Journal of Bioenergetics and Biomembranes, Vol. 16, Nos. 5/6, 1984

A Protein Activator of the Plasma Membrane Ca^{++}-ATPase of Heart Sarcolemma

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Received June 8, 1984; revised August 30, 1984

Abstract

A detergent extract of dog or beef heart sarcolemmal vesicles was prepared and found to have a stimulatory effect on the Ca^{++}-ATPase of plasma membranes from human erythrocyte and cardiac sarcolemma. A procedure is described which enriches the activating fraction. The protein nature of the preparation is illustrated by its sensitivity to boiling and to the proteolytic enzyme(s) trypsin and chymotrypsin. SDS[3] polyacrylamide gels indicate that the protein(s) involved have a molecular weight of 56 and 60 kDa. The sarcolemmal activator can stimulate the Ca^{++}-ATPase activity of the isolated enzyme more than 100% in the presence of saturating amounts of calmodulin. The activation is calcium dependent, being greatest at approximately 10μm Ca^{++}, free, but does not change the K_m for Ca^{++}. A possible physiological role for the activator is discussed.

Key Words: Sarcolemma; erythrocyte; Ca^{++}-ATPase; Ca^{++}-ATPase activation; plasma membrane; regulation; Ca^{++} transport.

Introduction

The plasma membrane Ca^{++}-ATPase is one of the two systems responsible for the transport of calcium out of the cell. Given the central role of calcium in fundamental processes such as muscle contraction and relaxation, cell shape, and cell division, it is obvious that the function of the ATPase is of key importance to the cell.

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[3] Abbreviations: DEAE, diethylaminoethyl sepharose 4B; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol-bis-(aminoethyl ether) *N*-*N*'-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; kDa, molecular mass in kilodaltons; IU, international unit; SDS, sodium dodecyl sulfate.

0145-479X/84/1200-0611$03.50/0

The Ca^{++}-ATPase has high affinity for calcium ($K_m \leq 1\mu M$) and relatively low maximal rate of pumping; thus, it can be considered as a fine tuner of cell calcium, in contrast to the other plasma membrane Ca^{++} transport system, the Na^+/Ca^{++} exchanger, which has opposite kinetic properties.

It is now well established that calmodulin directly modulates the activity of the plasma membrane ATPase through its effect on the V_{max} and K_m of the enzyme. Recent data have also suggested the regulation of the transporter through a phosphorylation–dephosphorylation system (Caroni and Carafoli, 1981b), possibly under the control of β-adrenergic stimulators. Other studies have uncovered regulatory protein candidates which could directly activate (Lotersztajn and Pecker, 1981; Au and Chan, 1981; Penniston and Dobbe, 1983) or inhibit (Lee and Au, 1983; Wuethrich, 1982) the Ca^{++}-ATPase, including a membrane-associated "calmodulin-binding" protein (Maudlin and Roufogalis, 1980). Our studies on enriched plasma membrane vesicles from heart muscle have led to the discovery of a protein preparation capable of stimulating the plasma membrane Ca^{++}-ATPase above the levels reached with calmodulin alone. The characteristics of the stimulatory activity and a procedure for its enrichment are discussed in this report.

Materials and Methods

All chemicals were of the highest grade commercially available.

The isolation of purified Ca^{++}-ATPase from human erythrocyte membranes was performed as described by Niggli *et al.* (1981b), with the use of a calmodulin affinity chromatography column. Sarcolemmal membranes were prepared from dog or beef heart as described by Jones *et al.* (1980). The purified sarcolemma was used for the isolation of Ca^{++}-ATPase with a method similar to that described for erythrocyte membranes (Caroni *et al.*, 1983) and for the enrichment of the Ca^{++}-ATPase activator as described below.

All procedures were carried out at 4°C. Sarcolemma, approximately 100 μg of protein, was diluted with distilled water to a concentration of 1 mg/ml. Triton-X-100 was then added from a 10% stock solution to a final concentration of 2.5%. The mixture was allowed to remain on ice for 30 min. After centrifuging at 104,000 *g* max, for 30 min, the supernatant was withdrawn and ammonium sulfate was added to it to a final concentration of 30% (0°C). After 30 min, the suspension was centrifuged again at 104,000 *g* max. for 30 min. The supernatant was withdrawn, and the ammonium sulfate concentration was raised to 60%. A 30-min incubation followed, and the suspension plus precipitate were centrifuged at 104,000 *g* max. for 30 min. The precipitate was carefully separated from the remaining solution and resuspended in

one-third the original volume of 30 mM imidazole, pH 8.0, and dialyzed overnight against 1000 volumes of the same buffer. A DEAE column (4 ml) was washed with 2–3 volumes of 30 mM imidazole, pH 8.0, and 0.1% Triton-X-100. The sample was then loaded on the column at a speed of 16 ml/hr. The final enriched activator fraction was eluted with 5–6 ml of 30 mM imidazole, pH 8.0, and 0.1% Triton-X-100. For SDS polyacrylamide gel electrophoresis of higher amounts of DEAE elutants, it was necessary to first concentrate the sample on Amicon PM 10 filters (Amicon Corp., Lexington, Massachusetts) in the presence of 30% ethylene glycol and 10% ethanol, as recommended by the company, to remove interfering Triton-X-100 (Frasch, 1976).

Calmodulin was isolated from bovine brain acetone powder (Sigma Chemical Co., St. Louis, Missouri) by the use of a phenyl sepharose column as described by Gopalakrishna and Anderson (1982). The protein purity was checked by SDS polyacrylamide gel electrophoresis. Calmodulin was iodinated by a modification of the procedure of LaPorte *et al.* (1980) for the use of lactoperoxidase and glucose oxidase. Enzymobeads from Biorad (Richmond, California) were used in place of the free enzymes.

The ATPase activity was assayed by use of a coupled enzyme system (Niggli *et al.*, 1979) at 37°C. One milliliter of medium contained 130 mM KCl, 20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.3 mM K_2ATP, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 1 IU lactate dehydrogenase, 1 IU pyruvate kinase per assay, and, unless otherwise specified, 15 μM free calcium. The change in absorbance of NADH was monitored spectropotometrically at 366–550 nm. Alternatively, hydrolysis of [^{32}P]-ATP was measured as follows: a 1-ml volume containing 130 mM KCl, 20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.1 mM [^{32}P]-ATP, 12 μg calmodulin, 10 μM Ca^{++} free, and 4 μg Ca^{++}-ATPase was maintained at 37°C. At appropriate time intervals, 100-μg samples were withdrawn with a Hamilton syringe and immediately mixed in a 400-μl Eppendorf tube with 50 μl of 1.5 M H_2SO_4, 1 mM NaH_2Pi, and 5% ammonium molybdate on ice. The sample was mixed with 100 μl isobutanol and centrifuged in a Beckman tabletop centrifuge at 12,000 *g* max.; the upper isobutanol layer was withdrawn, dissolved in 5 ml Instagel, and counted in a scintillation counter. Controls for spurious [^{32}P]-ATP splitting were also run, and found to be negligible.

The trypsinization of the Ca^{++}-ATPase and of the sarcolemma activator, when necessary, were performed as follows: 10 μg of protein were incubated, either on ice for the Ca^{++}-ATPase or at 37°C in the case of the sarcolemmal activator, with 0.25 mg/ml trypsin in a final volume of about 60 μl. The reaction was stopped after 15 min with trypsin inhibitor protein at a final concentration of 2.5 mg/ml.

The treatment of the sarcolemmal protein activator with chymotrypsin

was as follows: 5 μg sarcolemmal protein was incubated with 1 μg chymotrypsin and 1 mM $CaCl_2$ for 15 min at 37°C in 50 μl of 30 mM imidazole, pH 8.0, and 0.1% Triton-X-100. The reaction was stopped by the addition of 2.5 μl of 40 mM dithiothreitol. The assay medium also contained 1 mM dithiothreitol. For comparison, activator protein was treated in the same way without chymotrypsin.

Ten or 12% SDS polyacrylamide gel electrophoresis was performed with a 5% stacking gel after Laemmli (1970). Samples were first precipitated in 1 ml 6% trichloroacetate, spun down at 12,000 *g* max., and then washed in 1 ml chloroform/methanol (2:1), before solubilizing them in 25–50 μl SDS buffer. They were then applied to the gels.

Protein concentrations were determined by using the Biorad protein assay, which is a modification of the method of Bradford (1976).

Free calcium concentrations were determined in EGTA-buffered solutions by use of computer-generated calculations.

Results and Discussion

The search for regulators of the cardiac plasma membrane Ca^{++}-ATPase is best performed on the purified sarcolemmal Ca^{++}-ATPase. This, however, is severely hampered by the low protein yields of membrane preparations of this sort (0.15 mg/kg muscle; see, for example Jones *et al.*, 1980 and Caroni *et al.*, 1983). The Ca^{++}-ATPase of human erythrocytes, on the other hand, can be easily obtained in relatively high yield and excellent purity. Therefore, the erythrocyte plasma membrane enzyme was employed as a test system. The use of the erythrocyte enzyme is justified on a number of accounts. The heart and erythrocyte enzymes are activated in a common way by calcium and calmodulin (Caroni and Carafoli, 1981a), cross react with antibodies (Caroni *et al.*, 1982), and exhibit a similar fragmentation pattern by trypsin proteolysis (Caroni *et al.*, 1983). Indeed, preliminary activation experiments on the heart sarcolemmal Ca^{++}-ATPase have produced results in good agreement with those obtained using the erythrocyte system.

Figure 1b represents the activity of the Ca^{++}-ATPase from heart sarcolemma in the presence of Ca^{++}, calmodulin, and the heart sarcolemmal activator. The hydrolysis of ATP is highly stimulated by calmodulin. However, even in the presence of extremely high levels of calmodulin (350 mol/mol Ca^{++}-ATPase) the activity can be further stimulated by the addition of the sarcolemmal activator (0.5 μg/ml). The experiment of Fig. 1b is particularly important because the Ca^{++}-ATPase and the activator fraction were both extracted from the same membranes (i.e., the Ca^{++}-ATPase was first extracted in 0.5% Triton-X-100 and centrifuged at 100,000 × *g* max. for 30 min. The supernatant was run through a calmodulin affinity chromatography

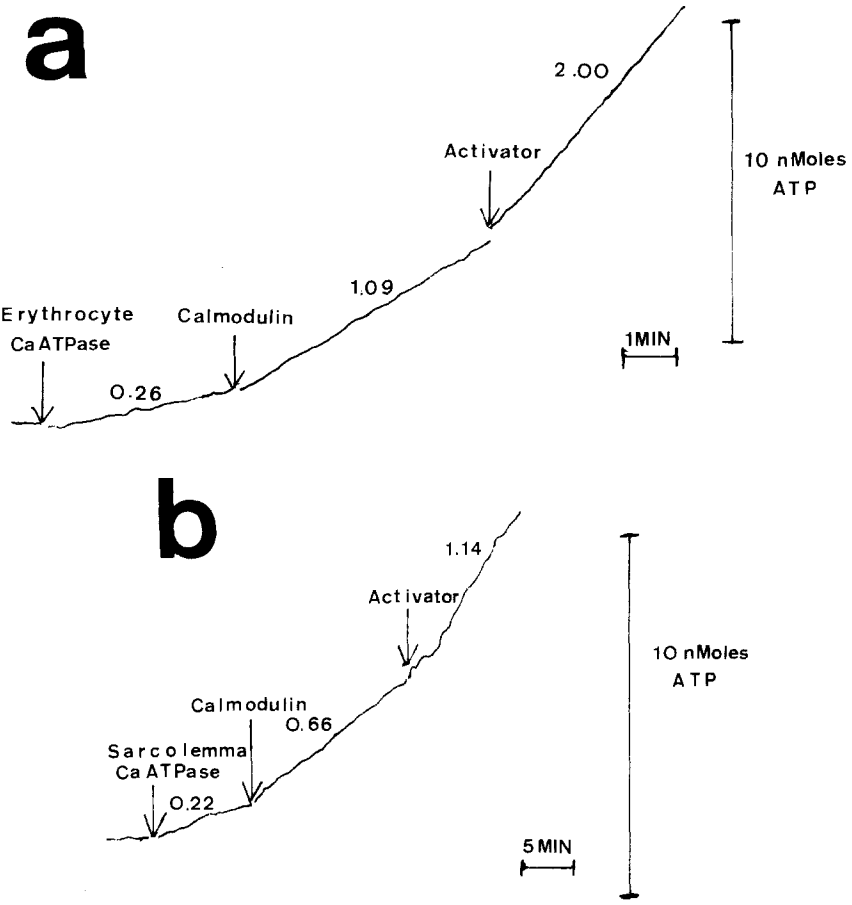

Fig. 1. The activation of the isolated Ca^{++}-ATPase by calmodulin and sarcolemmal protein activator. At the indicated points, 12 μg calmodulin or 0.5 μg sarcolemmal protein were added to the assay medium. (a) 0.3 μg Ca^{++}-ATPase of human erythrocyte plasma membrane; (b) 0.1 μg Ca^{++}-ATPase of dog heart sarcolemmal membranes. The figures give the activity in nmol/min.

column under the conditions described in Niggli *et al.*, (1981b), and the pure ATPase was isolated. The pellet was treated as described in the Materials and Methods section to obtain the activator fraction. Similar observations were made when the erythrocyte Ca^{++}-ATPase was used (Fig. 1a): both calmodulin and the sarcolemma activator stimulate the ATPase activity in a manner comparable to that found with sarcolemma enzyme. The stimulation of the erythrocyte enzyme was found to be Ca^{++} dependent (Fig. 2). In the absence of calmodulin, the activity of the ATPase could be increased to almost double even at Ca^{++} concentrations below 10 μM. This, however, represents a much

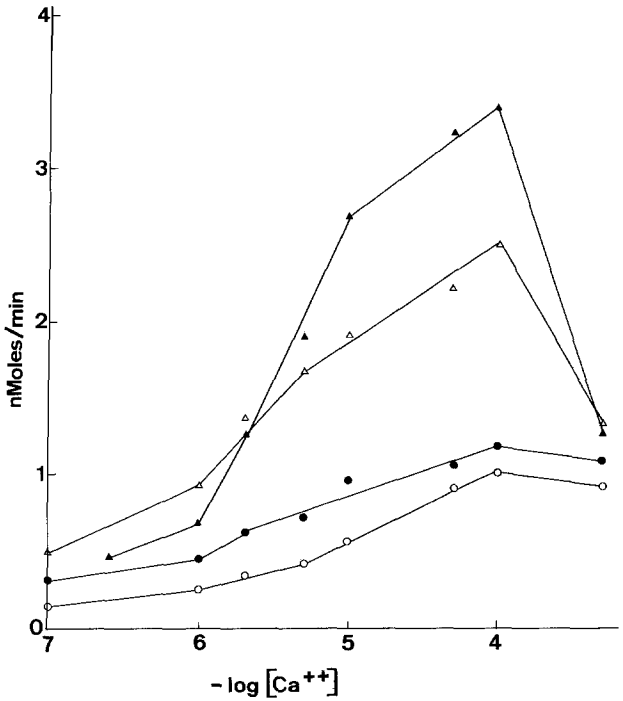

Fig. 2. The calcium dependence of ATPase activation. Free calcium concentrations were determined in assay media plus 10 μM EGTA. 0.3 μg Ca^{++}-ATPase of human erythrocyte were present per assay. Values are the mean of at least four trials. (○) control; (△) + 12 μg calmodulin; (●) + 0.5 μg sarcolemmal protein activator; (▲) + 0.5 μg sarcolemmal protein activator + 12 μg calmodulin.

lower absolute activation by the sarcolemma protein than in the presence of calmodulin. The data indicate an increase in ATPase activity of about 50% in the presence of calmodulin at about 15 μM free Ca^{++}. The K_m for Ca^{++} appears unchanged. This may indicate that the activator protein is in some way able to facilitate the action of calmodulin once bound.

As seen in Table I, only high concentrations of Triton-X-100 are suitable to extract the stimulatory factor from the sarcolemma. The activation can be further enriched by precipitation with ammonium sulfate, followed by DEAE column fractionation. An increase in "specific activity" (defined as the ratio of activation/mg sarcolemma protein) of more than 40 times was achieved. Unlike calmodulin (Roufogalis *et al.*, 1984) and the activator described by Lotersztajn and Pecker (1982), the sarcolemma protein considered here has little affinity for DEAE, even at pH 8.0.

SDS polyacrylamide gel electrophoresis (Fig. 3) performed on the

Table I. Solubilization and Enrichment of the Heart Sarcolemma Ca^{++}-ATPase Activator[a]

Fraction	μg Protein per assay	Stimulation ratio	Specific activity
Homogenate	15	1.2	80
1 mM EDTA extract	15	1.0	—
H_2O extract	5	0.8	—
0.1% TX-100 extract	10	0.9	—
1.0% TX-100 extract	18	1.1	61
2.5% TX-100 extract	18	1.5	83
60% $(NH_4)_2SO_4$ precipitate	20	3.7	185
DEAE eluate	0.5	1.7	3400

[a]The "extracts" refer to the supernatant recovered from a 30-min incubation of 100 μg sarcolemmal protein with the specified detergent or buffer, followed by a 30-min centrifugation at 104,000 × *g* max. Other details are given in the Materials and Methods section.

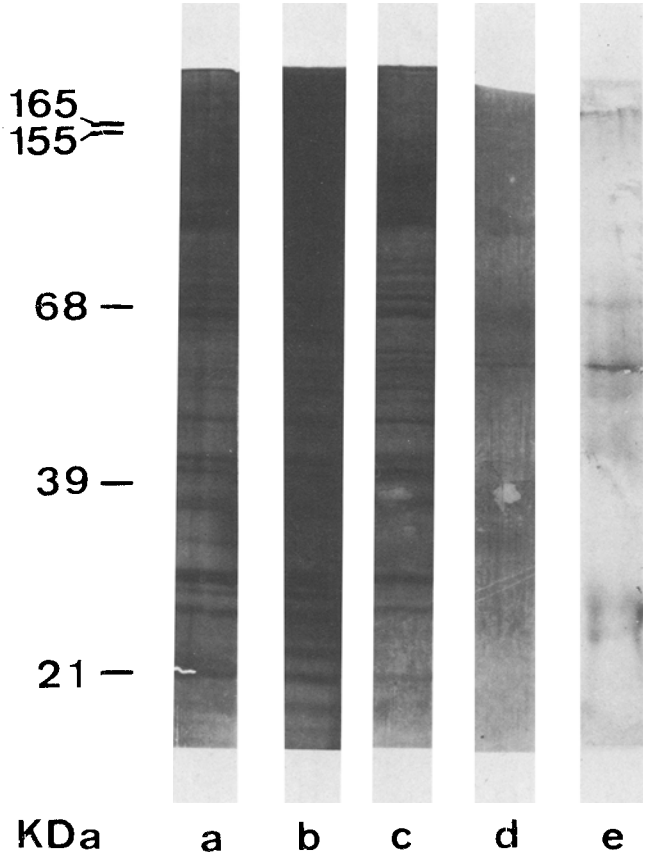

Fig. 3. The isolation of the sarcolemmal protein activator. A 10% SDS polyacrylamide gel was run as described in Materials and Methods. Lane a: 2.5% Triton-X-100 extract; lane b: 30% ammonium sulfate precipitate; lane c: 60% ammonium sulfate precipitate (after dialysis); lane d: DEAE column eluate (4 μg protein); lane e: DEAE column eluate (20 μg protein).

products of the various steps involved in the isolation of the activator documents the progress in its isolation. Lane a shows the Triton-X-100 extract in which all the major proteins are represented, in agreement with published data (see, for example, Flockerzi *et al.*, 1983; Lamers *et al.*, 1983; Jones *et al.*, 1979). Triton-X-100 (2.5%) was chosen for the reason that maximal stimulatory activity was extracted at this concentration, as seen from Table I. Lane b displays the proteins precipitated at 30% ammonium sulfate. This fraction had a low stimulatory activity, but also retained some Ca^{++}-independent ATPase activity, probably due to the Mg^{++}-ATPase. The use of 60% ammonium sulfate (lane c) separates the activating fraction from several low M_r proteins, as well as from the background activity. Running the material through a DEAE column at pH 8.0 in the presence of 0.1% Triton-X-100 results in the enrichment of three major proteins of 56, 60, and 70 kDa (lane d and e). One or two other proteins are also faintly visible. It is intriguing that no low-molecular-weight proteins (i.e., below 25 kDa) are visible in the final DEAE eluate.

Recently, several studies have focused on the role of phosphorylation in the activation of the Ca^{++}-ATPase. The primary observation is the presence in "purified sarcolemma" of a 21–22-kDa protein whose phosphorylation coincides with the stimulation of the Ca^{++}-ATPase (Lamers *et al.*, 1981; Rinaldi *et al.*, 1981). The similarity of this protein to phospholamban has been duly noted. Rinaldi *et al* (1981) have suggested that this protein may be the "calcium channel." However, this interpretation has recently been contested by Jones and Besch (1983) and it has been concluded that the 21–22-kDa protein may be adventitious sarcoplasmic reticulum phospholamban present in sarcolemma. On the other hand, the nature of the proteins isolated here may be worth some scrutiny. A 56-kDa sarcolemmal phosphate acceptor has already been described whose extent of phosphate incorporation was found to increase slightly in the presence of the C subunit of the cAMP-dependent protein kinase, or of cyclic nucleotides (Rinaldi *et al.*, 1981; Jones *et al.*, 1980). Preliminary tests were made to ascertain if the sarcolemmal protein discussed here is involved in a phosphorylation–dephosphorylation system. In the absence of exogenous protein kinase, no direct phosphorylation of the Ca^{++}-ATPase from either erythrocyte or sarcolemma was observed. Furthermore, no incorporation of ^{32}P into any of the bands which compose the activator fraction could be discerned (data not shown). This is in sharp contrast to the cAMP-dependent protein kinase of heart sarcolemma, which autophosphorylates to a high degree (Jones *et al.*, 1980). The possibility exists, however, that the activator fraction may contain proteins which are already completely phosphorylated, as appears to be the case for some proteins in the sarcolemmal membrane, as they are isolated (Caroni and Carafoli, 1981b). Therefore, exclusion of the sarcolemmal proteins discussed here from a phosphorylation–dephosphorylation system is not yet certain.

Both for the purpose of establishing that the activation of ATPase activity is due to a protein component, and to ascertain that minor contamination by calmodulin has no role in the effects observed, several experiments were carried out. First, the stimulating fraction was preincubated in the presence of trypsin or chymotrypsin. Table II shows a partial decrease in the activation ability of the sarcolemmal fraction after trypsinization. A substantial (50%) activation, however, is still observed. This may indicate either that proteolysis has not progressed to completion, or that a limit-peptide, still capable of activation, has been produced from the protein. Chymotrypsin, however, completely abolishes the activation by the sarcolemmal fraction (Table II).

A characteristic of calmodulin is its heat stability. The activating sarcolemma protein was heated to 91°C for 15 min in elution buffer plus 0.1 mM $CaCl_2$. As a control, an aliquot of purified calmodulin was also heated. As seen in Table II, the heat treatment reduces the activation by the sarcolemmal fraction from more than double to only about 30%. By contrast, there is no detectable inhibition of the stimulatory action of calmodulin.

It has been reported (Roufogalis *et al.*, 1984) that an activator of approximately 56 kDa can be extracted from erythrocyte membranes by mild treatment with EDTA. This activator binds tightly to calmodulin and has a low affinity for DEAE. To test whether a relationship exists between the protein of Roufogalis *et al.* and the one described here, the following set of experiments was carried out. Calmodulin isolated from bovine brain acetone powder was iodinated with ^{125}I as described in the Materials and Methods section. This was added to the crude detergent extract from sarcolemma, and the usual enrichment procedure was carried out to conclusion. The resulting DEAE eluant retained less than 0.3% ($\leq$ 26 ng calmodulin) of the radioactiv-

Table II. Stimulation of Erythrocyte Ca^{++}-ATPase by Calmodulin and the Sarcolemma Activator[a]

	ATPase activity (nmol/min)			
	Stimulator treatment			
	None	15 min at 91°C	15 min trypsin	15 min chymotrypsin
Erythrocyte ATPase	0.41 + 0.06	—	—	—
Calmodulin (12 μg)	1.50 + 0.18	1.96 + 0.18	1.78 + 0.08	1.08 + 0.08
Calmodulin + sarcolemma activator (1 μg)	3.37 + 0.56	2.56 + 0.38	2.67 + 0.57	1.16 + 0.10
Stimulation ratio	2.3 + 0.3	1.3 + 0.1	1.5 + 0.3	1.1 + 0.1

[a] Isolated erythrocyte Ca^{++}-ATPase (0.5 μg) was assayed at 37°C as described in Materials and Methods. Calmodulin or sarcolemmal protein was treated as indicated in the columns and added sequentially to the assay medium. Rates of ATP hydrolysis were measured in the first 2 min following the additions. Other experimental details are given in the Materials and Methods section.

ity originally layered on the column, as determined with the use of a gamma counter. Autoradiography of SDS polyacrylamide gels of the eluate was also carried out (Fig. 4). No radioactivity can be detected in the peak activating fractions (lanes c, d, and e) although each lane represents more than three times the volume of the 60% ammonium sulfate fraction (lane b) applied to the gel. Taken together with lane d of Fig. 3, which shows no loss of intensity in the 56-kDa band in the DEAE eluant, it can be inferred that the stimulation is not due to a calmodulin-binding protein.

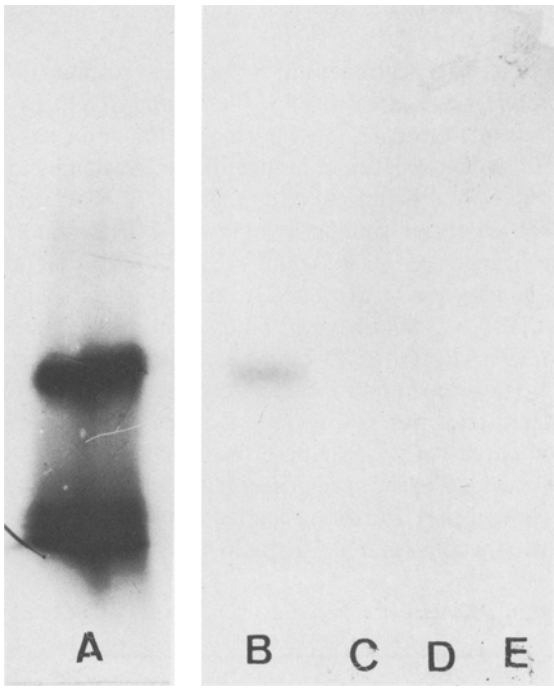

Fig. 4. The recovery of iodinated calmodulin from sarcolemmal activating fraction. 13 μg (1 μCi) of [^{125}I]calmodulin were added to 350 μg sarcolemma protein (following the 60% ammonium sulfate precipitation). After dialysis overnight, the sample contained approximately 70% of the original counts. The sample was applied to a DEAE column, as described in the Materials and Methods section, and the active fractions were collected. Samples were run on a 5% stacking gel with a 12% running gel. The gel was dried and incubated with x-ray film overnight. The autoradiogram is shown. Lane a: [^{125}I]calmodulin, 1.3 μg; lane b: 60% ammonium sulfate precipitate (after dialysis), 4.0 μg (20 μl); lane c–e: the three peak activating fractions recovered from DEAE column chromatography, 3.4 μg/lane (70 μl).

Table III. Activation of Trypsinized Erythrocyte Ca^{++}-ATPase by the Sarcolemma Activator

	ATPase activity (nmol/min)	
	Enzyme treatment	
	0 min trypsin	15 min trypsin
Erythrocyte ATPase (no additions)	0.60 + 0.05	1.33 + 0.08
Calmodulin (12 μg)	1.46 + 0.10	1.37 + 0.07
Calmodulin (12 μg) + activator (1 μg)	3.28 + 0.32	2.75 + 0.15
Stimulation ratio	2.2 + 0.3	2.0 + 0.2

[a]The Ca^{++}-ATPase of erythrocyte was trypsinized where indicated as described in the Materials and Methods section and kept on ice until ready for use in the presence of 2.5 mg/ml trypsin inhibitor protein. The assay mixture also contained the same concentration of trypsin inhibitor protein. Calmodulin and the protein activator were added sequentially and rates of ATP hydrolysis were measured. Other experimental details are given in the Materials and Methods section.

Finally, the sarcolemma protein activator was tested against the trypsinized Ca^{++}-ATPase of erythrocyte. Enzyme treated in this manner is known to achieve high velocities of ATP hydrolysis and, at the same time, to lose calmodulin sensitivity (Niggli *et al.* 1981a, b). As shown in Table III, the sarcolemmal protein was still able to stimulate the activity of the trypsinized ATPase. By contrast, and as expected, the stimulation by calmodulin was abolished under these conditions.

The evidence reported here indicates that a protein exists in mammalian heart sarcolemma which has a stimulatory effect on the plasma membrane Ca^{++}-ATPase. That 2.5% Triton-X-100 is required for its extraction suggests that the protein is intrinsic and highly hydrophobic. The possibility exists that it may be a component part of the cardiac Ca^{++}-ATPase complex. Preliminary experiments to test whether the protein fraction in question is part of the phosphorylation–dephosphorylation process involved in the regulation of the sarcolemmal Ca^{++}-ATPase have been inconclusive. However, the finding that the stimulation effects only the V_{max}, and not the K_m of the Ca^{++}-ATPase, may be taken as an indication that the effect occurs mainly at high ($\geq 10\ \mu M$) Ca^{++} concentrations. In heart, this may help potentiate the action of the Ca^{++}-ATPase, helping the heart cell control the internal Ca^{++} concentration during the repolarization phase following the contraction event.

Acknowledgments

The authors wish to thank Dr. Konrad Famulski for helpful discussions, and Mr. Rolf Moser for technical assistance. This project was supported by the Swiss Nationalfonds (Grant No. 3.189-0.82).

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