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Binding and elution of EGTA to anion exchange columns: implications for study of (Ca + Mg)-ATPase inhibitors

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Abstract

EGTA bound to DEAE-Sephadex and DEAE-cellulose at low ionic strength in the presence and absence of applied protein. It remained bound when the column was washed at low ionic strength, but was eluted as the ionic strength was increased. The amount of EGTA recovered at high ionic strength was 60 to 90% of that applied to the column. At the peak of its elution, the concentration of EGTA in the eluted fractions was 25 mM, over 10-fold higher than the concentration of EGTA applied to the column. Eluted fractions containing EGTA inhibited the (Ca + Mg)-ATPase by two mechanisms: (1) by chelating the Ca and (2) by affecting activity even when the free Ca was held constant. We suggest that at least some of the inhibitory effects previously attributed to a cytoplasmic inhibitor of the (Ca + Mg)-ATPase may in fact be due to contaminating amounts of EGTA.

Key words: Erythrocyte; ATPase, (Ca^{2+} + Mg^{2+})-; EGTA; Chromatography; Intracellular calcium ion; Calcium ion transport

1. Introduction

No one has yet purified or identified the proposed cytoplasmic inhibitor [1–9] of the red cell (Ca + Mg)-ATPase, the enzymatic basis of the Ca pump in the plasma membrane. The existence of a soluble inhibitor is of interest, because it could help regulate the Ca pump, one of the principal mechanisms that modulate intracellular Ca. During the purification of another cytoplasmic protein, we noted that a set of fractions isolated from red cell hemolysate by means of Ca-dependent hydrophobic interaction [8] and anion exchange chromatography both chelated Ca and inhibited the (Ca + Mg)-ATPase at constant Ca [9]. The isolated samples contained a protein of 500 to 1,000 MW which could explain the inhibition at constant Ca, but was present in amounts too small to account for the observed chelation [9]. We show here that EGTA, which we and others have used during attempts to isolate the proposed inhibitor, can account for the reduction in free Ca and at least a portion of the inhibition observed at constant free Ca.

2. Methods

2.1. Hemolysate

Human red blood cells were washed in 310 mosM phosphate buffer and the buffy coat removed by aspiration. The cells were then filtered through cellulose [10]. Hemolysate was prepared by hemolyzing 178 ml of packed cells in 20 volumes of ice-cold 20 mosM phosphate buffer (P20) containing 0.1 mM PMSF (pH 7.4) and removing the membranes using a Pellicon apparatus as previously described [11].

2.2. Ca-dependent hydrophobic interaction chromatography

Ca was slowly added to the hemolysate to a final concentration of 0.4 mM. 60 ml of phenyl-Sepharose

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Abbreviations: PMSF, phenylmethylsufonyl fluoride; PS, phenyl-Sepharose; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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equilibrated in 0.4 mM Ca and P20 was added to the hemolysate, the mixture was brought to room temperature, and was slowly stirred for 60 min. The phenyl-Sepharose was then collected on a sintered glass funnel and washed with 20 bed volumes of a solution containing 20 mM ammonium bicarbonate and 0.4 mM Ca (pH 7.7). The mixture was poured onto a 3.5×40 cm column and washed with the same solution until the absorbance at 275 nm reached baseline. The column was then eluted with a solution containing 20 mM ammonium bicarbonate, 2 mM EGTA (pH 7.7), yielding a sample of 288 ml which contained 21 mg of protein.

2.3. Anion exchange chromatography

DEAE-Sephadex (A-25) was swollen in 500 mM ammonium bicarbonate (pH 7.7), and then equilibrated in 20 mM ammonium bicarbonate (pH 7.7). After pouring and settling the column was washed with 20 mM ammonium bicarbonate to achieve a constant A_{206}. The sample was applied (see figure legends for individual experiments) and the column was washed with 20 mM ammonium bicarbonate until the A_{206} returned to baseline. Samples were eluted with a linear gradient of ammonium bicarbonate equal to approximately 4 bed volumes.

2.4. Preparation of human red cell membranes

Human red blood cell membranes were prepared by a modification of the Dodge procedure [12] as previously described [11].

2.5. ATPase assay

The (Ca + Mg)-ATPase assay was carried out using human red cell membranes stripped of endogenous calmodulin [11,13]. The assay medium contained 18 mM NaCl, 30 mM KCl, 112 mM choline chloride, 20 mM Hepes, 5 mM EDTA, 0.2% bovine serum albumin and 0.5 mM ouabain, and either 5.5 mM $MgCl_2$ (0 μM free Ca) or 1.84 mM $CaCl_2$, and 3.68 mM $MgCl_2$ (2 μM free Ca). The concentration of 2 μM free Ca was calculated from the total concentration of EDTA, Ca, Mg, and ATP by the iterative method of Perrin and Sayce [14]. Stability constants were from Martell and Smith [15]. The assay was conducted by first preincubating the membranes for 10 min at 37°C in the assay mixture without ATP. The reaction was started by adding ATP to a final concentration of 1 mM. The final assay volume was 0.3 ml. Thirty minutes later the reaction was stopped and the concentration of inorganic phosphate analyzed [16]. The amount of ATP hydrolyzed was 15% or less of the total. The (Ca + Mg)-ATPase activity was determined from the difference in P_i released with and without Ca. Enzyme activity is expressed as nanomoles P_i per milligram membrane protein per minute. Protein was determined according to Lowry et al. [17].

2.6. Arsenazo III measurements

To monitor the free Ca in the ATPase assay 200 μM arsenazo III was added to the assay solutions and the absorbance read at $A_{655} - A_{700}$ [18]. A volume of 0.05 ml of each fraction was added to the final assay solution (final volume of 0.3 ml) and the absorbance was read in a cuvette with a 1 mm pathlength. In fractions that reduced the absorbance of the arsenazo III, small volumes of concentrated $CaCl_2$ were added until the absorbance, as measured at a final volume of 0.3 ml, was the same as control values. The amount of Ca that restored the absorbance to control values is equal to the amount of Ca chelated by the added fraction. After the absorbance is restored, the free Ca in the sample is the same as that in the controls. The amount of EGTA present in the sample is equal to the amount of Ca added back to restore the original free Ca, because Ca binds to EGTA 1:1 at neutral pH.

2.7. Chemicals, chromatography media, and blood

All chemicals were reagent grade. Phenyl-Sepharose was purchased from Sigma (St. Louis, MO) and DEAE-Sephadex was obtained from Sigma and Pharmacia (Piscataway, NJ). Blood was obtained from the Red Cross within 3 days of collection.

3. Results

To purify and identify the proposed inhibitor of the (Ca + Mg)-ATPase, proteins were isolated from human red cell hemolysate using Ca-dependent hydrophobic interaction and applied to a DEAE-Sephadex column (see Methods and the legend to Fig. 1A for details). The applied protein was eluted in a number of broad peaks by a linear gradient of 20 to 500 mM ammonium bicarbonate (Fig. 1A).

Fractions 131 to 148 eluted between 250 and 500 mM ammonium bicarbonate (Fig. 1A) inhibited the (Ca + Mg)-ATPase measured at 2 μM free Ca in the absence of added calmodulin (Fig. 1B, solid line), these are conditions which partially activate the (Ca + Mg)-ATPase. Fractions that inhibited the (Ca + Mg)-ATPase reduced the free Ca present in the assay solutions as monitored by the absorbance of arsenazo III, a Ca-sensitive dye (Fig. 1C, solid line). The free Ca was reduced from its initial value of 2 μM to below the detection limit of the dye (≈ 0.1 μM). This reduction occurred in spite of the presence of 5 mM EDTA

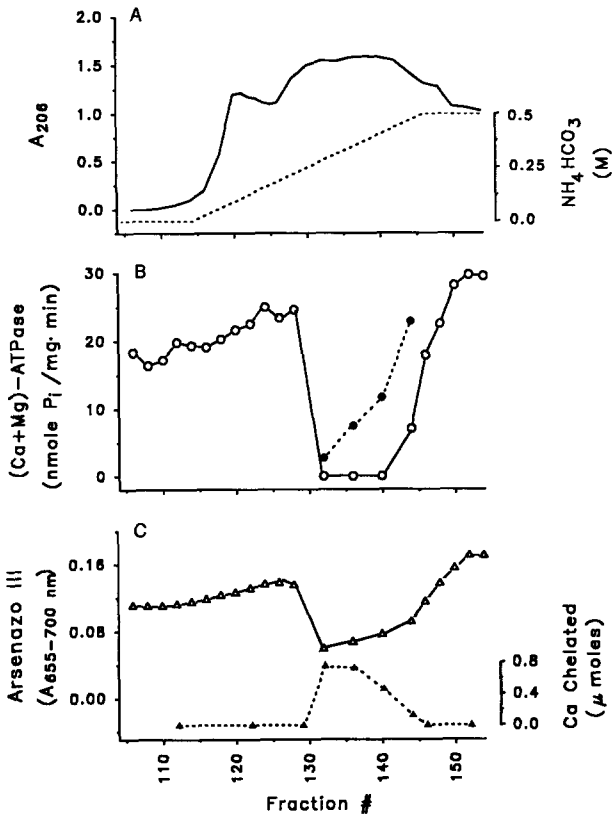

Fig. 1. (A) Elution profile of a DEAE-Sephadex column that was loaded with a solution containing protein and EGTA previously eluted from a phenyl-Sepharose column containing red cell proteins. Red cell hemolysate was applied to the phenyl-Sepharose column in the presence of Ca, unbound proteins were washed off, and the column was eluted in 288 ml of a solution containing 20 mM ammonium bicarbonate and 2 mM EGTA (pH 7.7) (see Methods). This solution containing 21 mg of protein was then pumped onto the DEAE column (20 ml bed vol.). The column was washed with 6 bed volumes of 20 mM ammonium bicarbonate (pH 7.7) and eluted with a linear gradient of 20 to 500 mM ammonium bicarbonate (total vol. = 90 ml) followed by 2 bed volumes of 500 mM ammonium bicarbonate (pH 7.7). Samples were collected in 2-ml fractions. Most of the protein was eluted in 70 ml giving an average protein concentration of 0.3 mg/ml. (B) The effect of the eluted samples on the activity of the (Ca + Mg)-ATPase initially measured at 2 μM free Ca. The solid line shows the effect of adding 0.05 ml of the above fractions to the ATPase assay (final volume of 0.3 ml). Some of these fractions reduced the free Ca in the assay (Fig. 1C). The dashed line shows the effect of these fractions after Ca was restored to 2 μM. (C) The absorbance (solid line) of arsenazo III in the solutions used to measure the activity of the (Ca + Mg)-ATPase in Fig. 1B. The graph (dotted line) shows the amount of Ca that had to be added back to the samples to restore the absorbance and free Ca to control levels. This is equal to the amount of Ca chelated by the added fractions. The absorbance in the lower numbered fractions (No. 110 and less) is primarily due to the presence of 2 μM free Ca in the assay solution. The absorbance increases gradually in higher numbered fractions due to the increasing ionic strength.

included in the assay solution to buffer the free Ca (see Methods), indicating that the fractions contained a strong chelator of Ca. To measure the amount of Ca chelated by the added fractions, Ca was added back to each sample to restore the original concentration of free Ca (see Methods). The amount of Ca chelated was the highest in the fractions that most decreased the absorbance of the arsenazo III (Fig. 1C, dotted line). The concentration of the added back Ca in the assay was approx. 2.7 mM in those samples that contained the fractions (131–137) that chelated the most Ca. On the assumption that the chelator bound Ca 1:1, the concentration of chelator in these fractions was over 16 mM.

To determine if the inhibition of the (Ca + Mg)-ATPase was all due to a reduction in free Ca, the activity of the (Ca + Mg)-ATPase was also measured after addition of Ca so that the free Ca was the same in all samples. Under these conditions fractions that had chelated Ca still inhibited the (Ca + Mg)-ATPase (Fig. 1B, dotted line). These results indicated that the eluted fractions contained a substance(s) that both chelated Ca and inhibited the (Ca + Mg)-ATPase, even when the free Ca was held constant.

To test if the substance that was chelating Ca came from the red cells, we repeated the above purification procedure using the same chromatographic media and solutions, but in the absence of red cell proteins. After loading the DEAE column with the solution eluted from phenyl-Sepharose, the DEAE column was again eluted with a linear gradient. Fractions eluted between 250 and 450 mM ammonium bicarbonate (Fig. 2A, solid line) again inhibited the (Ca + Mg)-ATPase (Fig. 2B, solid line) and chelated Ca (Fig. 2C, solid line). Fractions that reduced the absorbance, chelated the most Ca (Fig. 2C, dotted line). When fractions that chelated Ca were analyzed at constant free Ca (after back titrating with Ca) they still inhibited the (Ca + Mg)-ATPase (Fig. 2B, dotted line). The chelating capacity of the sample was similar to that observed in Fig. 1, because the amount of Ca required to bring the free Ca back to control levels was similar. The results shown in Fig. 2 indicate that EGTA or something eluted either from the phenyl-Sepharose or from the DEAE-Sephadex in the presence of EGTA chelated Ca and inhibited the (Ca + Mg)-ATPase.

To evaluate if the observed chelation and inhibition of the (Ca + Mg)-ATPase was due to something eluted from the phenyl-Sepharose in the presence of EGTA, a volume of solution containing only 2 mM EGTA equal to that used in Fig. 1 was pumped directly over the DEAE-Sephadex. The column was then washed with 20 mM ammonium bicarbonate, and eluted with a linear gradient of ammonium bicarbonate. Between 250 and 500 mM ammonium bicarbonate, a sample was eluted (Fig. 3A, solid line) which both inhibited the (Ca + Mg)-ATPase (Fig. 3B, solid line) and chelated Ca (Fig. 3C, solid line). The chelating capacity of this sample was similar to that observed in the other two types of experiments, because the amount of Ca re-

quired to restore the original concentration of 2 μM free Ca was similar (Fig. 3C, dotted line). Likewise, the sample inhibited the (Ca + Mg)-ATPase, even when the free Ca was adjusted to control levels (Fig. 3B, dotted line). The concentration of the added back Ca in the assay was 4 mM in those fractions (39–44) that chelated the most Ca. On the assumption that the chelation was due to EGTA, the EGTA concentration must also have been 4 mM, because EGTA binds Ca 1:1. Since these fractions were diluted 6-fold when put in the assay, the concentration of EGTA in the eluted fractions was 24 mM.

When a similar DEAE column was loaded with the same solutions without EGTA, a small peak was eluted

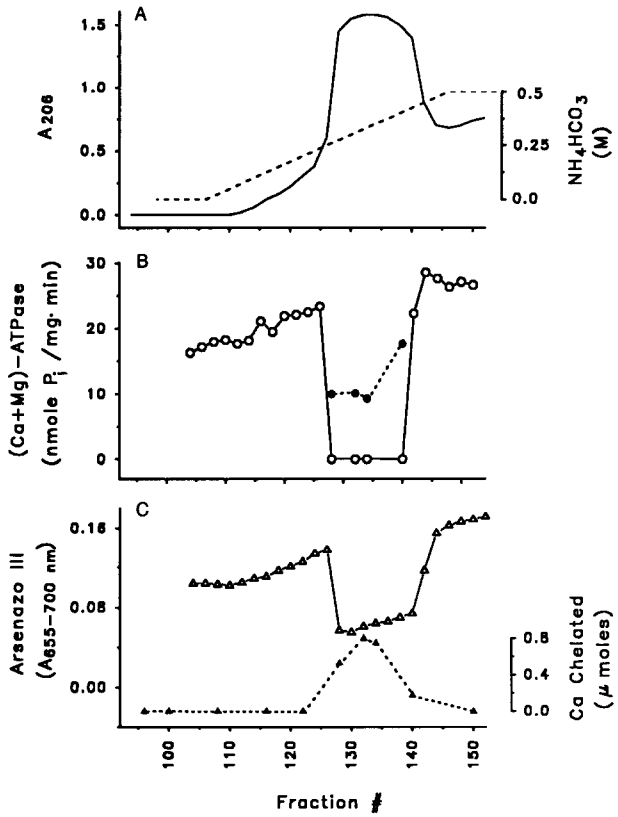

Fig. 2. (A) Elution profile of a DEAE-Sephadex column that was loaded with a solution containing EGTA (and no protein) previously eluted from a phenyl-Sepharose column not containing any protein. Otherwise, both the columns and the procedures were exactly as described in the legend to Fig. 1. (B) The effect of the eluted samples on the activity of the (Ca + Mg)-ATPase measured initially at 2 μM free Ca. The solid line shows the effect of adding 0.05 ml of the above fractions to the ATPase assay (0.3 ml final vol). Some of these fractions reduced the free Ca in the assay (Fig. 2C). The dashed line shows the effect of these fractions after Ca was restored to control levels. (C) The absorbance (solid line) of arsenazo III in the solutions used to measure the activity of the (Ca + Mg)-ATPase in Fig. 2B. The graph (dotted line) shows the amount of Ca that had to be added back to the samples to restore the absorbance and free Ca to control levels. This is equal to the amount of Ca chelated by the added fractions.

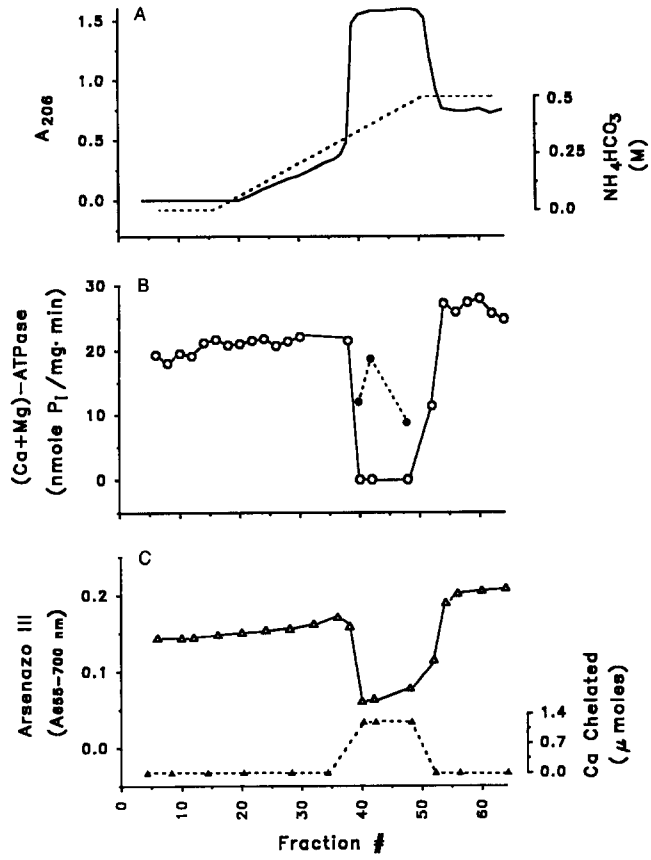

Fig. 3. (A) Elution profile of a DEAE-Sephadex column that was loaded with a solution containing EGTA not exposed to phenyl-Sepharose. A solution (288 ml) containing 20 mM ammonium bicarbonate, 2 mM EGTA (pH 7.7) was pumped directly onto the DEAE column. Otherwise, the DEAE column and the procedures were exactly as described in the legend to Fig. 1. (B) The effect of the eluted samples on the activity of the (Ca + Mg)-ATPase measured initially at 2 μM free Ca. The solid line shows the effect of adding 0.05 ml of the above fractions to the ATPase assay (0.3 ml final vol). Some of these fractions reduced the free Ca in the assay (Fig. 3C). The dashed line shows the effect of these fractions after Ca was restored to control levels. (C) The absorbance (solid line) of arsenazo III in the solutions used to measure the activity of the (Ca + Mg)-ATPase in Fig. 3A. The dashed line shows the amount of Ca added back to restore the absorbance and free Ca to control levels.

above 400 mM ammonium bicarbonate (Fig. 4A, solid line). Fractions containing 250 to 500 mM ammonium bicarbonate did not inhibit the (Ca + Mg)-ATPase (Fig. 4, solid line B) or chelate Ca (Fig. 4C, solid line). These data indicate that EGTA itself or something eluted from the DEAE column by EGTA is responsible for the absorbance at 206 nm, the chelation of Ca, and the inhibition of the (Ca + Mg)-ATPase even at constant Ca.

To determine if the inhibition was caused by the EGTA, and not by material eluted from DEAE-Sephadex by EGTA, EGTA was added directly to the (Ca + Mg)-ATPase assay. The addition of the EGTA inhibited the (Ca + Mg)-ATPase (Fig. 5, A open cir-

cles) and reduced the absorbance of arsenazo III (Fig. 5, B open triangles) due to a decrease in free Ca. The added EGTA also inhibited the (Ca + Mg)-ATPase when compensating Ca was added to maintain the free Ca constant at 2 μM (Fig. 5, A solid circles). The amount of Ca that had to be added to the assay to maintain the absorance of arsenazo III at a constant level was very similar to that added in the experiments shown in Figs. 1, 2, and 3 and was equal to the amount of EGTA added to the assay (Fig. 5, C). These results show that the inhibition was caused by the EGTA itself and not some factor removed from the DEAE by the EGTA.

Most of the EGTA applied to the columns (600 μmoles) was recovered in the eluted fractions. The percent recoveries ranged from 58% (Fig. 1) to 91% (Fig. 3). Furthermore, the concentration of EGTA in some of the eluted fractions was over 24 mM (Fig. 3), compared to 2 mM in the buffer applied to the column. These results demonstrate that the EGTA was binding

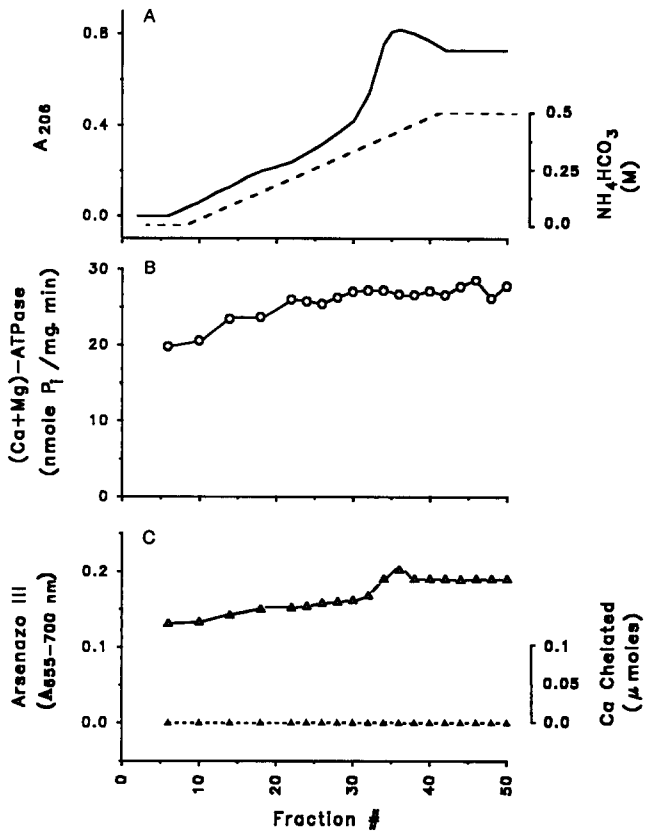

Fig. 4. (A) Elution profile of a DEAE-Sephadex column, previously loaded with the same solution used in Fig. 3, except that EGTA was absent. A solution (288 ml) containing 20 mM ammonium bicarbonate pH 7.7 was pumped onto the DEAE column. The column was then washed and eluted as described in the legend to Fig. 1. (B) The effect of the eluted samples on the activity of the (Ca + Mg)-ATPase at 2 μM free Ca. The solid line shows the effect of adding 0.05 ml of the above fractions to the ATPase assay. (C) The absorbance of arsenazo III in the solutions used to measure the activity of the (Ca + Mg)-ATPase in Fig. 4A.

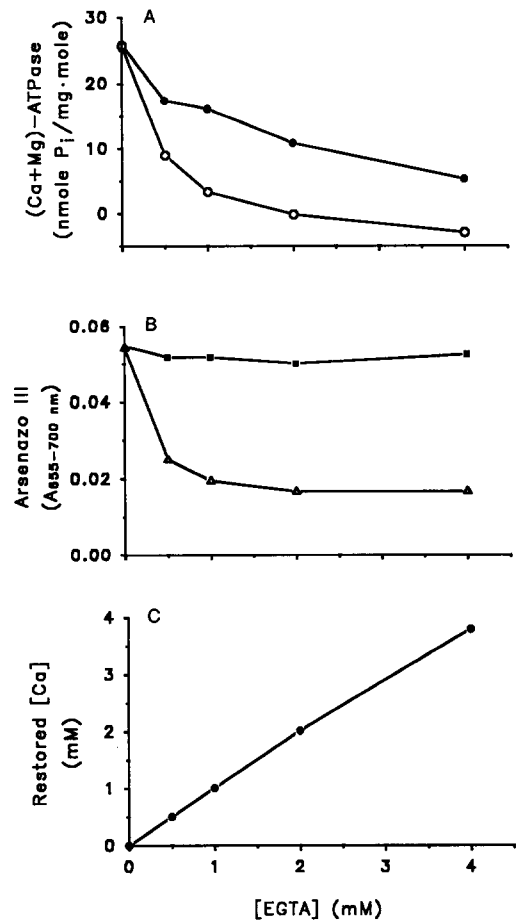

Fig. 5. (A) Effect on the (Ca + Mg)-ATPase activity of adding EGTA directly to the assay solution. The open circles (○) show the effect of adding EGTA without also adding Ca. The solid symbols (●) show the effect of EGTA when the free Ca was maintained at 2 μM by the concurrent addition of supplementary Ca. (B) The absorbance of arsenazo III in the solutions used to measure the activity of the (Ca + Mg)-ATPase in A. The absorbance in the solutions to which only EGTA was added are shown by the open triangles (△). The absorbance in the solutions to which both EGTA and supplementary Ca was added is shown by the closed squares (■). (C) The concentration of Ca that was required at each concentration of EGTA to maintain the absorbance of arsenazo III the same as the control to which no EGTA was added. This concentration of free Ca was 2 μM.

to the column and explain how the eluted fractions had such a marked effect on reducing the free Ca.

4. Discussion

We showed that EGTA bound to DEAE-Sephadex columns at low ionic strength in the presence and absence of protein and was eluted when the ionic strength was increased. The binding of EGTA was relatively strong, because it was not washed off at low ionic strength. Similar results were also found with DEAE-cellulose between pH 5 and 7.8 (data not

shown). These results suggest that EGTA could unexpectedly contaminate protein samples that are eluted from some types of anion exchange columns.

The copurification of EGTA along with red cell proteins on DEAE could account for some of the observations that red cells contain a cytoplasmic inhibitor of the (Ca + Mg)-ATPase. Either DEAE-Sephadex or similar anion exchange resins have been used in most of the attempts to purify the proposed inhibitor [2,3,5,7–9] and EGTA has been a common constituent of many purification buffers [4–6,8,9]. Even in studies where EGTA was not specifically mentioned, it is possible that trace amounts were inadvertently included and concentrated by the anion exchange resins. Contaminating EGTA may have subsequently been removed by dialysis [4,6]. On the other hand, we have found that EGTA stays on top of Amicon membranes (UM2 and YC05) with a 500 MW cutoff (data not shown) used to desalt samples [5].

The results presented here suggest that EGTA can account for the chelating effect observed in our earlier studies and at least some of the inhibition seen at constant Ca [9]. One expects that EGTA should inhibit the (Ca + Mg)-ATPase by reducing the free Ca, but we also observed that it inhibited the (Ca + Mg)-ATPase at constant Ca (2 μM). Inhibition of the (Ca + Mg)-ATPase at 2 μM free Ca is consistent with the known effects of EGTA: EGTA inhibits when the free Ca is low (< 4 μM) stimulates when the free Ca is intermediate, and inhibits again at higher concentrations (> 30 μM) [19]. The activating effect of EGTA is well known and is thought to be due to the Ca-EGTA complex interacting with the (Ca + Mg)-ATPase [19,20]. The inhibitory effects of EGTA are less well known and have not been adequately explained. Our results obtained by adding EGTA directly to the assay and testing at 2 μM free Ca suggest that the K_i for the EGTA effect is approx. 2 mM under our assay conditions (Fig. 5). This result is consistent with the concentration of the chelator present in the fractions that inhibited the (Ca + Mg)-ATPase at 2 μM free Ca in the experiments shown in Figs. 1, 2 and 3. They are also in the same range as previously reported [19]. It is not yet possible to determine if EGTA can account for all the inhibition observed by us or by others using partially purified samples of red cell hemolysate. Our red cell samples also contain a small peptide of 500 to 1000 M_r [9] which could contribute to the inhibition of the (Ca + Mg)-ATPase. We are further analyzing a peptide of this size isolated in the absence of EGTA to assess its effect on the (Ca + Mg)-ATPase.

Acknowledgments

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