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NATURE OF THE (Ca^{2+} + Mg^{2+})-ATPase ACTIVATOR PROTEIN WHICH ASSOCIATES WITH HUMAN ERYTHROCYTE MEMBRANES

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(Ca^{2+} + Mg^{2+})-ATPase activator protein associated with human erythrocyte membranes could be extracted with EDTA under isotonic condition at pH 7.6. No activator was released, however, using isotonic buffer alone. Like calmodulin, the activator in the EDTA extract migrated as a fast moving band on polyacrylamide gel electrophoresis. It was also heat-stable, was capable of stimulating active calcium transport and could stimulate (Ca^{2+} + Mg^{2+})-ATPase to the same extent. When chromatographed on a Sephacryl S-200 column, it was eluted in the same position as calmodulin and a membrane associated (Ca^{2+} + Mg^{2+})-ATPase activator prepared according to Mauldin and Roufogalis (Mauldin, D. and Roufogalis, B.D. (1980) Biochem. J. 187, 507–513). Furthermore, both Mauldin and Roufogalis protein and the activator in the EDTA extract exhibited calcium-dependent binding to a fluphenazine-Sepharose affinity column. On the basis of these data, it is concluded that the activator protein released from erythrocyte membranes by EDTA is calmodulin. A further pool of the ATPase activator could be released by boiling but not by Triton X-100 treatment of the EDTA-extracted membranes. This pool amounted to 8.9% of the EDTA-extractable pool.

Introduction

Calmodulin, a low molecular weight, heat-stable, acidic protein found in human erythrosol, is well established to be an activator of erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase [1,2]. Another pool of calmodulin, intimately associated with the red cell membranes, was reported by Lynch and Cheung [3]. Boiling of the membranes was required to release this residual pool of the activator from the membranes.

A protein activator of the red cell membrane ATPase, claimed to be distinct from calmodulin, was recently described by Mauldin and Roufogalis [4]. According to these investigators, this activator is more firmly associated with the membranes than is calmodulin. It also has a much higher molecular weight but is less active in stimulating the calcium transport enzyme. It can be extracted from the membranes by EDTA. Conditions for extraction of membrane bound (Ca^{2+} + Mg^{2+})-ATPase activator protein may, however, determine the type of protein activator that is released from erythrocyte membranes. Furthermore, the conditions employed by Mauldin and Roufogalis [4] could have resulted in the extraction of calmodulin but that it polymerized or became tightly bound to other protein(s) in the extract so giving it a higher molecular weight.

It is also not certain whether human erythrocyte possesses a membrane bound form of calmodulin extractable only by nonionic detergent and is reported to be present in brain, adrenal gland, liver, kidney and testis [5]. The present study was thus

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

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undertaken to clarify the still confused picture of the nature of the $(Ca^{2+} + Mg^{2+})$-ATPase activator protein which associates with human red cell membranes.

Materials

Vanadium-free Na_2ATP, SDS, EGTA, DL-dithiothreitol, epoxy-activated Sepharose 6B and bovine heart calmodulin were obtained from Sigma Chemical Co. (St. Louis, MO). Calmodulin from pig erythrocytes was also used. It was prepared by the method of Au [6]. Saponin was a product of Calbiochem (La Jolla, CA) while EDTA and L-histidine were purchased from E. Merck (F.R.G.). $[^{45}Ca]CaCl_2$ was purchased from the Amersham International, Amersham (U.K.) while Aquasol was from New England Nuclear (Massachusetts). Sephacryl S-200 Superfine was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) while PM-10 and UM-2 ultrafiltration membranes and microporous filters (25 mm diameter, 0.45 μm pore size) were from Amicon (Tokyo). Triton X-100 was from Tracerlab (Belgium). Bio-Beads SM-2 were from Bio-Rad Laboratories (Richmond, CA) while fluphenazine dihydrochloride was a gift from Byk Gulden, Konstanz, F.R.G.

Methods

Preparation of red cells and extraction of $(Ca^{2+} + Mg^{2+})$-ATPase activator protein from erythrocyte membranes. Fresh blood was drawn from healthy donors and collected in citrate-phosphate-dextrose. The blood was centrifuged to remove plasma and buffy coat from erythrocytes. 30 ml packed cells were then washed three times with isotonic 0.155 M NaCl/3 mM histidine (pH 7.6) before resuspending in the same buffer to give 10% hematocrit. The cells were then exposed to saponin at room temperature for 15 min at a final concentration of 0.1 mg saponin/ml of cell suspension. The resulting hemolysate was centrifuged at $48000 \times g$ for 30 min to sediment membranes. The pelleted membranes were then washed seven times with 5 vol. of the isotonic NaCl/histidine buffer. The last washing (isotonic buffer extract) was saved. The resulting membranes were white. These membranes (12 mg protein) were then extracted three times with 20 ml isotonic NaCl/histidine buffer containing 2 mM EDTA at a final pH of 7.6. Further extraction of membranes with EDTA could not remove any more $(Ca^{2+} + Mg^{2+})$-ATPase activator. The resulting extracts after sedimentation of membranes were pooled and all extracts dialyzed against deionised water. The dialyzed extracts were then concentrated 35-fold on an Amicon PM-10 filter. All operations were at 4°C. The concentrated extracts possessed no $(Ca^{2+} + Mg^{2+})$-ATPase activity. They were stored at −20°C till use. For comparison, membrane associated $(Ca^{2+} + Mg^{2+})$-ATPase activator protein was also prepared according to the method of Mauldin and Roufogalis [4]. Their procedure was closely followed and the preparation before Carboxymethyl-Sephadex chromatography was employed for the present study.

To see if there is still activator associated with erythrocyte membranes after EDTA extraction, the membranes were either boiled or subjected to Triton X-100 treatment to liberate residual activator protein. For Triton extraction, EDTA washed membranes (62 mg membrane protein) were extracted with 2% Triton X-100 for 2 h with mixing at 4°C. After sedimentation of the treated membranes by centrifugation, the supernatant of 10 ml was then extracted with 6 g Bio-Beads SM2 for 2 h followed by another extraction with 3 g SM2 beads for 1 h to remove Triton. The detergent concentration in the Triton X-100 extract was lowered to 0.05% and in the $(Ca^{2+} + Mg^{2+})$-ATPase assay medium, it amounted to only 0.009%. At this concentration, Triton was found to have no influence on $(Ca^{2+} + Mg^{2+})$-ATPase activity and calmodulin activation of the enzyme. Furthermore, the SM2 beads that were used did not adsorb calmodulin. The Bio-Bead treated Triton extract was then concentrated 10-fold by Amicon PM-10 filter before being assayed for the presence of calmodulin.

In experiments involving the release of calmodulin from membranes by boiling, EDTA extracted membranes derived from 20 ml packed cells were heated for 1 min at 100°C. Coagulated proteins were removed by centrifugation and the supernatant was concentrated to 2 ml by ultrafiltration through an Amicon PM-10 filter. The amount of calmodulin present in the concentrate

was compared with that present in an isotonic EDTA extract derived from the same batch of membranes. The assay of calmodulin was based on stimulation of calmodulin-deficient (Ca^{2+} $+Mg^{2+}$)-ATPase. No heat-labile calmodulin-binding protein was present in the membrane extracts to interfere with the assay.

Preparation of erythrocyte membranes deficient in ($Ca^{2+}+Mg^{2+}$)-ATPase activator. Hemolysate was prepared as described above. Membranes were pelleted by centrifugation and washed three times with 5 vol. of the isotonic EDTA-containing buffer to remove membrane bound activator before three further washes with the isotonic NaCl/histidine buffer to remove EDTA. The resulting white membranes were finally suspended in the same buffer at a concentration of 1.2 mg protein/ml and stored at −80°C till use. All protein determinations were by the method of Lowry et al. [7].

(Ca^{2+} $+Mg^{2+}$)-ATPase assay. The assay medium contained 80 mM NaCl, 28 mM KCl, 3.6 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM $CaCl_2$, 2.5 mM ATP, 0.01 mM ouabain and histidine, buffered to pH 7.6 and giving a final osmolarity of 290 mosM in the assay medium of 0.6 ml. Osmolarity was checked with an Advanced digimatic osmometer, Model 3 D II (Advanced Instruments Inc., Needham Heights, MA). Membranes (50 μg protein) were incubated for 0, 1 and 2 h at 44°C with or without addition of calmodulin or membrane extractable activator. The reaction was terminated by addition of 1 ml ice-cold 10% trichloroacetic acid. P_i liberated was determined essentially by the method of Fiske and SubbaRow [8]. Enzymatic activity was linear with time and proportional to the amount of membrane protein added. Data were analyzed for statistical significance by Student's *t*-test.

Polyacrylamide gel electrophoresis of the isotonic EDTA extract of erythrocyte membranes. The polyacrylamide gels used were composed of 7.5% acrylamide and 0.25% *N,N'*-methylenebisacrylamide. The upper buffer contained 0.052 M Tris/0.052 M glycine (pH 8.9) while the lower buffer contained 0.1 M Tris-HCl (pH 8.1). The gels were prerun for an hour using a constant current of 1 mA/gel while the actual run was at a constant current of 2 mA/gel. Electrophoresis was performed at 4°C. 180 μg protein was loaded onto each of two gels (5 × 60 mm). After the run, one gel was stained for protein using 0.1% Coomassie brilliant blue in 25% isopropanol/10% acetic acid while the other gel was sliced into 2-mm fractions. Each fraction was then extracted with 0.25 ml 0.155 M NaCl/3 mM histidine (pH 7.6) overnight at 22°C in a shaking water bath. 0.1 ml of the extract was then employed for assay of (Ca^{2+} $+Mg^{2+}$)-ATPase activator activity. For visualization of protein bands, the gel stained with Coomassie blue was destained with 7% acetic acid and then scanned at 580 nm with an ISCO gel scanner (Instrumentation Specialties CO., U.S.A.).

SDS-polyacrylamide gel electrophoresis of the isotonic EDTA extract of erythrocyte membranes. After treatment of membrane extract with 1% SDS-40 mM dithiothreitol for 30 min at 37°C, the extract (150 μg protein) was then loaded onto gels (5 × 65 mm). They were prerun for an hour at room temperature with a constant current of 4 mA/gel. Electrophoresis was then performed using essentially the method of Fairbanks et al. [9] except that 4.5% acrylamide was used. For the actual run, a constant current of 8 mA/gel was employed. After the run, the gels were stained with 0.1% Coomassie brilliant blue in 25% isopropanol/10% acetic acid. They were then destained in 7% acetic acid before scanning at 580 nm with an ISCO gel scanner.

Gel filtration of calmodulin and erythrocyte membrane extracts containing ($Ca^{2+}+Mg^{2+}$)-ATPase activator protein. Gel filtration was carried out in a 0.9 × 67 cm Sephacryl S-200 Superfine column. Elution was at 0.3 ml/min using 100 mM NaCl/30 mM histidine (pH 7.6) at 22°C. Fractions of 0.6 ml were collected. With bovine heart calmodulin and membrane extract prepared according to Mauldin and Roufogalis [4], 0.1 ml of each fraction was taken out directly for assay of (Ca^{2+} $+Mg^{2+}$)-ATPase activator activity. With the EDTA membrane extract prepared by our procedure, on the other hand, each fraction was diluted 1 in 40 before assay.

Fluphenazine-Sepharose affinity chromatography of erythrocyte membrane extracts containing (Ca^{2+} $+Mg^{2+}$)-ATPase activator protein. Fluphenazine-Sepharose was prepared essentially by the method of Kakiuchi et al. [10] and the amount of fluphenazine bound was estimated by the method

of Charbonneau and Cormier [11]. 9.7 μmol fluphenazine was coupled per g of suction-dried gel. The sample loaded on the fluphenazine-Sepharose 6B column (0.2 × 22 cm) was a membrane extract (2.2 mg protein) prepared according to Mauldin and Roufogalis [4] or an isotonic EDTA membrane extract (0.05 mg protein) prepared by our procedure and dialyzed against 20 mM Tris-HCl (pH 7.6) containing 500 mM NaCl and 0.2 mM $CaCl_2$. The sample was then eluted with the same buffer at a flow rate of 1.1 ml/h at 4°C till absorbance at 280 nm returned to zero. The buffer was then switched to 20 mM Tris-HCl (pH 7.6) containing 500 mM NaCl and 0.1 mM EGTA to elute calmodulin, if any, in the membane extracts.

Ca^{2+} uptake by inside-out vesicles. Inside-out membrane vesicles from fresh human red cells were prepared by the method of Steck and Kant [12]. These vesicles were further extracted with 0.5 mM histidine/2 mM EDTA (pH 7.6) to ensure removal of membrane-associated ($Ca^{2+} + Mg^{2+}$)-ATPase activator protein. EDTA was then removed by two further washes with 0.5 mM histidine (pH 7.6). The vesicles were kept at 4°C and used after 36 h of storage. Ca^{2+} uptake was studied in an incubation medium containing 20 mM Tris, 120 mM KCl, 5 mM $MgCl_2$, 2.5 mM Na_2ATP, 0.2 mM $CaCl_2$, 0.5 μCi ^{45}Ca/ml and inside-out vesicles (about 0.5 mg protein) in a final volume of 4.5 ml and pH 7.8. Membrane associated ($Ca^{2+} + Mg^{2+}$)-ATPase activator protein, when present, was derived from the peak activator fraction of the Sephacryl S-200 column. The incubation mixture was kept at 37°C in a shaking water bath. At specified times, 0.5 ml samples were removed and passed through Amicon microporous filters to collect membrane vesicles. The filters were washed three times with 15 ml ice-cold 0.155 M KCl. They were then dissolved in 10 ml Aquasol before being counted in a Beckman LS-250 liquid scintillation counter using the ^{14}C window.

Results

Identification of a ($Ca^{2+} + Mg^{2+}$)-ATPase activator in isotonic EDTA extract of human erythrocyte membranes

On extraction of human erythrocyte membranes with isotonic buffer (pH 7.6) containing 2 mM EDTA, an activator of membrane ($Ca^{2+} + Mg^{2+}$)-ATPase was released. This was evident from the ability of the extract in stimulating the ATPase of activator-deficient membranes (Table I). Enzyme activity was increased by 66% above the basal activity of 3.8 μmol P_i released/mg membrane protein per h. This was similar to the activation due to 100 ng pig erythrocyte calmodulin present in the assay medium. Extract derived from membranes treated with isotonic buffer alone, on the other hand, could not activate the enzyme.

Basal ($Ca^{2+} + Mg^{2+}$)-ATPase activity of erythrocyte membranes extracted with EDTA containing buffer was lower than that of membranes extracted with isotonic buffer alone. Furthermore, exogenous calmodulin or activator present in the EDTA extract could only activate the ATPase of EDTA-treated membranes. The activated activities were practically the same as the basal enzyme activity of isotonic buffer washed membranes. It could thus be concluded that in the presence of EDTA, membranes lost ($Ca^{2+} + Mg^{2+}$)-ATPase

TABLE I

EXTRACTION OF ($Ca^{2+} + Mg^{2+}$)-ATPase ACTIVATOR FROM HUMAN ERYTHROCYTE MEMBRANES BY EDTA PRESENT IN ISOTONIC BUFFER

White erythrocyte membranes prepared from fresh red cells were extracted with isotonic 0.155 M NaCl/3 mM histidine (pH 7.6) or with the same buffer containing 2 mM EDTA as described in Methods. The resulting extracts were compared with pig erythrocyte calmodulin (100 ng protein/assay) in their ability to stimulate ($Ca^{2+} + Mg^{2+}$)-ATPase. ($Ca^{2+} + Mg^{2+}$)-ATPase activities are expressed as μmol P_i released per mg membrane protein per h and presented as means of two experiments.

($Ca^{2+} + Mg^{2+}$)-ATPase activity	Extraction of erythrocyte membranes	
	With isotonic buffer	With isotonic buffer + EDTA
Basal	6.1	3.8
In the presence of		
Isotonic buffer extract	6.1	3.9
Isotonic buffer-EDTA extract	6.2	6.3
Calmodulin	6.2	6.3

activator so that its $(Ca^{2+} + Mg^{2+})$-ATPase activity was lowered. Presence of exogenous activator reactivated the enzyme to the level of the original isotonic membranes.

Analysis of isotonic EDTA extract of erythrocyte membranes

When the exract was subjected to analysis by polyacrylamide gel electrophoresis, a single fast migrating, negatively charged protein band moving just behind the tracking dye was detected. This band, on elution from the gel, was capable of stimulating erythrocyte membrane $(Ca^{2+} + Mg^{2+})$-ATPase by about 60% (Fig. 1). Calmodulin, eletrophoresed under the same conditions, is migrating as fast [1]. The eluted activator protein band was, however, only a minor component, contributing to a maximum of 1% of all proteins present in the extract. The major protein bands in the EDTA extract was one with a molecular weight of 71000 (peak I) and another one with a molecular weight of 30000 (peak II) (Fig. 2). Peak I has a molecular weight very similar to band 4.2 (72000) while peak II has a molecular weight close to that of band 6 (35000) of human erythrocyte membranes [13]. While in the study by Mauldin and Roufogalis [4], spectrin was identified as a major protein in the 0.1 mM EDTA-1 mM Tris (pH 8.0) extract of human erythrocyte membranes, under the present conditions of extraction, spectrin was not found.

Characteristics of $(Ca^{2+} + Mg^{2+})$-ATPase activator protein released from erythrocyte membranes by isotonic EDTA treatment as compared with that of calmodulin and activator prepared by the method of Mauldin and Roufogalis [4].

When chromatographed on a Sephacryl S-200

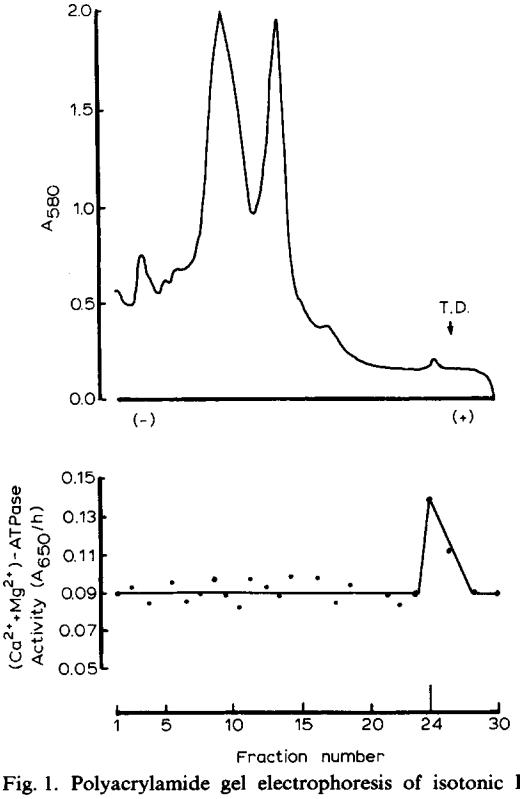

Fig. 1. Polyacrylamide gel electrophoresis of isotonic EDTA extract of erythrocyte membranes. EDTA extract (180 μg protein) was loaded onto each of two gels. One gel was stained with Coomassie blue and scanned at 580 nm to give the protein profile (top) while a similar gel was sliced and each fraction assayed for its ability to stimulate $(Ca^{2+} + Mg^{2+})$-ATPase (bottom). The tracking dye (T.D.) used was Bromophenol blue.

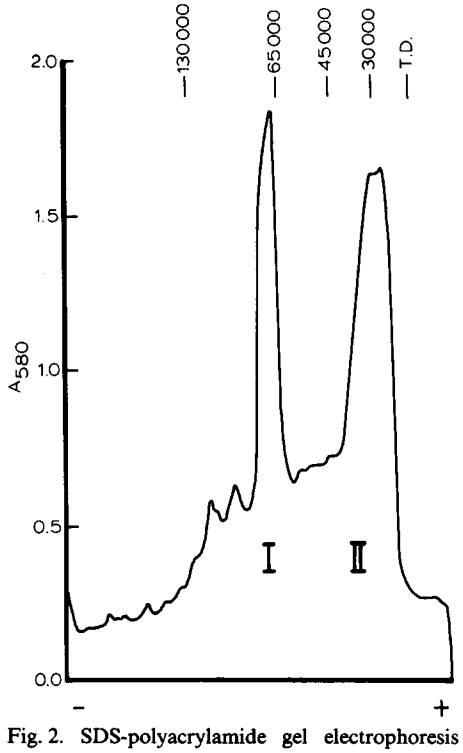

Fig. 2. SDS-polyacrylamide gel electrophoresis of isotonic EDTA extract of erythrocyte membranes. EDTA extract (150 μg protein) was treated with SDS-dithiothreitol before loading onto gel for electrophoresis as described in Methods. The gel was stained with Coomassie blue and then scanned at 580 m. The major protein peaks are numbered I and II and their positions compared with that of molecular weight markers and tracking dye.

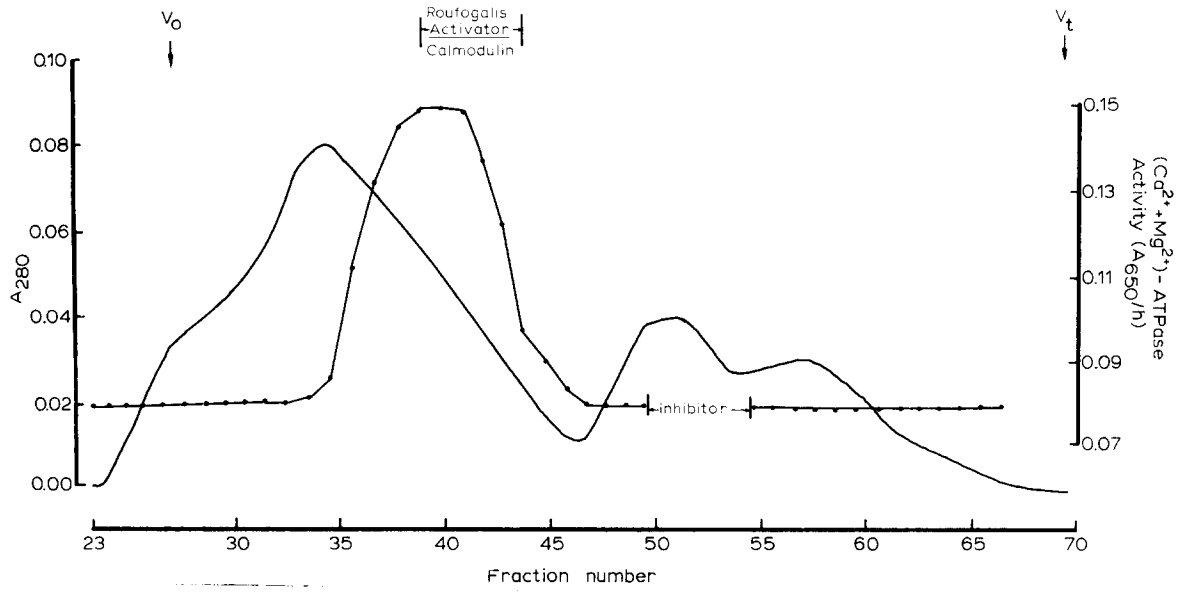

Fig. 3. Gel filtration of isotonic EDTA extract of human erythrocyte membranes on Sephacryl S-200 Superfine column. Isotonic EDTA extract (1.8 mg protein) was chromatographed on the column as described in Methods. The fractions collected were monitored for absorbance at 280 nm (———, A_{280}) and for ability to stimulate ($Ca^{2+}+Mg^{2+}$)-ATPase (●———●). V_o = void volume nad V_t = total bed volume. When bovine heart calmodulin was chromatographed on the same column, 25 units were loaded while with the activator prepared according to Mauldin and Roufogalis, 2.2 mg protein was loaded. The positions of fractions of calmodulin and Roufogalis activator showing activation and fractions of the isotonic EDTA extract showing inhibition of the ATPase are demarcated.

Superfine column, the isotonic EDTA extract of membranes gave one major and two minor protein peaks (Fig. 3). The activator was associated with the descending slope of the major peak while the minor protein peak immediately following was associated with a ($Ca^{2+}+Mg^{2+}$)-ATPase inhibi-

TABLE II

PROPERTIES OF ($Ca^{2+}+Mg^{2+}$)-ATPase ACTIVATOR PROTEIN RELEASED FROM ERYTHROCYTE MEMBRANES BY ISOTONIC EDTA TREATMENT – THERMAL STABILITY, STIMULATION OF ($Ca^{2+}+Mg^{2+}$)-ATPase TO THE SAME EXTENT AS CALMODULIN AND ACTIVATION OF Ca^{2+} UPTAKE BY HUMAN INSIDE-OUT VESICLES

The activator protein preparation used was the peak activator fraction isolated from the Sephacryl S-200 column (Fig. 3). When testing for thermal stability, the activator was heated for 3 min at 100°C in a sealed tube, cooled, before its activity was compared with unboiled control. The unboiled activator was also compared with calmodulin. In the transport study, a final activator concentration of 1.5 μg protein/ml was used. ($Ca^{2+}+Mg^{2+}$)-ATPase activity is expressed as A_{650}/h. A_{650} is the absorbance at 650 nm due to P_i liberated from ATP. The rate of ATP-dependent Ca^{2+} uptake is expressed as nmol Ca^{2+} uptake per mg protein of inside-out vesicles per min.

Activator preparation	Amount added (ng protein)	($Ca^{2+}+Mg^{2+}$)-ATPase activity	Rate of Ca^{2+} uptake
No	0	0.045	3.0
Unboiled activator	700	0.075	
	1400	0.090	
	3500	0.091	
	7000	0.090	
Boiled activator	7000	0.090	5.0
Calmodulin	40	0.090	
	80	0.090	
	200	0.091	
	400	0.090	

tor protein. A preliminary study on this protein inhibitor was reported elsewhere [14].

When $(Ca^{2+} + Mg^{2+})$-ATPase activator protein prepared by the method of Mauldin and Roufogalis [4] was chromatographed on the same column, two major followed by one minor protein peaks were resolved. The activator peak was associated with the descending slope of the second major peak (result not shown) and almost completely overlapped with the activator peak of the isotonic EDTA extract (Fig. 3). Calmodulin, when chromatographed on the same column, gave an activator peak also at the same position.

The $(Ca^{2+} + Mg^{2+})$-ATPase activator protein isolated by isotonic EDTA treatment also shared other properties of calmodulin (Table II). It was found to be heat stable after 3 min of boiling. It stimulated ATP dependent Ca^{2+} uptake by human inside-out vesicles by 66% and it maximally stimulated $(Ca^{2+} + Mg^{2+})$-ATPase to the same extent as calmodulin. According to Mauldin and Roufogalis, however, the $(Ca^{2+} + Mg^{2+})$-ATPase activator protein released from erythrocyte membranes maximally stimulated the ATPase less than calmodulin [4]. This could be due to the presence of trace amount of $(Ca^{2+} + Mg^{2+})$-ATPase inhibitor protein in their preparation. With our preparation of activator, on the other hand, the extract was separated from the protein inhibitor by Sephacryl S-200 chromatography. It was thus inhibitor free and could hence stimulate $(Ca^{2+} + Mg^{2+})$-ATPase to the same extent as calmodulin.

When chromatographed on a fluphenazine-Sepharose affinity column, $(Ca^{2+} + Mg^{2+})$-ATPase activator that was present in the isotonic EDTA extract or Mauldin and Roufogalis extract was eluted from the column only in the presence of EGTA. Proteins eluted before the inclusion of EGTA in the elution buffer, on the other hand, did not exhibit any calmodulin activity (Table III). The above findings could be interpreted to mean that there is only one type of $(Ca^{2+} + Mg^{2+})$-ATPase activator protein extractable by EDTA from human erythrocyte membranes and that the activator is calmodulin.

Lynch and Cheung [3] were able to release residual calmodulin by boiling human erythrocyte membanes previously extracted with 1 mM EGTA. Similarly, membranes treated with 2 mM EDTA also yielded calmodulin on boiling (Table IV). Note, however, that boiling pig erythrocyte mem-

TABLE III

FLUPHENAZINE-SEPHAROSE AFFINITY CHROMATOGRAPHY OF $(Ca^{2+} + Mg^{2+})$-ATPase ACTIVATOR PRESENT IN ISOTONIC EDTA EXTRACT AND MAULDIN AND ROUFOGALIS EXTRACT OF ERYTHROCYTE MEMBRANES

Isotonic EDTA extract (0.05 mg protein) and Mauldin and Roufogalis extract (2.2 mg protein) were chromatographed as described in Mehods. Fractions containing proteins eluted during the initial $NaCl/CaCl_2$ wash and the subsequent NaCl/EGTA wash were separately pooled, concentrated 8-fold by ultrafiltration on Amicon PM-10 filter and then assayed for their ability to stimulate calmodulin sensitive $(Ca^{2+} + Mg^{2+})$-ATPase of erythrocyte membranes. Basal $(Ca^{2+} + Mg^{2+})$-ATPase activity was 3.65 μmol P_i released/mg membrane protein per h.

Erythrocyte membrane extract	% stimulation of $(Ca^{2+} + Mg^{2+})$-ATPase	
	Proteins eluted by $NaCl/CaCl_2$ buffer	Proteins eluted by NaCl/EGTA buffer
Isotonic EDTA extract	0	41
Mauldin and Roufogalis extract	6	50

TABLE IV

RELEASE OF MEMBRANE BOUND CALMODULIN BY BOILING AND TRITON X-100

Erythrocyte membranes were extracted with isotonic 2 mM EDTA containing buffer and then boiled or further extracted with Triton X-100 as described in Methods. The extracts, after concentration, were then assayed for their ability to stimulate $(Ca^{2+} + Mg^{2+})$-ATPase. 1 unit of calmodulin is defined as the amount of the activator giving half-maximal activation of the ATPase. Unless specified, membrane extracts were derived from human red cells.

Erythrocyte membrane extract	Units of calmodulin per g membrane protein
Boiled membrane extract	9167
Boiled membrane extract (pig)	0
Isotonic EDTA extract	103375
Triton X-100 extract	0

branes extracted with EDTA did not yield any calmodulin. Presumably, in pig but not human erythrocyte membranes, calmodulin is bound onto membranes solely via calcium. A detergent extractable pool of calmodulin was also not found in human erythrocyte membranes (Table IV).

Discussion

The present study shows that the membrane bound activator protein of calcium transport ATPase in human red cells is calmodulin. The majority of this calmodulin pool can be extracted by EDTA thus suggesting that calmodulin interaction with membranes is mainly via divalent cations. 8.1% of the total calmodulin pool, however, is tightly bound onto membranes. It resists extraction by the calcium chelator and heating is required to release it from the membranes. Moreover, unlike calmodulin present in brain, adrenal gland, liver, kidney and testis [5], red cell calmodulin does not seem to interact hydrophobically with intrinsic membrane proteins since non-ionic detergent cannot solubilize any calmodulin from erythrocyte membranes.

The claim by Mauldin and Roufogalis [4] of the existence of yet another membrane bound (Ca^{2+} $+Mg^{2+}$)-ATPase activator protein having a molecular weight much higher than that of calmodulin could result from aggregation of calmodulin molecules when they are extracted from membranes with EDTA in dilute Tris buffer. The presence of Tris in the buffer might be responsible for the occurrence of high molecular weight (Ca^{2+} $+Mg^{2+}$)-ATPase activator since if the isotonic buffer made up of histidine for use in extracting erythrocyte membranes in the present study is replaced by Tris, (Ca^{2+} $+Mg^{2+}$)-ATPase activator proteins with molecular weights of 35000 and 58000 can be extracted along with calmodulin (Au, K.S., unpublished data). High molecular weight activators, however, were not seen with the preparation of Mauldin and Roufogalis when it was chromatographed on our Sephacryl S-200 column. This is probably because only very little Tris remained associated with the activator when it was eluted with non-Tris buffer in the column.

The case of calmodulin interaction with human erythrocyte membrane (Ca^{2+} $+Mg^{2+}$)-ATPase is probably similar to that of phosphorylase kinase [15]. With the latter enzyme, calmodulin either exists as a tightly associated subunit or is bound to another calmodulin-binding site on the enzyme via calcium. Release of the tightly bound calmodulin (δ-subunit) from the kinase also requires heat treatment. Neither EDTA nor EGTA can detach it from the enzyme. Whether the two different pools of calmodulin in human erythrocyte membrane have different functions is not known. When compared with the single pool of calmodulin that is present in pig red cell membrane, however, the situation in human might represent a step towards the acquirement of a more sophisticated method of controlling the red cell calcium pump by calmodulin.

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