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INFLUENCE OF EGTA ON THE APPARENT Ca^{2+} AFFINITY OF Mg^{2+}-DEPENDENT, Ca^{2+}-STIMULATED ATPase IN THE HUMAN ERYTHROCYTE MEMBRANE

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The apparent Ca^{2+} affinity of Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase ((Mg^{2+} + Ca^{2+})-ATPase) in human erythrocyte membranes increased with increasing concentrations of EGTA used to buffer free Ca^{2+}. The shift in apparent Ca^{2+} affinity was seen in membranes prepared by hypotonic hemolysis and in membranes depleted of endogenous activators by EDTA treatment. The effect of EGTA differed from that of calmodulin, as it increased Ca^{2+} affinity without increasing V. EGTA also increased the apparent Ca^{2+} affinity when calmodulin was present in the assay medium. ATP-stimulated calcium binding to membranes was greater at 1 mM EGTA than at 0.1 mM EGTA. Similarly to ATPase activation, whereas binding decreased as Ca^{2+} was raised above 35 μM at 1.0 mM EGTA, binding progressively increased up to 100 μM or more free Ca^{2+} at 0.1 mM EGTA. EGTA also increased the Ca^{2+} affinity of Triton X-100-solubilized (Mg^{2+} + Ca^{2+})-ATPase, indicating that its effect did not depend on an intact membrane. Analysis of the kinetic data by a computerized nonlinear curve fitting procedure showed that a low Ca^{2+} affinity state of the enzyme was converted to a high Ca^{2+} affinity state in the presence of EGTA. The species associated with the enzyme interconversion appeared to be $[CaEGTA]^{2-}$.

Introduction

The reported Ca^{2+} affinity of the Ca^{2+}-transport complex in human erythrocytes varies considerably [1]. Resealed ghosts loaded with calcium in 5 mM EGTA buffer [2], or intact cells loaded with calcium in the presence of 10 μM ionophore A23187 [3], transport Ca^{2+} with a high Ca^{2+} affinity (1–4 μM). By contrast, resealed ghosts loaded with Ca^{2+} in the absence of EGTA buffers [4,5] and intact cells loaded with Ca^{2+} in the cold [6] or with 2 μM A23187 [7] transport Ca^{2+} with a predominantly low Ca^{2+} affinity (0.05–1 mM). Similar differences in the Ca^{2+} affinity of (Mg^{2+} + Ca^{2+})-ATPase, the biochemical expression of the Ca^{2+}-transport pump, have also been reported. The apparent Ca^{2+} affinity of (Mg^{2+} + Ca^{2+})-ATPase in resealed ghosts loaded with calcium in 2.5 mM EGTA was 2–3 orders of magnitude greater than that in ghosts loaded in the absence of EGTA [2].

The apparent Ca^{2+} affinity of (Mg^{2+} + Ca^{2+})-ATPase in the erythrocyte membrane is influenced by the concentration of Ca^{2+} in the hemolysis medium [8], the presence of Ca^{2+} ionophore A23187 [9] and the extent of association of the membrane with calmodulin [9,10,11], a Ca^{2+}-binding protein found in erythrocytes. In the present study we have examined the influence of EGTA on the kinetics of Ca^{2+} activation of (Mg^{2+} + Ca^{2+})-ATPase in erythrocyte membranes. The apparent Ca^{2+} affinity

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Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

of $(Mg^{2+} + Ca^{2+})$-ATPase was shown to increase as the total EGTA concentration in the reaction medium was increased from 0.1 to 3.0 mM. The mechanism of the EGTA effect, and the possible contribution of calmodulin, have been investigated.

Materials and Methods

Preparation of erythrocyte membranes

Packed red blood cells, 4 days old or less, were obtained from the local Red Cross in acid-citrate-dextrose. Cells were washed three times in normal saline and the top one-third layer removed before preparation of the membranes. Membranes were prepared by a hypotonic hemolysis procedure, as follows: 1 vol of cells was hemolysed in 14 vol of 20 imosM sodium phosphate buffer, pH 7.4 at 4°C, followed by washing and storage in the same buffer (Dodge ghosts) [12]. The procedures were described in detail previously [13].

EDTA-extracted erythrocyte membranes containing predominantly low affinity $(Mg^{2+} + Ca^{2+})$-ATPase activity were prepared by incubating Dodge ghosts in 5 vol of 1 mM Tris-0.1 mM EDTA, pH 8.0 with shaking at 37°C for 30 min, as previously described [14]. Membranes were separated by centrifugation at 20 000 × *g* for 20 min and resuspended in 20 imosM sodium phosphate buffer, pH 7.4 at 4°C, to the original packed cell volume. Since this procedure removes 25% or more of membrane proteins, the ATPase activity of ghosts and extracted membranes were expressed per ml of ghosts (or packed cells, assuming 100% recovery).

Measurement of $(Mg^{2+} + Ca^{2+})$-ATPase activity

$(Mg^{2+} + Ca^{2+})$-ATPase activity was determined from $^{32}P_i$ released from [γ-^{32}P]ATP [15]. The medium contained 55 mM Tris-maleate, 66 mM NaCl, 0.1 mM ouabain, 6.5 mM $MgCl_2$, 0.1–3.0 mM EGTA, various amounts of $CaCl_2$ and 50 µl of membrane suspension (4 mg protein/ml) in a total volume of 0.6 ml at pH 6.9. The reaction was started by addition of ATP (disodium salt) to a final concentration of 2 mM, and the mixture was incubated with shaking for 60 min at 37°C. Free Ca^{2+} was controlled by varying the $CaCl_2$/EGTA ratio [16]. The free Ca^{2+} concentrations were calculated by the method of Katz et al. [17], using association constants

TABLE I

COMPARISON OF CALCULATED AND MEASURED FREE Ca^{2+}, EGTA AND ATP CONCENTRATIONS AT VARIOUS EGTA CONCENTRATIONS

Total $CaCl_2$ (µM)	Total EGTA (mM)	Free Ca^{2+} (µM)		$[Ca \cdot EGTA]^{2-}$ (µM)	$EGTA^{4-}$ (µM)	ATP^{4-} (µM)	$MgATP^{2-}$ (µM)	$CaATP^{2-}$ (µM)
		Calculated	Measured					
66.7	0.1	1.37	1.42	65.1	34.9	5.0	1.99	0.22
80.0	0.1	2.48	3.98	77.1	22.9	5.0	1.99	0.39
93.2	0.1	5.07	5.01	87.3	12.7	5.0	1.99	0.80
100.0	0.1	7.61	8.91	91.2	8.8	5.0	1.99	1.20
116.6	0.1	17.77	14.2	96.0	4.0	5.0	1.99	2.80
133.4	0.1	30.87	31.6	97.7	2.3	5.0	1.98	4.86
166.6	0.1	58.64	56.2	98.8	1.2	5.0	1.98	9.20
200.0	0.1	87.21	79.4	99.2	0.8	5.0	1.98	13.63
300.0	0.1	173.55	158.5	99.6	0.4	4.9	1.96	26.87
667	1.0	1.46	1.95	665.3	334.7	5.0	1.99	0.23
800	1.0	2.88	3.16	796.7	203.3	5.0	1.99	0.45
932	1.0	8.68	8.91	921.9	78.1	5.0	1.99	1.37
1000	1.0	24.83	31.6	971.3	28.7	5.0	1.99	3.91
1033	1.0	43.22	39.8	983.3	16.7	5.0	1.98	6.79
1067	1.0	67.04	63.1	989.2	10.8	5.0	1.98	10.50
1100	1.0	93.25	89.1	992.2	7.8	5.0	1.98	14.57
1200	1.0	176.78	177.5	995.9	4.1	4.9	1.96	27.36

of $1.3568 \cdot 10^6$ M, $3.15 \cdot 10^4$ M and $8.8 \cdot 10^4$ M at pH 6.9 for $[Ca \cdot EGTA]^{2-}$, $[Ca \cdot ATP]^{2-}$ and $[Mg \cdot ATP]^{2-}$, respectively. Free Ca^{2+} was also measured directly by a calcium selective electrode (IS 561-Ca^{2+}) calibrated in the complete assay medium minus Mg^{2+}, EGTA and ATP at 37°C. Buffered Ca^{2+} concentrations were measured at 37°C in the same medium plust EGTA. Calibration curves (mV vs. pCa^{2+}) were linear between 10^{-6} and 10^{-3} M Ca^{2+}. A small increase in the electrode potential due to the presence of EGTA was observed, and subtracted from the readings in the presence of EGTA. The calculated and observed Ca^{2+} values were similar (Table I); calculated values have been plotted in the figures. Table I also shows the calculated values of the various EGTA and ATP species in the reaction medium.

Determination of membrane-bound calcium

ATP-dependent Ca^{2+} binding at two EGTA concentrations was measured by atomic absorption spectrophotometry and by $^{45}CaCl_2$ equilibration.

Atomic absorption. Dodge ghosts (0.25 ml), frozen and thawed once at −20°C, were incubated with 0.1 mM or 1.0 mM EGTA at various $CaCl_2$ concentrations in the complete medium of the $(Mg^{2+} + Ca^{2+})$-ATPase assay, with or without ATP, in a final volume of 3 ml. The membranes were centrifuged at $14\,000 \times g$ for 5 min at 4°C and washed twice with the above incubation medium minus EGTA and $CaCl_2$. Membrane calcium was extracted with 0.5 ml of 3 M trichloroacetic acid and glacial acetic acid (1 : 1, v/v). $LaCl_3$ and KCl were added to a final concentration of 10 mM and 5 mg K/ml, respectively, and distilled water was added to a total volume of 2.5 ml. The suspension was centrifuged at $14\,000 \times g$ for 20 min. Calcium contents in the supernatant were determined by atomic absorption spectroscopy (Perkin Elmer model 360).

^{45}Ca-binding assay. Dodge ghosts were incubated as above, except that 2 μCi of ^{45}Ca was added to each tube and ^{45}Ca-binding was determined by the method of Porzig and Stoffel [18]. Membranes were centrifuged and a sample of the supernatant (0.5 ml) was counted in a scintillation counter. The rest of the supernatant was removed by aspiration and the inner sides of the tubes were wiped carefully. The wet weight of the pellet was determined. Since the dry weight of the pellet was negligible, it was assumed that the wet weight of the pellet represented the weight of supernatant trapped in the pellet. The ^{45}Ca trapped in the pellet was counted and expressed as cpm. Membrane bound ^{45}Ca was determined from the difference in counts between the pellet and the supernatant (cpm) above the pellet. The pellet was counted by dissolving it in 0.5 ml of 5% Triton X-100 and quantitatively transferring it to counting vials (three washes with 0.5 ml deionized distilled water). The specific activity in the incubation medium was determined at each calcium concentration and used for the calculation of calcium binding.

Solubilization of $(Mg^{2+} + Ca^{2+})$-ATPase

$(Mg^{2+} + Ca^{2+})$-ATPase was solubilized from hypotonic ghosts [19] (2–4 mg protein/ml) with Triton X-100 (1.5 mg/mg protein) at 5°C for 10 min with shaking. The suspension was centrifuged at $100\,000 \times g$ for 30 min at 2°C. The supernatant was separated and kept on ice for determination of $(Mg^{2+} + Ca^{2+})$-ATPase activity. The $(Mg^{2+} + Ca^{2+})$-ATPase assay was performed as above, except that Tris-ATP was added to a final concentration of 0.5 mM, the pH of the medium was 7.2 and the mixture was incubated for 5 min at 37°C. The activity was expressed as nmol $^{32}P_i$ release/min per mg protein.

Protein determination

Protein content of the solubilized ATPase was determined by the method of Lowry et al. [20]. The protein was precipitated free of interfering materials with deoxycholate and trichloroacetic acid prior to assay [21].

Materials

Inorganic salts and solvents were Analytical-Reagent grade. [γ-^{32}P]ATP (10–40 Ci/mmol) and ^{45}Ca as $CaCl_2$ (0.5 mCi/29.75 μg ^{45}Ca) were from Amersham. Charcoal (Norit A) was from Fisher Chemical Co., Triton X-100 and EGTA (sodium salt) were from Sigma. Purified calmodulin from bovine brain was kindly supplied by Dr. J. Penniston, Mayo Clinic, Rochester, Minnesota. The lyophilized preparation contained some low molecular weight protein (10 000 or less) in addition

to the predominant calmodulin band when analyzed by 0.1% SDS-gel electrophoresis, as previously described [13].

Results

The apparent Ca^{2+} sensitivity of $(Mg^{2+} + Ca^{2+})$-ATPase in 'Dodge ghost' membranes [12] increased as the total EGTA concentration was increased from 0.1 mM to 3 mM (Fig. 1). A similar shift in Ca^{2+} sensitivity was seen in membranes prepared by a stepwise hemolysis procedure [14] (results not shown). The Ca^{2+} activation curves showed complex kinetics. At low EGTA concentrations (0.1–0.4 mM) the Ca^{2+} activation was biphasic, with an apparent high Ca^{2+} affinity component which tended to saturated at 10–30 μM Ca^{2+}, and an apparent low Ca^{2+} affinity component which was not saturated at the highest Ca^{2+} concentration studied (176 μM). At higher total EGTA concentrations (0.8 mM to 3 mM) the high Ca^{2+} affinity component was increased relative to the total activity, and it was saturated at progressively lower Ca^{2+} concentrations as the EGTA concentration was increased from 0.8 mM to 3 mM. At the high EGTA concentrations the enzyme was inhibited by high Ca^{2+} concentrations.

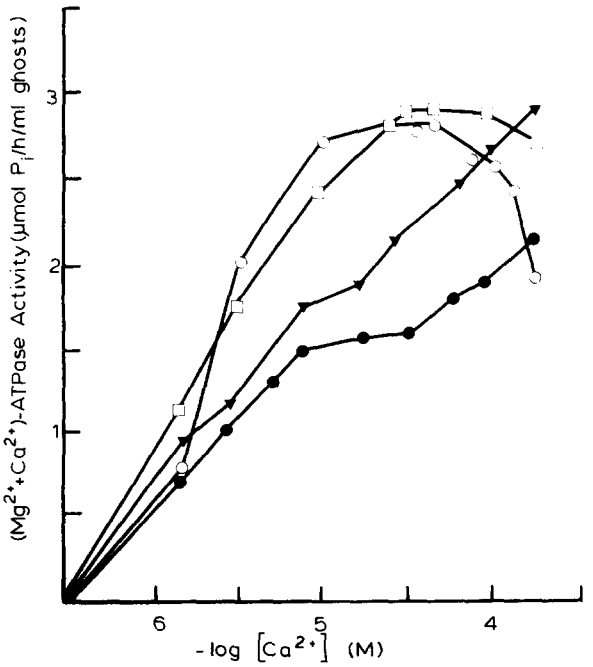


Fig. 1. Ca^{2+} activation of $(Mg^{2+} + Ca^{2+})$-ATPase activity in Dodge ghosts in the presence of different concentrations of EGTA: •———•, 0.1 mM; ▾———▾, 0.4 mM; □———□, 1.0 mM; ○———○, 3.0 mM. Mean values of three experiments are shown. The mean standard error in the observations was around 0.2. Error bars are omitted for clarity.

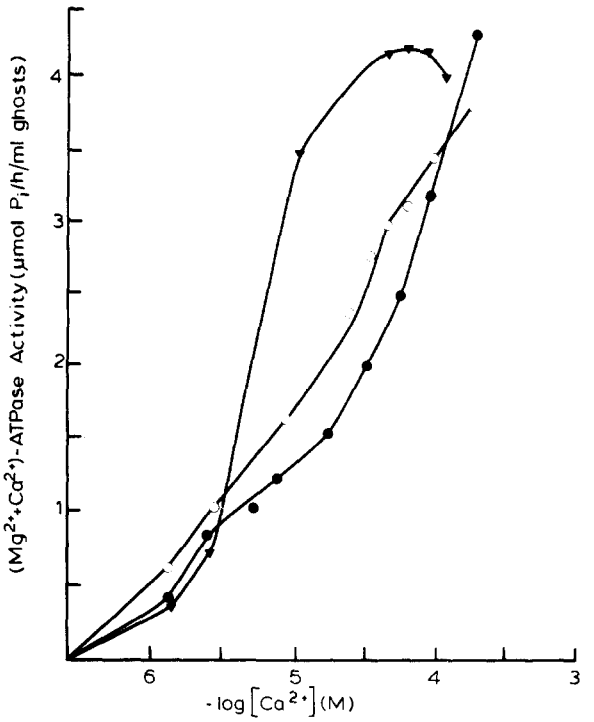


Fig. 2. Ca^{2+} activation of $(Mg^{2+} + Ca^{2+})$-ATPase activity in EDTA-treated membranes in the presence of different concentrations of EGTA: •———•, 0.1 mM; ○———○, 1.0 mM; ▾———▾, 3.0 mM. Mean values of three experiments are shown. The mean standard error of the determinations was around 0.5.

Increasing total EGTA concentration also increased the Ca^{2+} sensitivity of $(Mg^{2+} + Ca^{2+})$-ATPase in EDTA-treated membranes (Fig. 2). The total ATPase activity cannot be compared to that in untreated ghosts, as we have evidence that in the present series of experiments the ghosts contain latent ATPase activity in a population presumed to be sealed (unpublished results). From 0.1 mM to 1.0 mM EGTA the proportion of high Ca^{2+} affinity component to the total ATPase activity in the EDTA-treated membranes was less than in Dodge ghosts; thus these membranes showed mainly a low Ca^{2+} affinity component. At 3 mM EGTA the EDTA-

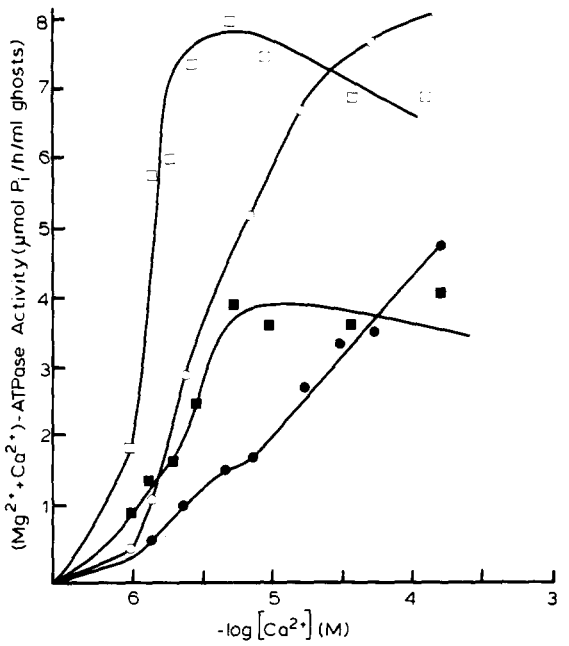


Fig. 3. Ca^{2+} activation of $(Mg^{2+} + Ca^{2+})$-ATPase activity in Dodge ghosts in the presence of 2 μg calmodulin (open symbols) and the absence of calmodulin (closed symbols) at different concentrations of EGTA: (●, ○) 0.1 mM; (■, □) 3.0 mM. The points represent a single experiment.

treated membranes showed a predominantly high Ca^{2+} affinity component, and a pronounced sigmoidal Ca^{2+} activation curve.

The EGTA-dependent shift in Ca^{2+} sensitivity also occurred in the presence of saturating concentrations of calmodulin (Fig. 3). Calmodulin increased the apparent Ca^{2+} sensitivity and maximum velocity of the enzyme at both 0.1 mM and 3.0 mM EGTA.

The effects of EGTA on the activity and Ca^{2+}-affinity of $(Mg^{2+} + Ca^{2+})$-ATPase were quantitated by nonlinear curve fitting analysis (see Appendix). The simplest model compatible with most of the data was a two-state model, where the enzyme exists in high Ca^{2+} affinity (E_H) or low Ca^{2+} affinity states (E_L), depending on the EGTA concentration. At 1.0 mM EGTA, Ca^{2+} activation in Dodge ghosts could be approximate by a Langmuir isotherm (r = 0.998 (Table II); see Fig. 6), described by Eqn. 1.

$$v_1 = \frac{V_1 \cdot [Ca^{2+}]}{[Ca^{2+}] + K_1} \quad (1)$$

where v_1, V_1 and K_1 are the velocity, maximum velocity and Ca^{2+} dissociation constant of the high Ca^{2+} affinity component, respectively.

Analysis of the Ca^{2+} activation curve for EDTA-treated membranes at 0.1 mM EGTA (Fig. 2) indicated that the low Ca^{2+} affinity component had not reached saturation by 175 μM free Ca^{2+} concentration. This means that the Ca^{2+} dissociation constant of the low Ca^{2+} affinity component (K_2) is large and $K_2 > [Ca^{2+}]$, so that the Ca^{2+} activation is described by Equation 2:

$$v_2 = K_2' \cdot [Ca^{2+}] \quad (2)$$

where K_2' is equal to V_2/K_2 when $K_2 > [Ca^{2+}]$. Assuming that the observed velocity (v) in a system containing both high and low Ca^{2+} affinity components is the sum of the velocities of the individual components, v is described by Equation 3.

$$v = \frac{V_1 \cdot [Ca^{2+}]}{[Ca^{2+}] + K_1} + K_2' \cdot [Ca^{2+}] \quad (3)$$

This equation closely fitted the data at low EGTA concentrations (Table II) *. At the high EGTA concentrations the data was best fitted by Eqn. 1 (Table II). (No attempt was made to model the inhibition at high Ca^{2+} concentrations and these points were not included in the computerized fitting.) The fit of representative data to the models is shown in the Appendix (Fig. 6).

The kinetic constants and their standard deviations derived from the nonlinear curve-fitting analysis are summarized in Table II.

The effect of EGTA on calcium binding to Dodge ghost membranes in the ATPase assay medium was determined by atomic absorption spectroscopy (Fig. 4A) or by ^{45}Ca equilibration (Fig. 4B). No

* Analysis of the data in ghosts by the equation

$$v = \frac{V_1 \cdot [Ca^{2+}]}{[Ca^{2+}] + K_1} + \frac{V_2 \cdot [Ca^{2+}]}{[Ca^{2+}] + K_2},$$

where V_2 and K_2 are the maximum velocity and Ca^{2+} dissociation constant of the low Ca^{2+} affinity component, gave a high correlation coefficient (r = 0.992). However, the standard deviations of the constants were large and hence more uncertain than those obtained from Eqn. 3.

TABLE II

KINETIC PARAMETERS OF (Mg^{2+} + Ca^{2+})-ATPase IN DIFFERENT MEMBRANE PREPARATIONS AT VARIOUS Ca-EGTA CONCENTRATIONS

The parameters V_1, K_1 and K'_2 were estimated by the computer curve fitting analysis (see Methods). The units of maximum velocity (V_1, V_2) and the Ca^{2+}-dissociation constant (K_1) are μmol P_i/h per ml packed cells and μM, respectively. The absolute velocities of Dodge ghosts and EDTA-treated membranes are not directly comparable. n.d., not determined

Total EGTA (mM)	Dodge ghosts				EDTA-treated membrane			
	V_1 (±S.D.)	K_1 (±S.D.)	K'_2 (±S.D.)	r	V_1 (±S.D.)	K_1 (±S.D.)	K'_2 (±S.D.)	r
0.1	1.7 ± 0.05	1.6 ± 0.2	0.003 ± 0.001	0.995 *	1.8 ± 0.17	4.6 ± 1.3	0.02 ± 0.001	0.996 *
0.2	1.8 ± 0.05	2.0 ± 0.2	0.004 ± 0.001	0.996 *	n.d.	n.d.	n.d.	n.d.
0.4	2.2 ± 0.11	2.6 ± 0.50	0.005 ± 0.001	0.989 *	4.25 ± 0.94	20.0 ± 8.1	0.001 ± 0.005	0.986 *
0.8	2.2 ± 0.20	1.8 ± 0.50	0.014 ± 0.004	0.989 *	3.64 ± 0.60	15.9 ± 5.2	0.002 ± 0.003	0.991 *
1.0	3.1 ± 0.04	2.6 ± 0.16	–	0.998 **	3.20 ± 0.20	7.8 ± 1.5	0.005 ± 0.002	0.995 *
3.0	3.14 ± 0.45 ***	2.5 ± 1.3	–	0.938 **	4.80 ± 0.50 ***	7.7 ± 3.5	–	0.967 **
								0.953 *

* Best fit by equation 3.

** Best fit by equation 1.

*** This data exhibited an apparent positive cooperativity (Figs. 1 and 2) not taken into account by eqn. 1 or 3: this accounts for the lower correlation coefficient (r) observed in this data.

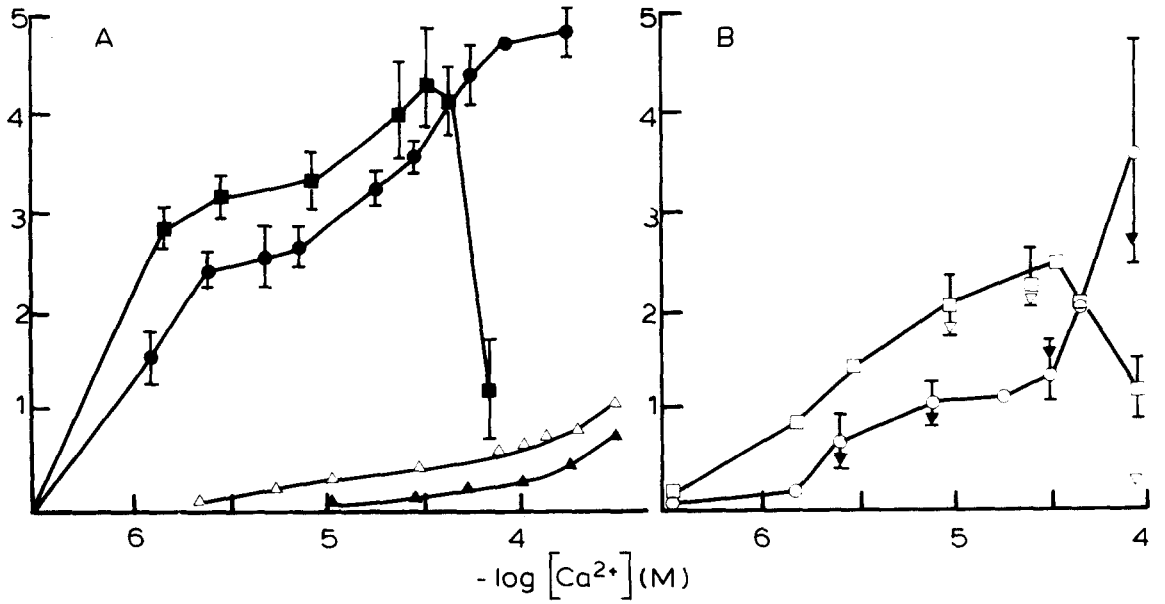


Fig. 4. Calcium binding in the presence of different [Ca^{2+}]. Ca^{2+} was determined (A) by atomic absorption, (B) by $^{45}Ca^{2+}$ equilibrium. EGTA was 0.1 mM (●, ○) or 1.0 mM (■, □). Ionophore A23187 (2 μM) was added in some experiments in (B) at 0.1 mM EGTA (▼) and 1.0 mM EGTA (▽). Calcium binding in the absence of ATP was determined in the presence of 0.1 mM EGTA (▲) and 1.0 mM EGTA (△). Points represent means ± S.E. of at least three experiments. Ordinate: membrane-bound Ca (nmol/mg protein).

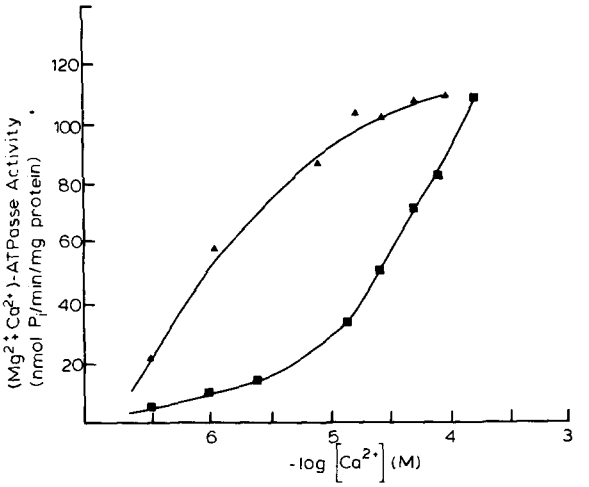


Fig. 5. Ca^{2+} activation of Triton X-100 solubilized (Mg^{2+} + Ca^{2+})-ATPase at different EGTA concentrations. The EGTA concentrations were 0.1 mM (■———■) and 3.0 mM (▲———▲). Points represent means of two experiments; the mean range was 0.1.

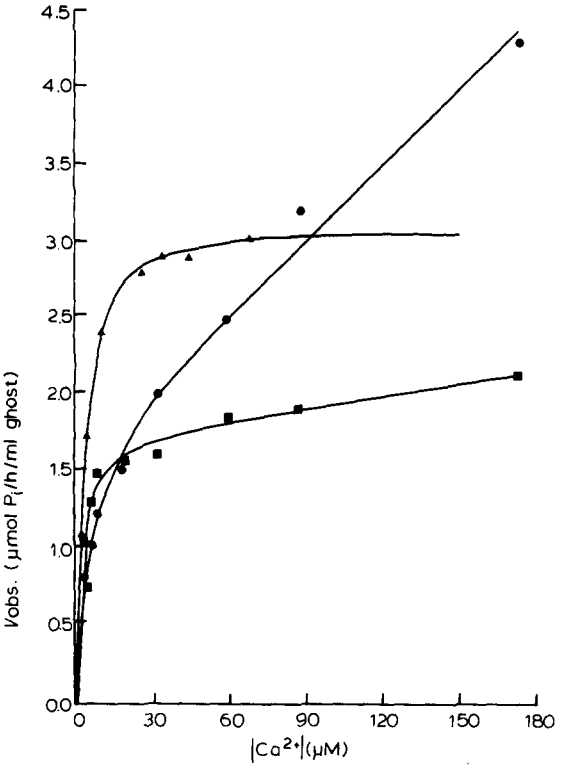


Fig. 6. Computer curve fitting of Ca^{2+} activation of (Mg^{2+} + Ca^{2+})-ATPase. Curves were fitted to the data by the curve-fitting program described in the Appendix. (■), Dodge ghosts, 0.1 mM EGTA; (▲), Dodge ghosts, 1.0 mM EGTA; (●), EDTA-extracted membranes, 0.1 mM EGTA.

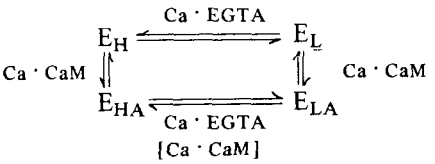
endogenous calcium was detected either at 0.1 mM or 1.0 mM EGTA when 2 μM Ca^{2+} ionophore A23187 was included in the assay medium (results not shown). Calcium binding was significantly increased by ATP (Fig. 4A), as also reported by Porzig and Stoffel [18]. The Ca^{2+} dependence of binding was biphasic, similar to that observed for (Mg^{2+} + Ca^{2+})-ATPase activation. At 0.1 mM EGTA, both high and low Ca^{2+} affinity components were observed by both methods (Fig. 4A and 4B). The low Ca^{2+} affinity component was not saturated by 100 μM or more Ca^{2+} in the medium. More calcium was bound at 1.0 mM EGTA than at 0.1 mM EGTA up to 35 μM free Ca^{2+}. At 1.0 mM EGTA, Ca^{2+} binding peaked around 35 μM Ca^{2+} and declined at higher Ca^{2+} concentrations. The calcium bound was not trapped inside vesicles, as A23187 did not decrease the ^{45}Ca levels (Fig. 4B), probably because Ca^{2+} did not reseal the ghosts, which were freeze-thawed before the binding studies were performed.

The shift in Ca^{2+} affinity with EGTA was also found in Triton X-100 solubilized (Mg^{2+} + Ca^{2+})-ATPase (Fig. 5). Whereas at 0.1 mM EGTA Ca^{2+} activation was mainly of a low Ca^{2+} affinity, a high Ca^{2+} affinity was obtained at 1.0 mM EGTA.

Discussion

In this study we have confirmed earlier observations that the apparent Ca^{2+} affinity of the Ca^{2+}-transport ATPase in human erythrocyte membranes is increased by the presence of EGTA in the assay medium. The apparent Ca^{2+} sensitivity increased progressively as the total EGTA in the medium was increased systematically from 0.1 to 3 mM. This occurred in membranes prepared by hypotonic hemolysis and in membranes in which a predominantly low Ca^{2+} affinity ATPase was induced by EDTA-treatment. More EGTA was required to shift the Ca^{2+} affinity in EDTA-treated membranes than in Dodge ghosts. EGTA did not increase the maximum activity of the (Mg^{2+} + Ca^{2+})-ATPase, and hence its action differs from calmodulin, which increases maximum velocity as well as the apparent

Ca^{2+} affinity of the enzyme ([9,10] and Fig. 3). This observation also makes it unlikely that the effect of EGTA is due to an alteration in the extent of association of the enzyme with endogenous calmodulin, known to be present to varying degrees in isolated membranes [11,13]. Furthermore, the shift in apparent Ca^{2+} affinity produced by EGTA also occurred in the presence of saturating levels of added calmodulin. The data suggests that (Mg^{2+} + Ca^{2+})-ATPase exists in a number of functional states,



as depicted in Model I, where E_L is a low Ca^{2+} affinity state in the absence of EGTA [9], E_H is a high Ca^{2+} affinity, low V state in the presence of saturating amounts of EGTA, and E_A is a high V state in the presence of Ca^{2+} and calmodulin (CaM). Calmodulin, like EGTA, also increases the apparent Ca^{2+} affinity of the enzyme [9,10], but in some conditions EGTA can further increase the Ca^{2+} affinity of the calmodulin-activated state. The differences in the Ca^{2+} affinity of the Ca^{2+} transport ATPase in human erythrocytes reported in the literature (see Ref. 1) may therefore be explained in part by the presence or absence of EGTA in the assays, as well as the extent of association of the transport complex with its endogenous activators.

At low EGTA concentrations the data was best fitted by two (high and low) Ca^{2+} affinity states, whereas at high EGTA concentrations a saturable, single high Ca^{2+} affinity state best described the experimental data (Table II). The major effect of EGTA was to increase the proportion of the E_H state and to abolish the low Ca^{2+} affinity (E_L) component. Thus we propose that in the presence of EGTA the enzyme is converted to a high Ca^{2+} affinity state. Free Ca^{2+} in excess of 35 μM inhibits the enzyme in the high Ca^{2+} affinity state, by as yet unidentified mechanisms which may correspond to the inhibition of total membrane calcium-binding (Fig. 4).

The increase in total calcium, which accompanied increased EGTA concentrations, could not account for the EGTA effect, as the activity at 66.7 μM $CaCl_2$ (0.1 mM EGTA) was of the same order as that at 667 μM $CaCl_2$ (at 1.0 mM EGTA) (Fig. 1). $[ATP]^{4-}$, $[MgATP]^{2-}$ and $[CaATP]^{2-}$ remained constant as EGTA (and $CaCl_2$) were increased. Both $[CaEGTA]^{2-}$ and $[EGTA]^{4-}$ increased as the total EGTA concentration was increased, but only the former increased progressively with increasing free Ca^{2+} (Table I). We propose that free Ca^{2+} is required for activation, while $[CaEGTA]^{2-}$ regulates the Ca^{2+} affinity of the enzyme. At 0.1 mM EGTA there is insufficient $[CaEGTA]^{2-}$ to induce fully the high Ca^{2+} affinity state, resulting in mixed high and low Ca^{2+} affinity states. The proposed role of $[Ca \cdot EGTA]^{2-}$ is consistent with the higher total calcium-binding observed at 1.0 mM EGTA compared to 0.1 mM (Fig. 4) and suggests that $[Ca \cdot EGTA]^{2-}$ combines with the enzyme-membrane complex. The biphasic Ca^{2+} binding observed at 0.1 mM EGTA (Fig. 4A and B) is similar to the biphasic Ca^{2+} activation of (Mg^{2+} + Ca^{2+})-ATPase under these conditions (Fig. 1 and 2). Although the calcium binding was significantly stimulated by ATP, the maximum amount of calcium bound (2.5–5.5 nmol/mg) represents more than 500 000 sites per erythrocyte [18], well in excess of the 700 (Mg^{2+} + Ca^{2+})-ATPase sites reported to be present in the red blood cell [22]. The nature of the ATP-stimulated Ca^{2+} binding on the membrane is unknown [18]. However, it is likely that the (Mg^{2+} + Ca^{2+})-ATPase involves only a small fraction of the binding sites measured. The effect of EGTA was also observed in detergent-solubilized (Mg^{2+} + Ca^{2+})-ATPase, where the membrane organization is disrupted (Fig. 5).

Sarkadi et al. [23] proposed that both Ca^{2+} and Ca · EGTA bind simultaneously to stimulate the Ca^{2+}-transport complex, the Ca · EGTA substituting for Ca^{2+} at a low Ca^{2+} affinity site which must be occupied for Ca^{2+} activation. An alternative mechanism may involve $[Ca \cdot EGTA]^{2-}$ at a regulatory site which converts the enzyme to a high Ca^{2+} affinity state. In support of this mechanism, we have found that a number of other anions stimulate (Mg^{2+} + Ca^{2+})-ATPase activity in both membrane and detergent-solubilized preparations at a fixed total calcium concentration (Al-Jobore, A., Minocherhomjee, A. and Roufogalis, B.D., unpublished results). However, it is also possible that the increase in total calcium

with increasing EGTA concentration in some way enhanced the diffusion of Ca^{2+} to its active site, which could account for the results if the Ca^{2+} site on the transport complex is poorly accessible.

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Appendix

The curve fitting program used was NONLIN, written by Metzler, C.M., and described in Metzler, C.M. (1969) A User's Manual for NONLIN, the Upjohn Company Technical Report 7292/69/7292/005, Kalamazoo, MI; the 1974 update was used in this work. The correlation coefficient, r, is a measure of the portion of the total variation (variance) in the data which is predicted by the model, with the parameters which were chosen in the program.

The biphasic Ca^{2+}-activation kinetics of (Mg^{2+} + Ca^{2+})-ATPase at 0.1 mM EGTA was described by a two state model, assuming that the enzyme existed in a high Ca^{2+} affinity state in the presence of saturating EGTA and in a low Ca^{2+} affinity state in the absence of EGTA. This is the simplest model which fitted the data, assuming that the observed velocity at any Ca^{2+} concentration is the sum of the velocity of the two individual states. This model assumes rapid equilibration of Ca^{2+} with the two states, and that the affinity of substrate for the two states does not change at different Ca^{2+} concentrations. While the model predicts the intermediary plateau region (but not a dip) between the two components observed experimentally, a better fit will probably require extension of the model to include more than one Ca^{2+} binding site [24], and cooperativity between Ca^{2+} sites [21], particularly at very high EGTA concentrations (Figs. 1 and 2). Application of a more complex model than that used here is not justified by the data presently available.

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