observations are consistent with the notion that calmodulin has a site at which $Mg^{2+}$ must be bound for its activity but at which $Ca^{2+}$ can displace $Mg^{2+}$. Such a mechanism is consistent with Seamon's [34] NMR studies with purified calmodulin and with data of Wolff et al. [17] on the binding of $Ca^{2+}$ and $Mg^{2+}$ to purified calmodulin. Wolff et al. [17] have shown that there are three sites that bind $Ca^{2+}$ with a high affinity and $Mg^{2+}$ with a low affinity (A-sites) and one site at which $Mg^{2+}$ and $Ca^{2+}$ compete having dissociation constants of 20 $\mu$M and 1 $\mu$M, respectively (B-sites). We assume that the active calmodulin species (the form that stimulates $Ca^{2+}$ flux) is the $X\text{Ca}_\text{A}$-$1\text{Mg}_\text{B}$-calmodulin complex (where $X$ may be 1, 2 or 3). Under our experimental conditions, the $X\text{Ca}_\text{A}$-$1\text{Mg}_\text{B}$-calmodulin complex would predominate at low $Ca^{2+}$ concentrations. As $Ca^{2+}$ levels increased, $Mg^{2+}$ on the B-site would be displaced by $Ca^{2+}$ and the active $X\text{Ca}_\text{A}$-$1\text{Mg}_\text{B}$-calmodulin complex would be converted to the $X\text{Ca}_\text{A}$-$\text{Ca}_\text{B}$-calmodulin complex. In order to explain the deactivation of fluxes at high $Ca^{2+}$ concentrations we assume that the all $Ca^{2+}$-form of calmodulin is unable to activate the pump. This explanation for the deactivation of $Ca^{2+}$ fluxes observed in Figs. 3 and 4 also explains why $Mg^{2+}$ antagonized the deactivation at high $Ca^{2+}$ concentrations. At a given $Ca^{2+}$ concentration at higher $Mg^{2+}$ concentration would increase the fraction of calmodulin in the active configuration: the $X\text{Ca}_\text{A}$-$1\text{Mg}_\text{B}$-calmodulin complex. Elevations of $Mg^{2+}$ concentrations to similar levels in the absence of calmodulin have been found to be without detectable effect on $Ca^{2+}$ flux (Macintyre, J.D. and Gunn, R.B., unpublished observations).

It can be shown with a single site for competition between $Mg^{2+}$ and $Ca^{2+}$ ions on the activator that the calcium concentrations required to reduce the flux to half of the value obtained by linear extrapolation back to the ordinate, $Ca^{\text{I}}_{1/2}$, is given by:

$$Ca^{\text{I}}_{1/2} = K^{\text{Ca}}_{\text{D}} \left(1 + \frac{Mg^{2+}}{K^{\text{Mg}}_{\text{D}}} \frac{K^{\text{Ca}}_{\text{AT}}}{K^{\text{Ca}}_{\text{Mg-AT}}}\right) \qquad \text{(See Appendix)}$$

If we assume the values obtained by Wolff and colleagues [17] for $K^{\text{Mg}}_{\text{D}} = 2 \cdot 10^{-5}$ M, $K^{\text{Ca}}_{\text{D}} = 10^{-6}$ M and assume that the free $Mg^{2+}$ concentration is the total concentration minus the ATP concentration (Mg – ATP) that is; $1.5 \cdot 10^{-3}$ or $3.5 \cdot 10^{-3}$ when the total Mg was $2 \cdot 10^{-3}$ and $4 \cdot 10^{-3}$, respectively; and, further, if we assume a 2.4-fold enhancement of $Ca^{2+}$ affinity to the transport site by the Mg-activator, then $Ca^{\text{I}}_{1/2} = 180$ $\mu$M, which is the value

calculated from the graph of experiment B minus experiment W in Fig. 4. The increase in $Mg^{2+}$ concentration in experiment C of Fig. 3 and Fig. 4 would result in a $Ca^{I}_{1/2} = 420$ $\mu$M, which is approximately that observed in those experiments. These calculations demonstrate the consistency of our results with the notion that $Ca^{2+}$ binding to a site on calmodulin which requires $Mg^{2+}$ for activity is responsible for the failure of calmodulin to sustain activation at $Ca^{2+}$ concentrations above 90 $\mu$M. Therefore, as a first approximation, the dissociation constants calculated from the data of Wolff et al. [17], for Ca-Mg binding to sites of class B on calmodulin offer a reasonable explanation for the results shown in Fig. 3 and 4 of this present paper. Scharff [33] has made a similar suggestion to explain the kinetics of $Ca^{2+}$ activation of $(Ca^{2+} + Mg^{2+})$-ATPase measured in the presence of calmodulin in open erythrocyte ghosts.

The final point is to compare theoretical and experimental values for the association between calmodulin (the activator, A) and the pump (the transporter, T) i.e., the reaction of $A + T \rightleftharpoons A \cdot T$. From the data obtained by the purification of calmodulin by Jarrett and Penniston [15], the concentration of cytoplasmic calmodulin can be calculated to be 1.5 $\mu$mol per l packed cells or 2.5 $\mu$M in the cell water. Jarrett and Kyte [29] reported a single class of calmodulin binding sites in human erythrocyte membranes for activation of $(Ca^{2+} + Mg^{2+})$-ATPase. The number of sites was 4500 per ghost. Another group studying phosphorylation of red cell membranes estimates 700 $Ca^{2+}$ pumps per cell [35]. For the sake of calculation we will assume 2000 $Ca^{2+}$ pumps per cell and $10^{13}$ cells per l packed cells. Using a simple mass action relationship then, the $K_D$ for calmodulin dissociation must be smaller than 1% of 2.5 $\mu$M or 25 nM for greater than 99% saturation, in vivo.

Previously Larsen and Vincenzi [12] have reported that half-maximal activation of red blood cell activator-sensitive $Ca^{2+}$ fluxes in inside-out vesicles is 4.4 nM. In a detailed analysis of activator-sensitive $(Ca^{2+} + Mg^{2+})$-ATPase Jarrett and Kyte [29] reported the calmodulin half-maximal activation as 14.5 nM. In this study we found a value of 30 nM for the amount of calmodulin needed to give half-maximal stimulation of activator-sensitive $Ca^{2+}$ fluxes in inside-out vesicles. All of these values are consistent with the value calculated for 99% saturation. These differences in measured $K_{1/2}$ values appear to be due to differences in experimental conditions and procedure. For example, we initiated our fluxes by the addition of membranes. Since the on-rate of calmodulin is measured in minutes at low calmodulin concentrations [33], our $Ca^{2+}$ fluxes may be compromised somewhat at low calmodulin concentrations. Such an effect would tend to elevate the $K_{1/2}$ observed by our method. Thus we believe the determinations of the $K_{1/2}$ for calmodulin are in reasonable agreement.

Near the beginning of this discussion we pointed out that the $V$ of $Ca^{2+}$ efflux in intact human red cell was approx. 60% of the $V$ value in inside-out vesicles reported in this paper, 14 nmol $Ca^{2+}$/unit of AChE per min. Two factors may contribute to this difference: (1) subsaturating free $Mg^{2+}$ concentrations in the intact cell (400 $\mu$M) and (2) the very high free calcium concentration in $Ca^{2+}$ loaded cells (500 $\mu$M). Using the equation above, and assuming a free $Mg^{2+}$ concentration of 400 $\mu$M, $Ca^{I}_{1/2}$ would equal 50 $\mu$M. Thus at 500 $\mu$M free $Ca^{2+}$ nearly all of the calmodulin would be inactivated by displacement of $Mg^{2+}$ from the B-site. (See Appendix, reaction 4.) Therefore, the extent of calmodulin activation in these $Ca^{2+}$-loaded cells could be insignificant, in which case the $V$ in erythrocytes should be the same as in inside-out vesicles free of exogenous activator (experiment W in Figs. 2 and 4) as is the case.

In summary, calmodulin activates $Ca^{2+}$ fluxes in inside-out vesicles in a complex fashion. Calmodulin appears to have a site which binds either $Mg^{2+}$ or $Ca^{2+}$. When $Mg^{2+}$ binds to this site, calmodulin activates $Ca^{2+}$ flux. If $Ca^{2+}$ binds to this site, calmodulin is an ineffective activator of $Ca^{2+}$ flux.

**Acknowledgements**

We are grateful to Dr. T. Steck for suggesting the method for the separation of leaky and sealed vesicles as described in the methods section. We are also grateful for the excellent technical assistance of Mr. Gary L. Hansen. This work was supported in part by USPHS grants F32-HL-05733 and HL-20365.

## Appendix

A simple scheme which is consistent with the broad outline of Ca, Mg, calmodulin (A) and transporter (T) interactions is that the calmodulin-transporter complex, AT, may have either Mg or Ca on the B-site of calmodulin, Mg-AT or Ca-AT, and that for transport of a calcium ion it must bind to the Mg-AT complex to form Mg-AT-Ca while Ca-AT-Ca is not a transport activated form for the calcium.

$$AT + Mg \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Mg - AT \quad (1)$$

$$MgAT + Ca \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Mg\text{-}AT\text{-}Ca \rightarrow \text{transport} \quad (2)$$

$$AT + Ca \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} AT\text{-}Ca \quad (3)$$

$$AT\text{-}Ca + Ca \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} Ca\text{-}AT\text{-}Ca \quad (4)$$

This set of equilibrium reactions together with the equation for conservation of forms ($AT + Mg\text{-}AT + AT\text{-}Ca + Ca\text{-}AT\text{-}Ca + Mg\text{-}AT\text{-}Ca = AT_{total}$) can be solved and the slope of the deactivation curve calculated. The calcium concentration at which the flux is reduced to half of the value obtained by linear extrapolation back to the zero ordinate is $Ca^{I}_{1/2}$ and is given by:

$$Ca^{I}_{1/2} = K_D^{Ca}\left(1 + \frac{Mg^{2+}}{K_D^{Mg}}\,\frac{K_{AT}^{Ca}}{K_{Mg\text{-}AT}^{Ca}}\right)$$

where

$$K_D^{Ca} = \frac{k_{-4}}{k_4}, \quad K_D^{Mg} = \frac{k_{-1}}{k_1}, \quad K_{Mg\text{-}AT}^{Ca} = \frac{k_{-2}}{k_2},$$

$$K_{AT}^{Ca} = \frac{k_{-3}}{k_3}$$

If Mg at the B-site of calmodulin had no effect on the binding of Ca at the transport site, $K_{Mg\text{-}AT}^{Ca}$ would equal $K_{AT}^{Ca}$ and the $Ca^{I}_{1/2}$ would have the usual relationship of an apparent dissociation constant to the true dissociation constant in the presence of a competitive inhibitor: $K_{app} = K_D(1 + Mg/K_D)$. Our data require that $K_{AT}^{Ca}/K_{Mg\text{-}AT}^{Ca} = 2.4$.

## References


1 Schatzmann, H.J. (1973) J. Physiol. 235, 551–569
2 Schatzmann, H.J. (1975) Curr. Topics Membranes Transport 6, 125–161
3 Weed, R.I., LaCelle, P.L. and Merrill, E.W. (1969) J. Clin. Invest. 48, 795–811
4 Siefring, G.E., Jr., Apostol, A.B., Velasco, P.T. and Lorand, L. (1978) Biochemistry 17, 2598–2604
5 Sarkadi, D., Szász, I., Gerlóczy, A. and Gárdos, G. (1977) Biochim. Biophys. Acta 464, 93–107
6 Gárdos, G. (1958) Biochim. Biophys. Acta 30, 653–654
7 Low, P.S. (1978) Biochim. Biophys. Acta 514, 264–273
8 Gunn, R.B., Fröhlich, O., Macintyre, J.D. and Low, P.S. (1979) Biophys. J. 25, 106a
9 Larsen, F.L., Hinds, T.R. and Vincenzi, F.F. (1978) J. Membrane Biol. 41, 361–376
10 Macintyre, J.D. and Green, J.W. (1978) Biochim. Biophys. Acta 510, 373–377
11 Sarkadi, B., Macintyre, J.D. and Gárdos, G. (1978) FEBS Lett. 89, 78–82
12 Larsen, F.L. and Vincenzi, F.F. (1979) Science 204, 306–309
13 Scharff, W. and Foder, B. (1978) Biochim. Biophys. Acta 509, 67–77
14 Bond, G. and Clough, D.L. (1973) Biochim. Biophys. Acta 323, 592–599
15 Jarrett, H.W. and Penniston, J.T. (1978) J. Biol. Chem. 253, 4676–4682
16 Brostrom, M.A., Brostrom, C.O., Breckenridge, B.M. and Wolff, D.J. (1976) J. Biol. Chem. 251, 4744–4750
17 Wolff, D.J., Poirier, P.G., Brostrom, C.O. and Brostrom, M.A. (1977) J. Biol. Chem. 252, 4108–4117
18 Cheung, W.Y., Lynch, J.J. and Wallace, R.W. (1978) Adv. Cyclic Nucleotide Res. 9, 233
19 Dedman, J.R., Potter, J.D., Jackson, R.L., Johnson, J.D. and Means, A.R. (1977) J. Biol. Chem. 252, 8415–8422
20 Lin, Y.M., Liu, Y.P. and Cheung, W.Y. (1974) J. Biol. Chem. 249, 4943–4954
21 Teo, T.S. and Wang, J.H. (1973) J. Biol. Chem. 284, 5950–5955
22 Watterson, D.M., Harrelson, W.G., Jr., Keller, P.M., Sharief, J. and Vanaman, T.C. (1976) J. Biol. Chem. 251, 4501–4513
23 Steck, T.L. and Kant, J.A. (1974) Methods Enzymol. 31, A, 172–180
24 Ellman, G.L., Courtney, D., Andres, V., Jr. and Featherstone, R.M. (1961) Biochem. Pharm. 7, 88–95
25 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
26 Van Kampen, E.J. and Zijlstra, W.G. (1961) Clin. Chim. Acta 6, 538–544
27 Macintyre, J.D. (1979) Biophys. J. 25, 103a
28 Wilkinson, G.N. (1961) Biochem. J. 80, 324–332

29 Jarrett, H.W. and Kyte, J. (1979) J. Biol. Chem. 254, 8237–8244
30 Cohen, C.M. and Solomon, A.K. (1976) J. Membrane Biol. 29, 345–372
31 Flatman, P. and Lew, V.L. (1977) Nature 267, 360–362
32 Ferriera, H.G. and Lew, V.L. (1977) Nature 259, 47–59
33 Scharff, O. (1980) in Membrane Transport in Erythrocytes (Lassen, U.V., Ussing, H.H. and Wieth, J.O., eds.), pp. 236–254, Munksgaard, Copenhagen
34 Seamon, K.B. (1980) Biochem. 19, 207–215
35 Rega, A.F. and Garrahan, P.J. (1975) J. Memb. Biol. 22, 313–327