Calmodulin regulation of Ca^{2+} transport in human erythrocytes

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Inside-out vesicles of human erythrocytes took up Ca^{2+} against an electrochemical gradient. This Ca^{2+} uptake was dependent on ATP and was stimulated by calmodulin. Treatment of vesicles with 1 mM-EDTA exposed an apparent low- Ca^{2+} -affinity Ca^{2+} -transport component with K_d of about $100\ \mu\text{M}\text{-Ca}^{2+}$ or more. This was converted into a single high- Ca^{2+} -affinity transport activity of K_d about $2.5\ \mu\text{M}\text{-Ca}^{2+}$ in the presence of $2\ \mu\text{g}$ of calmodulin/ml, showing that the decrease in transport activity after EDTA treatment was reversible. Vesicles not extracted with EDTA showed mainly apparent high- Ca^{2+} -affinity kinetics even in the absence of added calmodulin. Trifluoperazine ($30\ \mu\text{M}$) and calmodulin-binding protein ($20\ \mu\text{g}/\text{ml}$) inhibited about 50% of the high-affinity Ca^{2+} uptake and $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{-ATPase}$ (Ca^{2+} -activated, Mg^{2+} -dependent ATPase) activity of these vesicles, indicating that the vesicles isolated by the procedure used retained some calmodulin from the erythrocytes. Comparison of Ca^{2+} transport and $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{-ATPase}$ activities in inside-out vesicles yielded a variable $\text{Ca}^{2+}/\text{P}_i$ stoichiometric ratio. At low free Ca^{2+} concentrations (below $20\ \mu\text{M}\text{-Ca}^{2+}$), a $\text{Ca}^{2+}/\text{P}_i$ ratio of about 2 was found, whereas at higher Ca^{2+} concentrations the stoichiometry was approx. 1. The stoichiometry was not significantly altered by calmodulin.

Ca^{2+} is an important 'second messenger' for the initiation of a variety of intracellular processes in many animal cells (Rasmussen, 1970). Excitation of cells transiently increases intracellular Ca^{2+} from resting concentrations of around $0.01\text{--}1.0\ \mu\text{M}$ to $10\ \mu\text{M}$ or higher (see Cheung, 1980). The increased concentrations of Ca^{2+} are considered to initiate various physiological responses by interaction with specific intracellular Ca^{2+} -binding proteins, of which calmodulin is perhaps the most ubiquitous. The return of Ca^{2+} to its resting concentration is accomplished by a variety of mechanisms, including an active Ca^{2+} pump, which uses ATP to transport Ca^{2+} across the plasma membrane. Erythrocytes have frequently been used as model plasma membranes for the study of transport, because they are isolated relatively easily in large quantities and free of membranes from intracellular organelles. Ca^{2+} is toxic to the human erythrocyte (see Roufogalis, 1979). Low intracellular Ca^{2+} in the erythrocytes is maintained, at least in part, at $0.1\ \mu\text{M}$ or less by a high-capacity Ca^{2+} -transport system that uses ATP to extrude Ca^{2+} against a large electrochemical

gradient. Associated with the transport of Ca^{2+} is a $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{-ATPase}$ (Ca^{2+} -activated Mg^{2+} -dependent ATPase) activity (Schatzmann & Vincenzi, 1969). Estimates of the ratio of Ca^{2+} pumped to phosphate (P_i) liberated have varied from 2 in some instances (Quist & Roufogalis, 1975a; Sarkadi *et al.*, 1977) to 1 in others (Schatzmann, 1973; Larsen *et al.*, 1978).

Calmodulin, shown to be present in the erythrocyte (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977), stimulates active uptake of Ca^{2+} into inside-out vesicles of human erythrocytes (Sarkadi *et al.*, 1978; Hinds *et al.*, 1978; Larsen & Vincenzi, 1979). Inside-out vesicles are particularly useful for investigating active cation transport because they can be prepared to be impermeable to Ca^{2+} and other polar substances (Sarkadi *et al.*, 1978; see Fig. 1) and they allow precise control of the concentrations of Ca^{2+} , Mg^{2+} , ATP and other regulators at the cytosolic face of the membrane. Ca^{2+} -uptake rates determined in inside-out vesicles closely approximate to Ca^{2+} -transport rates in other more conventional preparations (Larsen & Vincenzi, 1979). Much of the previous information on the effects of calmodulin on human erythrocyte

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Ca^{2+} transport was derived from studies on its effect on $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity in permeable membrane preparations. Calmodulin is reported to stimulate the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity in isolated erythrocyte membranes by increasing both the V_{max} and the affinity of the enzyme for Ca^{2+} (Scharff & Foder, 1978; Hanahan *et al.*, 1978). Calmodulin-rich membranes show a single high- Ca^{2+} -affinity $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity ($K_d = 1\text{--}4\ \mu\text{M}\text{-Ca}^{2+}$), whereas calmodulin-deficient membranes show a low- Ca^{2+} -affinity component together with the high-affinity component (Quist & Roufogalis, 1975b; Scharff, 1976; Scharff & Foder, 1978; Katz *et al.*, 1979).

The present study reports a more extensive analysis of the effects of calmodulin on inside-out vesicles of human erythrocytes. Kinetic plots show that calmodulin has similar effects on the activation of Ca^{2+} transport and $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity, and that concomitant estimates of transport and ATPase activity demonstrate a variable stoichiometry of the $\text{Ca}^{2+}/\text{P}_i$ ratio dependent on the Ca^{2+} concentration.

Parts of this work appeared in abstract form in the Conference on Calmodulin and Cell Functions (Larsen *et al.*, 1980).

Experimental

Preparation of inside-out vesicles

Inside-out vesicles of erythrocyte membranes were made by the methods of Steck & Kant (1974) as modified by Larsen & Vincenzi (1979). Fresh erythrocytes were collected by venipuncture from a healthy volunteer. Sidedness of the vesicles was determined by an acetylcholinesterase assay in the absence and presence of Triton X-100 (Steck & Kant, 1974). These vesicles were 45–60% inside-out, except in the experiment shown in Fig. 5, where a fraction of higher-density vesicles was 75–80% inside-out. Vesicles were used on the day of preparation (fresh vesicles) or after storage at 4°C for 1 or 2 days (see Figure legends). Stored vesicles lost about 10% of their activity per day, but gave qualitatively similar kinetics.

Measurement of Ca^{2+} transport

Transport experiments were conducted at 25°C and 37°C as described previously (Larsen & Vincenzi, 1979), with some modifications. Vesicles were preincubated for 30 min in a solution at pH 6.9 that contained: 40 mM-NaCl, 7.5 mM-KCl, 10 mM-histidine, 10 mM-imidazole, 0.6 mM-glycylglycine, 3 mM-MgCl₂, 0.1 mM-ouabain, either 1 or 0.1 mM-EGTA (see Figure legends) and various amounts of CaCl_2 , $^{45}\text{CaCl}_2$ (specific radioactivity $1.2 \times 10^{12}\text{--}8.7 \times 10^{12}$ c.p.m./mol of Ca^{2+}) and calmodulin. Each reaction vessel contained 0.12–0.16 mg of vesicle

membrane protein. Free concentrations of Ca^{2+} were calculated at pH 6.9 by using an EGTA- Ca^{2+} stability constant of $10^{11.00}$ at 20°C [$d(\log K)/dT = -0.0205/\text{K}$; Bjerrum *et al.*, 1957]. The apparent stability constants were calculated to be $1.357 \times 10^6\text{ M}$ for $[\text{CaEGTA}]^{2-}$ and $3.15 \times 10^4\text{ M}$ for $[\text{CaATP}]^{2-}$ at pH 6.9. The calculated Ca^{2+} concentrations were verified by direct measurement with a Ca^{2+} -selective electrode, as previously described (Vincenzi & Larsen, 1980).

Active uptake of Ca^{2+} into inside-out vesicles was initiated by adding 3 mM-ATP (disodium salt). At 25°C , 150 μl samples were removed 8 s after the addition of ATP and every 2 min thereafter for 8 min. The total volume of the reaction medium was 1 ml. At 37°C , the total volume of the reaction medium was 2 ml and 400 μl samples were removed 8 s after the addition of ATP and every 1 min thereafter for 4 min. The samples were quenched in ice-cold 40 mM-glycylglycine buffer (pH adjusted with Tris base to pH 7.1) and 0.1 mM-MgCl₂ and trapped on 0.45 μm microporous filters (Amicon). The filters were washed with $2 \times 2\text{ ml}$ of the Tris/glycylglycine buffer, dissolved in scintillation fluid (ACS; Amersham) and counted for ^{45}Ca radioactivity. No vesicle membrane escaped entrapment on the filters, as protein was undetectable in the filtrate. Rates of Ca^{2+} uptake were determined from linear regression analysis of at least four data points for 3–4 min (37°C) or 8 min (25°C). In every instance the correlation coefficient, r , was greater than 0.97. Ca^{2+} transport rates were calculated per mg of inside-out-vesicle protein.

Measurement of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase in vesicles

ATPase activities were determined in vesicles incubated in the same media used for transport experiments. ATPase activities were determined in vesicles incubated at 37°C for 1 h, except for experiments designed to estimate the stoichiometry of transport; in the latter case the time of incubation was 20 min. ATPase activities were linear over these time periods at all Ca^{2+} concentrations tested. ATPase reactions were stopped by the addition of 2% (w/v) sodium dodecyl sulphate (final concn.). P_i was measured by an automated Fiske & SubbaRow (1925) method. $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity was defined as the activity remaining after subtracting the Mg^{2+} -ATPase activity from the ouabain-insensitive ATPase activity.

Preparation of calmodulin

Calmodulin was purified from human erythrocytes by the following method adapted from Raess & Vincenzi (1980). Fresh erythrocytes, obtained from the Red Cross and washed three times in 0.9% (w/v) NaCl, were lysed in 5 vol. of 20 mM-imidazole

(pH 6.8) that contained 1 mM-EDTA and 50 μM -phenylmethanesulphonyl fluoride. The haemolysate was centrifuged at 27000g for 30 min and the membrane pellet was discarded. Pre-swollen DEAE-Sephadex (pH adjusted to 6.8) was poured slowly into the haemolysate and stirred slowly for 1.5 h at room temp. The mixture was then filtered through a nylon cloth and the filtrate discarded. The Sephadex beads were washed with 100 mM-NaCl/20 mM-imidazole (pH 6.8)/1 mM-EDTA (soln. A), and then packed into a column and eluted with soln. A until the remaining haemoglobin was washed from the column. Calmodulin was then eluted with a linear salt gradient (500 ml) of 0.1–1 M-NaCl in soln. A. The calmodulin-containing fractions were combined and heated to 70°C for 30 min. The precipitated protein was removed by centrifugation (2700g for 30 min) and the supernatant was concentrated to about 10 ml by ultrafiltration (UM-10 Amicon filters), dialysed overnight in soln. A and placed on a Sephadex G-75 (Pharmacia) column (100 cm $\times$ 3 cm) that had been equilibrated in soln. A. Calmodulin was eluted from the G-75 column with an apparent mol.wt. of 29 700, consistent with previously reported values in the presence of EDTA. Calmodulin-containing fractions were concentrated as above, dialysed extensively against 20 mM-imidazole (pH 6.8) and kept frozen at -20°C . The calmodulin was found to be homogeneous by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (mol.wt. 16 570). Bovine brain calmodulin from the laboratory of Dr. T. C. Vanaman, Duke University, gave virtually identical results. Calmodulin-binding protein from bovine brain, kindly supplied by Dr. K. Sharma, in the laboratory of Dr. J. Wang, University of Manitoba, was extensively dialysed against 20 mM-imidazole at 0–4°C before use.

Protein content of membranes and calmodulin was determined by an automated version of the Lowry *et al.* (1951) method.

Results

A time course of $^{45}\text{Ca}^{2+}$ uptake into inside-out vesicles is shown in Fig. 1. Uptake was linear over the sampling period (3 min) under all conditions. Correlation coefficients of 0.97 or greater were obtained by linear regression of data such as these (uptake was linear for 8 min at 25°C and 4 min at 37°C). Addition of 2 mM-EGTA or 0.2 mM- LaCl_3 (final concn.) to the reaction media stopped the uptake of $^{45}\text{Ca}^{2+}$ (see Fig. 1). As $^{45}\text{Ca}^{2+}$ was maintained in the vesicles under these conditions, they were impermeable to the passive leak of Ca^{2+} over the time course of these experiments. This is also supported by the lack of Ca^{2+} uptake into control vesicles in the absence of ATP. The Ca^{2+}

Fig. 1. Rate of $^{45}\text{Ca}^{2+}$ uptake into inside-out vesicles; effects of ATP, EGTA, La^{3+} and ionophore A23187. Ca^{2+} uptake was determined at 37°C as described in the Experimental section. The free Ca^{2+} concentration was 22 μM in all cases. Experiments were performed in the absence of ATP (○), in the presence of 3 mM-ATP (□) and 3 mM-ATP plus 1 μg of calmodulin/ml (●, ■). EGTA (2 mM), La^{3+} (0.2 mM) and ionophore A23187 (I; 2 μM) were added to the reaction media at the times indicated by the arrows (all concentrations are final). Data are from a single experiment on vesicles prepared on the same day.

taken up was trapped inside the vesicles, as it was released by adding the Ca^{2+} -selective ionophore A23187 (2 μM final concn.). A small but consistent increase in $^{45}\text{Ca}^{2+}$ was seen when ionophore A23187 was added in the control vesicles incubated without ATP (Fig. 1). Thus the results in Fig. 1 show that Ca^{2+} uptake is an active process.

Calmodulin stimulated the active uptake of Ca^{2+} into inside-out vesicles above basal uptake rates (in the absence of calmodulin) at all Ca^{2+} concentrations tested, except at 0.2 μM - Ca^{2+} (Fig. 2). The results in Fig. 2 were obtained from fresh vesicles prepared on the same day and tested at 25°C. In the presence of calmodulin, Ca^{2+} uptake was maximal at 20 μM - Ca^{2+} . The greatest activation by calmodulin occurred at 5 μM - Ca^{2+} and was typically about 4-fold (Fig. 2). In the presence of added calmodulin Ca^{2+} uptake decreased at high Ca^{2+} concentrations (above 20 μM - Ca^{2+} ; Figs. 2 and 3), which was not observed for basal uptake. The apparent K_d for Ca^{2+} uptake into these vesicles was about 1.5–4 μM - Ca^{2+}

Fig. 2. Comparison of Ca^{2+} activation of Ca^{2+} transport in EDTA-extracted vesicles and non-extracted vesicles in the presence and absence of added calmodulin

Uptake experiments were conducted at 25°C in the presence of 1 mM-EGTA as described in the Experimental section. EDTA extraction was performed by incubating vesicles in 40 mM-Tris/glycylglycine buffer (pH 7.4) with 1 mM-EDTA for 30 min at 30°C . Non-EDTA-extracted vesicles (O, ●) and EDTA-extracted vesicles (□, ■) were tested in the presence (solid symbols) and in the absence (open symbols) of added calmodulin ($2\mu\text{g}/\text{ml}$). Data on EDTA-extracted vesicles are from one experiment typical of two such experiments performed on fresh vesicles. Data on non-EDTA-extracted vesicles are the means $\pm$ S.D. of three experiments on fresh vesicles.

in the presence and absence of added calmodulin (Table 1). Without added calmodulin a low- Ca^{2+} -affinity transport component was also seen in the vesicles under these conditions (Fig. 2, Table 1).

To determine if the high- Ca^{2+} -affinity kinetics displayed in Fig. 2 for control vesicles may have been due to contamination of the vesicles by endogenous calmodulin, vesicles were treated with EDTA to extract Ca^{2+} -bound calmodulin. Vesicles were suspended in 35 vol. of 40 mM-Tris/glycylglycine buffer (pH 7.4) containing 1 mM-EDTA for 30 min at 30°C (Farrance & Vincenzi, 1977). The Ca^{2+} uptake obtained in EDTA-extracted vesicles is compared with that in control vesicles in Fig. 2. In the absence of added calmodulin, EDTA-extracted vesicles took up less Ca^{2+} than did non-extracted vesicles and displayed a more pronounced low-

Fig. 3. Effects of calmodulin-binding protein on basal Ca^{2+} uptake into non-EDTA-extracted vesicles

Uptake experiments were conducted at 37°C as described in the Experimental section. Ca^{2+} concentrations were buffered with 0.1 mM-EGTA. Non-EDTA-extracted vesicles were assayed in the presence (●) and in the absence (○) of added calmodulin ($2\mu\text{g}/\text{ml}$) and in the presence of calmodulin-binding protein ($20\mu\text{g}/\text{ml}$) without added calmodulin (□). Vesicles were preincubated in the same assay medium for 1 h before addition of ATP. Data are from a single experiment on vesicles stored for 2 days at 4°C . Qualitatively similar results were obtained at 25°C and on $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity at 37°C (results not shown).

Ca^{2+} -affinity component. Ca^{2+} uptake into EDTA-extracted vesicles showed two distinct Ca^{2+} affinities, with apparent K_d values $0.7\mu\text{M}$ - Ca^{2+} for the high- Ca^{2+} -affinity component and $100\mu\text{M}$ or more [depending on the estimated maximum-velocity (V_2) value] for the low- Ca^{2+} -affinity component (see Table 1). No difference in transport rates or Ca^{2+} affinity was apparent between EDTA-extracted and non-extracted vesicles in the presence of calmodulin (Fig. 2), indicating that the decrease in Ca^{2+} -transport rate was reversible.

Similar results were obtained for Ca^{2+} transport into non-EDTA-treated inside-out vesicles at 37°C (Fig. 3, Table 1). Calmodulin-binding protein ($20\mu\text{g}/\text{ml}$) decreased the basal uptake of Ca^{2+} (Fig. 3); this was reversed when excess calmodulin was added to an assay mixture that contained calmodulin-binding protein (results not shown), suggesting that calmodulin-binding protein inhibited basal Ca^{2+} uptake by interacting with residual membrane-bound calmodulin. Calmodulin-binding protein also inhibited $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity similarly (results not shown).

Experiments on the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity of inside-out vesicles produced similar kinetic

Table 1. *Calculated V_{max} and apparent K_d values of Ca^{2+} -activation of Ca^{2+} transport in inside-out vesicles*
The kinetic constants were calculated by a non-linear curve-fitting program assuming the equation $v = \{V_1 \cdot [Ca^{2+}] / (K_1 + [Ca^{2+}])\} + K'_2 \cdot [Ca^{2+}]$, where V_1 (nmol of Ca^{2+} /min per mg of inside-out-vesicle protein) and K_1 (μM) are the maximum velocity and Ca^{2+} dissociation constant, respectively, of the high- Ca^{2+} -affinity component and $K'_2 = V_2/K_2$, where V_2 and K_2 are the corresponding constants for the low- Ca^{2+} -affinity component, assuming $K_2 > [Ca^{2+}]$ (Al-Jobore & Roufogalis, 1981). In the presence of calmodulin the data were best fitted by assuming $K'_2 = 0$ (correlation coefficient $r = 0.999$), whereas data obtained from EDTA-extracted vesicles in the absence of calmodulin were best fitted by the inclusion of a low- Ca^{2+} -affinity component into the equation ($r = 0.999$), where $K'_2 = 0.024$. Assuming a V_2 of 3 nmol/min per mg of protein at 25°C (Fig. 2), the estimate of K_2 for the low- Ca^{2+} -affinity component of EDTA-extracted vesicles in the absence of calmodulin was 125 μM . The equation used assumes a Hill coefficient of 1.0. Estimates of the kinetic constants $\pm$ the standard deviation of the variance of these estimates were obtained from data combined from three experiments in non-EDTA-extracted vesicles and duplicate experiments in EDTA-extracted vesicles.

Calculated kinetic constants	Non-extracted vesicles		EDTA-extracted vesicles	
	No calmodulin	With calmodulin	No calmodulin	With calmodulin
V_1	6.7 ± 0.13 (25°C) 17.1 ± 1.1 (37°C)	20.7 ± 0.5 (25°C) 41.6 ± 5.7 (37°C)	4.2 ± 0.36 (25°C)	21.2 ± 0.8 (25°C)
K_1	1.4 ± 0.2 (25°C) 4.1 ± 0.9 (37°C)	2.9 ± 0.2 (25°C) 1.8 ± 0.9 (37°C)	0.7 ± 0.2 (25°C)	2.5 ± 0.3 (25°C)
K'_2	0.005 ± 0.007 (25°C)		0.024 ± 0.005 (25°C)

Fig. 4. *Effects of trifluoperazine on calmodulin-activated and basal ($Ca^{2+} + Mg^{2+}$)-ATPase activities of non-EDTA-extracted vesicles*
($Ca^{2+} + Mg^{2+}$)-ATPase activities were determined at 37°C in non-EDTA-extracted vesicles at 0.1 mM-EGTA as described in the Experimental section. The vesicles were preincubated for 1 h before addition of ATP. The ATPase activities were determined in the absence of trifluoperazine (○, ●) and in the presence of 30 μM -trifluoperazine (△, ▲) and in the presence (solid symbols) and absence (open symbols) of calmodulin (0.2 μg /ml). Data are from vesicles stored for 2 days and are representative of two similar experiments.

Fig. 5. *Ca^{2+} transport and ($Ca^{2+} + Mg^{2+}$)-ATPase activities of non-EDTA-extracted vesicles*
 Ca^{2+} transport (solid symbols) and ($Ca^{2+} + Mg^{2+}$)-ATPase activities (open symbols) were measured at 37°C at 0.1 mM-EGTA on non-EDTA-extracted vesicles (see the Experimental section) in the presence (△, ▲) and in the absence (○, ●) of added calmodulin (2 μg /ml). Data are from fresh vesicles and are representative of three similar experiments. The transport and ATPase rates are expressed per mg of total protein. No correction was made for unsealed vesicles (10–15%; F. Larsen, S. Katz & B. D. Roufogalis, unpublished work), which will tend to underestimate the stoichiometry.

plots to those of Ca^{2+} transport (see Figs. 4 and 5). Ca^{2+} activated the ($Ca^{2+} + Mg^{2+}$)-ATPase with a single high Ca^{2+} affinity in the presence of calmodulin and a mixed high and low Ca^{2+} affinity in

the absence of added calmodulin (Fig. 4). Trifluoperazine, which has been shown to bind to the calmodulin- Ca^{2+} complex (Levin & Weiss, 1979),

Table 2. *Stoichiometry of Ca²⁺ transport at various Ca²⁺ concentrations*

Stoichiometry was calculated from experiments of the type shown in Fig. 5 in untreated vesicles. The results represent the mean $\pm$ s.d. from three experiments, in the absence or in the presence of 2 μ g of calmodulin/ml.

[Ca ²⁺] _{free} (μ M)	Ca ²⁺ /P _i	
	(-calmodulin)	(+calmodulin)
0.2	1.8 $\pm$ 0.2	1.6 $\pm$ 0.2
0.7	2.1 $\pm$ 0.3	1.8 $\pm$ 0.5
2	2.0 $\pm$ 0.2	1.3 $\pm$ 0.3
8	1.7 $\pm$ 0.3	1.4 $\pm$ 0.3
24	1.3 $\pm$ 0.2	1.1 $\pm$ 0.1
240	0.9 $\pm$ 0.2	1.0 $\pm$ 0.2

blocked stimulation by added calmodulin at all Ca²⁺ concentrations tested and inhibited about 50% of the basal (Ca²⁺ + Mg²⁺)-ATPase activity at 30 μ M (Fig. 4). The high-Ca²⁺-affinity component was particularly sensitive to trifluoperazine treatment, consistent with the suggestion that residual calmodulin contaminated the vesicles. No inhibition of the (Na⁺ + K⁺ + Mg²⁺)-ATPase (Mg²⁺-dependent, Na⁺- and K⁺-stimulated ATPase) activity was observed at 30 μ M-trifluoperazine (results not shown).

Ca²⁺ transport and (Ca²⁺ + Mg²⁺)-ATPase were compared under identical conditions (Fig. 5), except that transport was terminated after 8 min, whereas ATPase activities was continued for up to 20 min. [The (Ca²⁺ + Mg²⁺)-ATPase activities were linear over the 20 min time period.] At Ca²⁺ concentrations below 20 μ M-Ca²⁺, the ratio of Ca²⁺ pumped to P_i liberated varied from 1.6 to 2.1 in the absence or in the presence of calmodulin (2 μ g/ml). At free Ca²⁺ concentrations above 20 μ M, the Ca²⁺/P_i ratio changed to about 1. Mean estimates of stoichiometry are listed in Table 2 at the various Ca²⁺ concentrations tested.

Discussion

Previous studies on human unsealed erythrocyte membranes have shown biphasic Ca²⁺ activation of (Ca²⁺ + Mg²⁺)-ATPase (Quist & Roufogalis, 1975b). Extraction of Ca²⁺ and peripheral proteins with EDTA treatment increased the low-Ca²⁺-affinity component (Quist & Roufogalis, 1975b; Roufogalis & Mauldin, 1980), and the addition of calmodulin converted the enzyme into a high-Ca²⁺-affinity state (Scharff & Foder, 1978; Roufogalis & Mauldin, 1980). It was of interest to find similar modulation of Ca²⁺ transport into inside-out vesicles in the present study. Inside-out vesicles prepared by a modification of the procedure of Steck & Kant (1974) appeared to contain endogenous calmodulin from

the erythrocyte, as Ca²⁺ transport was partially inhibited by trifluoperazine (30 μ M) and calmodulin-binding protein (20 μ g/ml), particularly in the high-Ca²⁺-affinity region. Ca²⁺ transport proceeded with a pronounced low Ca²⁺ affinity ($K_d = 100 \mu$ M or more) after treatment of the vesicles with 1 mM-EDTA for 30 min. Addition of calmodulin to EDTA-extracted vesicles increased both the V_{max} and the apparent Ca²⁺ affinity ($K_d = 1-2 \mu$ M) of Ca²⁺ transport. These results show for the first time that Ca²⁺ transport can proceed when the Ca²⁺ pump is operating predominantly in the low-Ca²⁺-affinity mode. This suggests that calmodulin acts as a 'switch' (Vincenzi, 1978) to increase the Ca²⁺ affinity and velocity of the Ca²⁺ pump, rather than being required as an integral subunit of the Ca²⁺-transport system, assuming that Ca²⁺-independent binding of calmodulin to the vesicles (Klinger *et al.*, 1980) is not required for Ca²⁺ transport. The present study also shows that EDTA treatment can be used to remove calmodulin, and possibly other endogenous activators (Mauldin & Roufogalis, 1980), from vesicles, without irreversible inactivation of the Ca²⁺ pump (Fig. 2) or a change in the leakiness (C. Akyempon & B. D. Roufogalis, unpublished work) or sidedness (results not shown) of the vesicle preparation.

Our results suggest a variable stoichiometry for the Ca²⁺ pump. The stoichiometry changed from about 2 Ca²⁺ ions pumped per ATP molecule hydrolysed at low free Ca²⁺ concentrations to about 1 Ca²⁺ per ATP at high Ca²⁺ concentrations (see Fig. 5 and Table 2). Whether the change in stoichiometry reflects a direct effect of high Ca²⁺ on the Ca²⁺-pump mechanism or an indirect effect on another ATP-consuming process (Quist & Roufogalis, 1975a) is unclear at present. Calmodulin had little effect on the stoichiometry at either low or high Ca²⁺ concentrations. These findings may help to resolve eventually why some investigators found a Ca²⁺/P_i ratio of 2 (Sarkadi *et al.*, 1977; Quist & Roufogalis, 1975a), whereas others found a ratio of 1 (Schatzmann, 1973; Larsen *et al.*, 1978). Although in our protocol we measured P_i release over a longer time period than Ca²⁺ transport, both transport and ATPase activities were measured as initial rates over a linear time course. Under these conditions it is unlikely that back-diffusion of Ca²⁺ or reversal of the Ca²⁺ pump could have a major influence on the results obtained.

One further finding of this study needs elaboration. We consistently found (see Figs. 2-5) that at 0.2 μ M-Ca²⁺, a concentration close to the estimated free Ca²⁺ concentration in human erythrocytes (Simons, 1974), calmodulin had no effect on either Ca²⁺ transport or (Ca²⁺ + Mg²⁺)-ATPase activity. At this Ca²⁺ concentration transport of Ca²⁺ at 37°C (about 0.8 nmol of Ca²⁺/min per mg of

inside-out-vesicle protein) represents $352\text{ }\mu\text{mol}$ of Ca^{2+}/h per litre of cells (assuming a conversion factor of 0.66 pg of membrane protein per erythrocyte; Dodge *et al.*, 1956). This efflux rate is about 60 times the passive influx rate of Ca^{2+} reported by Ferreira & Lew (1977) under static conditions in ATP-depleted erythrocytes ($6\text{ }\mu\text{mol}$ of Ca^{2+}/h per litre of cells at 37°C). An increase in free Ca^{2+} concentration to, for example, $7\text{ }\mu\text{M}-\text{Ca}^{2+}$, increases transport another 8-fold in the absence of calmodulin. It therefore appears possible that calmodulin would be needed to maintain Ca^{2+} homeostasis of erythrocytes only under pathological conditions or in old or senescent cells, where passive Ca^{2+} influx may be elevated. It is possible, however, that determinations of passive Ca^{2+} influx under static conditions do not adequately reflect the membrane permeability of the normal mature erythrocyte to Ca^{2+} *in vivo*. Preliminary evidence from tests designed to determine whether shear stress of physiological magnitudes affects the permeability of the erythrocyte membrane to Ca^{2+} indicates that low magnitudes of shear stress increase the permeability of the cell to Ca^{2+} (Larsen *et al.*, 1981).

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