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A Protein Activator of Mg^{2+}-Dependent, Ca^{2+}-Stimulated ATPase in Human Erythrocyte Membranes Distinct from Calmodulin

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Treatment of extensively washed erythrocyte membranes with 0.1 mM-EDTA decreased their Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase [$(Mg^{2+} + Ca^{2+})$-ATPase] activity. An activator released by this treatment restored the $(Mg^{2+} + Ca^{2+})$-ATPase to its original value in a Ca^{2+}-dependent manner. This activator was different from calmodulin, as determined by a number of criteria. It was retained on an Amicon XM-100 ultrafiltration membrane (molecular-weight cut-off 100000); it appeared in the void volume of Sephadex G-100 and G-75 columns; it was not retained on a DEAE-cellulose ion-exchange column at ionic strengths similar to those used to retain calmodulin; and it maximally activated $(Mg^{2+} + Ca^{2+})$-ATPase activity less than calmodulin and at a higher Ca^{2+} concentration. Like calmodulin, the activator is heat-stable. The activator fraction isolated on a 2.5–15% sucrose gradient in 0.16 M-KCl showed a single band of mol.wt. 63000 and no calmodulin on 10%-polyacrylamide/sodium dodecyl sulphate gels. A trace amount of calmodulin was detected in the activator fraction by radioimmunoassay (approx. 10 pg/ml of 'ghosts'), but this amount was insufficient to account for the $(Mg^{2+} + Ca^{2+})$-ATPase activation. Furthermore, calmodulin-binding protein failed to inhibit $(Mg^{2+} + Ca^{2+})$-ATPase activity by more than 10–20% in the membrane preparations from which the activator was extracted. It was concluded that erythrocyte membranes contain a $(Mg^{2+} + Ca^{2+})$-ATPase activator that may attenuate the activation of the Ca^{2+}-transport ATPase by calmodulin.

The cytosol of human erythrocytes, or a low-molecular-weight protein isolated from it, stimulates $(Mg^{2+} + Ca^{2+})$-ATPase (Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase) activity of haemolysed erythrocyte membranes (Bond & Clough, 1973; Luthra *et al.*, 1976). This heat-stable activator has been purified (Jarrett & Penniston, 1977) and shown to be indistinguishable from Ca^{2+}-binding proteins isolated from a variety of other tissues and species (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977; Jarrett & Kyte, 1979), and collectively termed calmodulin (Cheung *et al.*, 1978). It is not known at present whether calmodulin is bound to the membrane of intact erythrocytes in physiological conditions (Scharff & Foder, 1978; Roufogalis, 1979), since the amount of calmodulin found in isolated membranes is dependent on the method of membrane isolation (Hanahan & Ekholm, 1978; Katz *et al.*, 1979). The erythrocyte appears to contain calmodulin in more than 100-fold excess over that needed to saturate the $(Mg^{2+} + Ca^{2+})$-ATPase (Farrance & Vincenzi, 1977*b*). Calmodulin binds to the membrane in a Ca^{2+}-dependent manner (Farrance & Vincenzi, 1977*a*; Scharff & Foder, 1978; Lynch & Cheung, 1979).

Treatment of erythrocyte membranes with EDTA decreases the activity and Ca^{2+}-affinity of the $(Mg^{2+} + Ca^{2+})$-ATPase in the membrane (Quist & Roufogalis 1975; Scharff & Foder, 1978). Addition to the membrane of the proteins extracted by EDTA restores the $(Mg^{2+} + Ca^{2+})$-ATPase activity to its original state (Quist & Roufogalis, 1975; Scharff & Foder, 1978). In the present study we describe the isolation of an activator of $(Mg^{2+} + Ca^{2+})$-ATPase from the mixture of proteins extracted from extensively washed human erythrocyte membranes by the EDTA treatment. Evidence is presented that the activator is not calmodulin. We suggest that the activator described is more firmly associated with the membrane than is calmodulin, and may modulate the activation of the Ca^{2+}-transport ATPase by Ca^{2+} and calmodulin.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Experimental

Materials

[γ-^{32}P]ATP (10–40Ci/mmol) was purchased from New England Nuclear Canada, Lachine, Quebec, Canada. ATP (vanadate-free, disodium salt), ouabain, EGTA, EDTA, fatty acid-free crystalline bovine serum albumin, catalase and DEAE-cellulose were from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex gels and CM-Sephadex A50 were from Pharmacia, Uppsala, Sweden. Sucrose was 'Baker Analyzed' grade from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. All other salts were analytical-reagent grade.

Preparation of 'ghosts'

'Ghosts' were prepared by a method similar to that described by Dodge *et al.* (1963). Human erythrocytes less than 5 days old and packed in acid–citrate–dextrose solution were supplied by the Red Cross. The packed cells were washed three times by centrifugation at 2000*g* for 6min in equal volumes of 310 ideal mosM-sodium phosphate buffer, pH7.4 (P310 buffer). The buffy coat was carefully aspirated after each wash. The washed cells were suspended in an equal volume of P310 buffer and 10ml of the suspension was rapidly pipetted into 140ml of 20 ideal mosM-sodium phosphate buffer, pH7.4 (P20 buffer), followed immediately by swirling. The haemolysed cells were centrifuged at 22000*g* for 40min and the haemolysate was carefully decanted. The tubes were rotated through 180° to expose a small 'whitish button', which was removed. The cells were washed twice more in 140ml portions of P20 buffer. The resultant 'ghosts' from individual centrifuge bottles were pooled and washed once more by centrifugation at 40000*g* for 20min in an equal volume of P20 buffer. The 'ghosts' were either used fresh or frozen at −12°C, as specified in the Figure legends.

($Mg^{2+} + Ca^{2+}$)-ATPase was assayed by a radiometric method with [γ-^{32}P]ATP, as previously described (Katz *et al.*, 1979). Protein was determined by the method of Lowry *et al.* (1951), with fatty acid-free bovine serum albumin as a standard. Free Ca^{2+} concentrations were estimated from the Ca–EGTA equilibrium constant, as previously described (Katz *et al.*, 1979).

Partial purification of activator on CM-Sephadex

'Ghosts' (1vol.) were incubated with agitation in 5vol. of 0.1mM-EDTA/1.0mM-Tris, pH8, at 37°C for 30min. The mixture was cooled in ice and centrifuged at 20000*g* for 20min. The membranes were resuspended to their original starting volume with 5mM-Tris/HCl/15mM-NaCl, pH7.2, and used fresh for further study (EDTA-treated membranes). The supernatant was concentrated 6-fold on an Amicon XM 100 filter at 103.5kPa. The concentrated soluble fraction was adjusted to pH6.8 with HCl and loaded on a CM-Sephadex A-50 column (1.6cm × 28cm) equilibrated with 20 ideal mosM-Tris/HCl, pH6.8. The column was washed with the same buffer until A_{280} returned to zero. The fractions with the highest A_{280} values were pooled and concentrated on an Amicon XM 100 membrane to the original volume of 'ghosts' from which the fraction was derived.

Sucrose gradient centrifugation

Activator was extracted from 'ghosts' with 0.1mM-EDTA as described above, with the following modifications: 'ghosts' (12.5ml) were incubated with 62.5ml of 0.1mM-EDTA/1.0mM-Tris, pH6.5, for 35min at 37°C. After the membranes were pelleted by centrifugation at 20000*g* for 20min, the supernatant was centrifuged at 105000*g* for 70min to remove any remaining particulate material. The resulting supernatant was concentrated 20-fold on an Amicon PM 10 membrane. KCl was added to the concentrated activator solution to a final concentration of 0.16M. Samples (0.5ml) were layered on linear sucrose gradients (2.5–15%, w/v) in 0.16M-KCl, which were centrifuged at 177000*g* for 16h in a swinging-bucket rotor (SW Ti41). Fractions (17 drops or 0.5ml) were collected by aspiration of the gradient from the bottom, by using a Desaga peristaltic pump and a Gilson microfraction collector, except for the topmost fraction (fraction 1), which was aspirated from the top. The refractive index of each fraction was determined in a Fisher refractometer. Samples (usually 200μl) were assayed for activation of ($Mg^{2+} + Ca^{2+}$)-ATPase activity in the presence of Ca^{2+} from 0.6 to 100μM.

SDS/polyacrylamide-gel electrophoresis

This was performed essentially by the method of Weber & Osborn (1969) on 10% acrylamide gels containing 0.27% bisacrylamide. Protein samples (70–200μl) in 5% β-mercaptoethanol, 0.1% SDS and 0.1M-sodium phosphate, pH7.0, were immersed in a boiling-water bath for 5min before application to the gels. The gels were run at 8mA per tube in 0.1M-sodium phosphate buffer and 0.1% SDS, pH7, for 8.5h, at which time the tracking dye (Bromophenol Blue, included in the protein sample) migrated to the end of the tube. The gels were stained first in 10% (v/v) acetic acid/25% (v/v) propan-2-ol containing 0.05% Coomassie Blue, then transferred to a similar staining solution containing 10% acetic acid in a convection destainer (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). The destained gels were scanned at 560mm with a Gelman ACD 16 densitometer before storage in 10% acetic acid/75% propan-2-ol.

Results

Incubation of 'ghosts' from human erythrocytes in 0.1 mM-EDTA for 30 min at 37°C decreased both the activity and Ca^{2+}-affinity of the membrane-bound $(Mg^{2+} + Ca^{2+})$-ATPase (Fig. 1*a*). The soluble proteins released from the membranes were passed through a CM-Sephadex column and concentrated on an Amicon XM-100 filter. The resulting protein fraction stimulated $(Mg^{2+} + Ca^{2+})$-ATPase in both ordinary and EDTA-treated 'ghosts' (Fig. 1*a*). The activation of 'ghost' $(Mg^{2+} + Ca^{2+})$-ATPase (1.5-fold) was not dependent on Ca^{2+}, but that of the EDTA-treated 'ghosts' was maximally activated 2-fold in a Ca^{2+}-dependent manner (Fig. 1*b*). The activator fraction isolated by CM-Sephadex chromatography showed a number of proteins, including spectrin, on SDS/polyacrylamide gels (results not shown).

The activator extracted by treatment of 'ghosts' with 0.1 mM-EDTA/1.0 mM-Tris, pH 6.5, for 35 min at 37°C was purified further on a 2.5–15% sucrose gradient in 0.16 M-KCl. The gradient profile of the proteins is shown in Fig 2. Maximum activation of $(Mg^{2+} + Ca^{2+})$-ATPase activity occurred in the two fractions at the top of the gradient (fractions 1 and 2). Densitometry scans of SDS/polyacrylamide gels of fractions 1 and 2, pooled from six sucrose gradients and concentrated on an Amicon XM-100 ultrafilter, showed a single protein of mol.wt. 63000 (Fig. 3). The protein content was greater in fraction 2 than in fraction 1. In these experiments no other low-molecular-weight proteins were evident, but in some other experiments a band of mol.wt. 32000

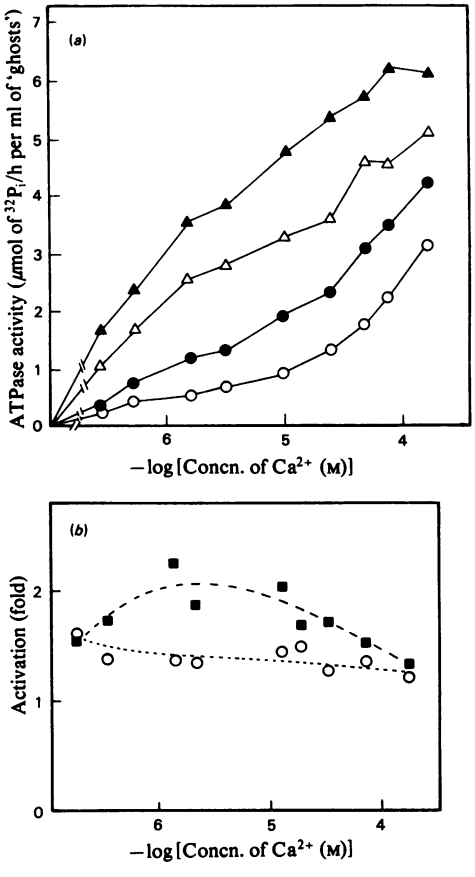

Fig. 1. *Effect of CM-Sephadex-treated activator from erythrocyte membranes on $(Mg^{2+} + Ca^{2+})$-ATPase activity*

(*a*) Freshly prepared 'ghost' membranes (△) were treated with 0.1 mM-EDTA/1.0 mM-Tris, pH 8, for 30 min at 37°C (○). Proteins extracted from the membrane by the EDTA treatment were passed through CM-Sephadex and added to 'ghost' membranes (▲) or to EDTA-treated membranes (●), and assayed for $(Mg^{2+} + Ca^{2+})$-ATPase activity at different Ca^{2+} concentrations. The data points are the means of three experiments, with a standard error of about ±15%. (*b*) Ca^{2+}-dependence of the activation by the CM-Sephadex-treated activator of $(Mg^{2+} + Ca^{2+})$-ATPase in 'ghost' membranes (○) and EDTA-treated membranes (■) was calculated from the data in (*a*).

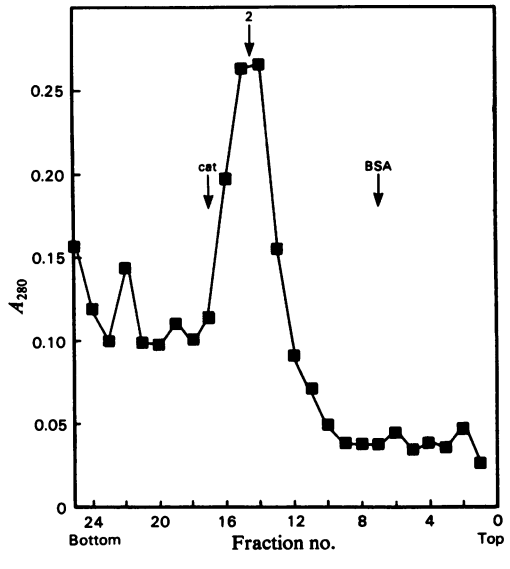

Fig. 2. *Sucrose-gradient protein profile of soluble activator fraction extracted from membranes with 0.1 mM-EDTA/1.0 mM-Tris/HCl, pH 6.5*

Protein was monitored at 280 nm. The gradient was calibrated with bovine serum albumin (BSA) (4.4S) and catalase (cat) (11.4S), as shown by the arrows. The major protein fraction (peak 2) corresponds to 9.2S.

was also found. However, proteins corresponding to calmodulin in the low-molecular-weight region were always absent.

Both fractions 1 and 2 from the sucrose gradients described above stimulated $(Mg^{2+} + Ca^{2+})$-ATPase activity in 'ghosts'. The stimulation by activator in fraction 2 is shown in Fig. 4(*a*); the stimulation (1.5-fold) was essentially independent of Ca^{2+}. The activation by fraction 2 was concentration-dependent and reached saturation at higher concentrations (Fig. 4*b*).

SDS/polyacrylamide-gel electrophoresis of the major protein peak of 9.2S in the sucrose gradient (peak 2) gave a number of proteins, including a major band corresponding to spectrin. Peak 2 neither activated $(Mg^{2+} + Ca^{2+})$-ATPase activity in 'ghosts' (results not shown) nor antagonized the stimulation of $(Mg^{2+} + Ca^{2+})$-ATPase by calmodulin (Fig. 5).

The effect of combining the activator in fraction 2 with saturating concentrations of calmodulin was investigated in 'ghosts'. The activator did not antagonize the action of calmodulin and the activation was not additive under these conditions (Fig. 6).

The possibility that the activator fraction extracted from the membranes contained small amounts of calmodulin was examined. The activator fraction extracted with 0.1mM-EDTA/1.0mM-Tris, pH6.5, and boiled for 5min in 8M-urea, maximally stimulated $(Mg^{2+} + Ca^{2+})$-ATPase activity in 'ghost' membranes 1.5-fold, compared with 1.6-fold before heating (three experiments; results not shown). The supernatant resulting from boiling 'ghosts' for 5min in 8M-urea also stimulated $(Mg^{2+} + Ca^{2+})$-ATPase 1.5-fold (results not shown). A protein fraction extracted from 12.5ml of 'ghosts' by 0.1mM-EDTA/1.0mM-Tris, pH6.5, was concentrated 20-fold, layered on a 2.5–15% (w/v) sucrose gradient in 0.16M-KCl and centrifuged at 177000*g* for 16h. The second of two 0.5ml fractions from the top of the gradients (fraction 2) was pooled,

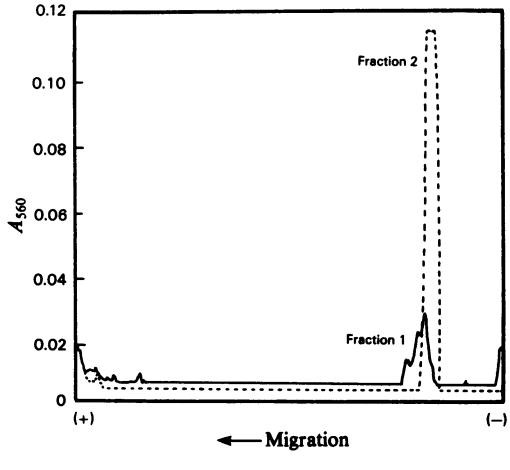

Fig. 3. *Scan of a SDS/10% polyacrylamide gel of protein fractions in Fig. 2*

The migration of the major peak corresponds to a mol.wt. of 63000. The gels were calibrated with standard proteins. Scans represent the protein profiles of fraction 1 (——) and fraction 2 (– – – –) obtained from the sucrose gradients in Fig. 2.

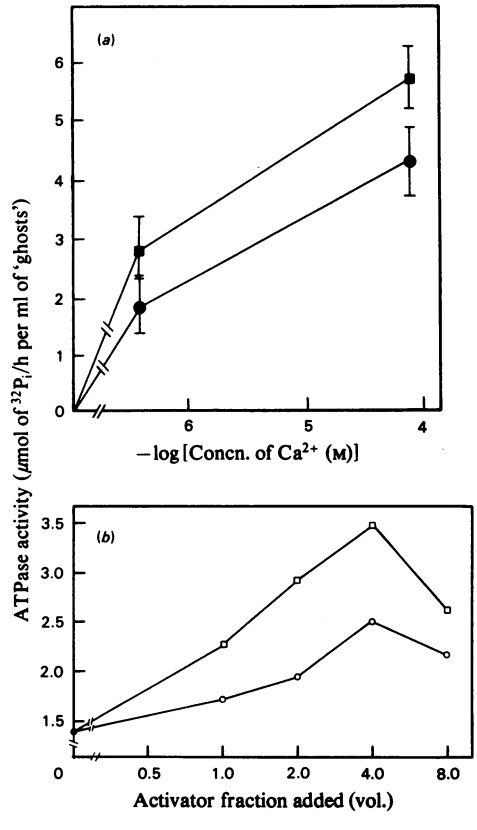

Fig. 4. *Stimulation of $(Mg^{2+} + Ca^{2+})$-ATPase in 'ghost' membranes by activator (fraction 2) isolated on sucrose gradients, as in Fig. 2*

(*a*) Ca^{2+}-dependence of 'ghost' membranes in the absence (●) and in the presence (■) of 1vol. of fraction-2 activator (200μl). 'Ghosts' were frozen and thawed three times before assay. Each point is the mean ± S.E.M. of three experiments. (*b*) Concentration-dependence of $(Mg^{2+} + Ca^{2+})$-ATPase activation by fraction 1 (○) and fraction 2 (□) at 3.6μM-Ca^{2+}. Fractions 1 and 2 were pooled from three sucrose-gradient experiments, and concentrated 8-fold by freeze-drying. One vol. is equivalent to 200μl of fraction 2 used in (*a*). The data are the means of duplicate determinations in one experiment.

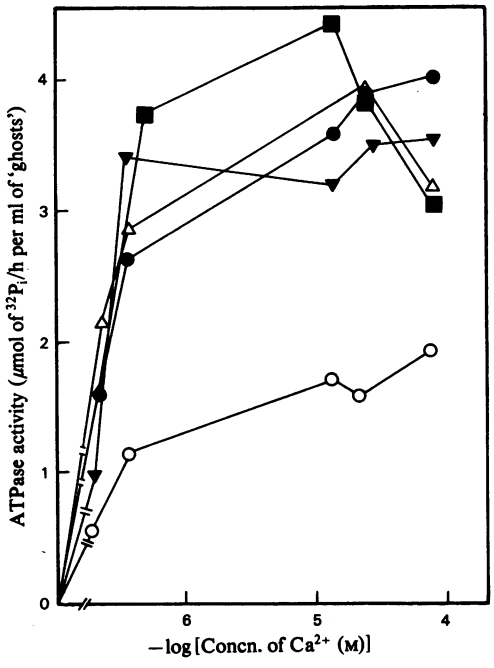

Fig. 5. *Effect of a spectrin-enriched protein fraction on the activation of $(Mg^{2+} + Ca^{2+})$-ATPase by bovine brain calmodulin*

The spectrin-enriched fraction (peak 2) was the major protein peak obtained from sucrose gradients similar to that shown in Fig. 2. $(Mg^{2+} + Ca^{2+})$-ATPase activity was assayed in three-times freeze-thawed 'ghost' membranes (○), with the following additions; 4μg of bovine brain calmodulin (●); 4μg of calmodulin + peak 2 (50μl) (△); 4μg of calmodulin + peak 2 (100μl) (▲); 4μg of calmodulin + peak 2 (200μl) (■). Results of a single experiment are shown.

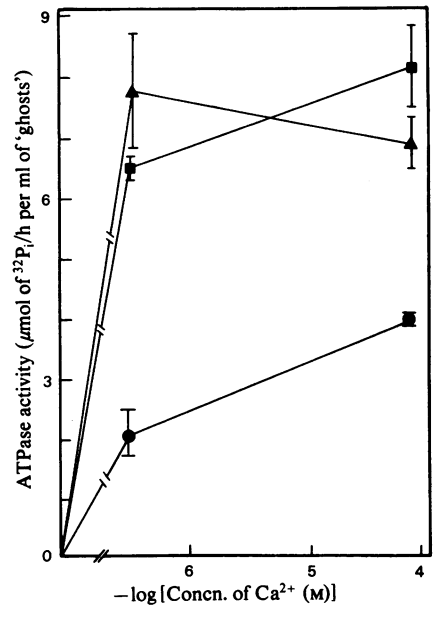

Fig. 6. *Effect of fraction-2 activator on $(Mg^{2+} + Ca^{2+})$-ATPase in the presence of calmodulin*

$(Mg^{2+} + Ca^{2+})$-ATPase activity was assayed in 'ghost' membranes (●) frozen and thawed three times with the following additions: 4μg of bovine brain calmodulin (■); 4μg of calmodulin + 200μl (1 vol.) of fraction-2 activator (▲). Each point is the mean ± the range of duplicate experiments.

freeze-dried and tested for calmodulin by radioimmunoassay by Dr. J. G. Chafouleas in the laboratory of Dr. A. R. Means, University of Texas. The calmodulin content, approx. 10pg/ml of 'ghosts', was at the detection limit of the assay. Calmodulin-binding protein (5μg, kindly supplied by Dr. J. H. Wang, University of Manitoba) inhibited $(Mg^{2+} + Ca^{2+})$-ATPase activity in 'ghosts' by 10–20% at 100μM-Ca^{2+}, but not at lower Ca^{2+} concentrations (results not shown).

Discussion

Treatment of erythrocyte membranes with 0.1mM-EDTA for 30min at pH6.5 or pH8 resulted in a decrease in the activity and Ca^{2+}-affinity of $(Mg^{2+} + Ca^{2+})$-ATPase. This activity was restored by the addition of the extracted proteins to the membranes. The activator extracted from the membranes was a heat-stable, acidic protein, but was shown to differ from calmodulin as determined by a number of criteria. The activator did not pass through an XM-100 ultramembrane filter of mol.wt. cut-off 100000 and was eluted in the void volume of Sephadex G-100 and Sephadex G-75, in the presence or absence of 5mM-EDTA, properties inconsistent with those of calmodulin of mol.wt. 16700 (see Wang, 1977). Similarly to calmodulin (Luthra *et al.*, 1976), the activator was not retained by a CM-Sephadex column, but in contrast with calmodulin (Jarrett & Penniston, 1977) it was not retained on a DEAE-cellulose column at high ionic strength (D. Mauldin & B. D. Roufogalis, unpublished work).

The activator extracted from erythrocyte membranes stimulated $(Mg^{2+} + Ca^{2+})$-ATPase activity to a lesser extent, and with a different Ca^{2+}-dependence, than calmodulin. Although calmodulin maximally stimulated $(Mg^{2+} + Ca^{2+})$-ATPase activity in EDTA-treated membranes 36-fold at around 0.1μM-Ca^{2+} (B. D. Roufogalis & D. Mauldin, unpublished work), a maximal 3-fold activation was produced at around 3.6μM-Ca^{2+}

by saturating concentrations of the activator in the present study. We considered the possibility that the activation observed was due to contamination by small amounts of calmodulin undetectable by SDS/polyacrylamide-gel electrophoresis. A number of findings made this unlikely. The $(Mg^{2+} + Ca^{2+})$-ATPase in the membranes from which the activator was extracted was inhibited by only 10–20% by calmodulin-binding protein, and only at high Ca^{2+} concentrations; at a similar concentration, calmodulin-binding protein almost totally inhibited calmodulin-stimulated Ca^{2+} transport into inside-out vesicles (F. Larsen, S. Katz & B. D. Roufogalis, unpublished work) and $(Mg^{2+} + Ca^{2+})$-ATPase activity in calmodulin-containing membranes (Lynch & Cheung, 1979). The quantity of calmodulin detected by radioimmunoassay in the sucrose-gradient-purified activator fraction (approx. 10pg/ml of 'ghosts') is one-thousandth or less than the threshold amount of calmodulin required to stimulate significantly $(Mg^{2+} + Ca^{2+})$-ATPase in EDTA-treated membranes.

The possibility that the low activation was due to calmodulin tightly bound to another higher-molecular-weight protein was also considered. A protein fraction of approx. 9.2S isolated from the sucrose gradient and enriched in spectrin failed to activate $(Mg^{2+} + Ca^{2+})$-ATPase activity and did not antagonize the stimulation of this activity by calmodulin. Thus spectrin, the major protein extracted from membranes by EDTA treatment (Fairbanks *et al.*, 1971), did not appear to bind calmodulin significantly. We also treated EDTA-extracted activator fraction with a variety of chaotropic agents and in salt conditions designed to dissociate a possible calmodulin-binding-protein complex. Activator preparations treated in this way were tested after dialysis or after XM-100 membrane ultrafiltration, concentration and dialysis. Treatment with 6M-urea (with and without heating), 2.5M-guanidine hydrochloride or 0.3–0.5M-NaCl failed to alter the Ca^{2+}-dependence or the maximum extent of $(Mg^{2+} + Ca^{2+})$-ATPase activation.

Finally, SDS/polyacrylamide-gel electrophoresis of the activator fraction isolated on sucrose density gradients showed a protein of apparent mol.wt. 63000, but was devoid of any protein in the molecular-weight region expected for calmodulin (16000–21000). A 32000-mol.wt. protein was found in some activator preparations in addition to the 63000-mol.wt. protein, but it does not appear to be derived from calmodulin, as it was never detected in pure calmodulin samples under the same electrophoretic conditions. A final estimate of the properties of the activator requires its large-scale purification.

It is noteworthy that membranes used in the present study were extensively diluted and washed thoroughly with phosphate buffer in the absence of Ca^{2+}. Calmodulin was found in membrane preparations isolated by different procedures (Lynch & Cheung, 1979; Scharff & Foder, 1978), and indeed in occasional membranes isolated in our laboratory by the Dodge *et al.* (1963) procedure used in this study.

The $(Mg^{2+} + Ca^{2+})$-ATPase activator isolated from essentially calmodulin-free membranes, as prepared in the present study, appears to be more firmly associated with the membrane than calmodulin. The function of this activator is unknown at present. It appears to be aggregated or highly asymmetric, as determined from its behaviour on gel chromatography. Although it does not cross-react with calmodulin in the calmodulin immunoassay, the possibility that it is a membrane-modified calmodulin with low cross-reactivity cannot be excluded at present. We tentatively suggest that when bound to the membrane the activator may serve to modulate the effect of calmodulin on the Ca^{2+}-transport ATPase in the human erythrocyte. Its effect on Ca^{2+} transport in activator-depleted membranes in the presence and absence of calmodulin requires examination.

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