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CHARACTERIZATION OF A β-ACTININ-LIKE PROTEIN IN PURIFIED NON-MUSCLE CELL MEMBRANES

ITS ACTIVITY ON $(Na^+ + K^+)$-ATPase

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Treatment by EDTA of purified plasma membranes from MF_2S cells (a variant of the murine plasmacytoma MOPC 173) solubilized proteins and increased by a 1000-fold the sensitivity of $(Na^+ + K^+)$-ATPase to ouabain. When added back with Ca^{2+} to treated plasma membranes, these EDTA-solubilized proteins restored the initial sensitivity of the enzyme to its inhibitor. We report the purification of a protein of M_r 32000, isolated from the EDTA-treated membrane supernatant. This protein was purified by a one-step procedure involving a preparative polyacrylamide gel electrophoresis without detergent. In the presence of Ca^{2+} it was able to restore the original sensitivity to ouabain of $(Na^+ + K^+)$-ATPase from EDTA-treated membrane. This protein was shown to be similar to the β-actinin described by Maruyama by the following criteria: (1) molecular weight and amino acid composition; (2) cross-reactivity with their respective antisera; (3) in the presence of Ca^{2+} the same quantitative biological activity on ouabain sensitivity of the $(Na^+ + K^+)$-ATPase. A possible interaction between β-actinin, calmodulin and membrane-bound $(Na^+ + K^+)$-ATPase is discussed.

Introduction

The stimulation of membrane-bound enzymes, such as $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) [1] acetyl and methyl transferases [2,3] has been observed during the mitogenic response in lymphocytes. It is well established that microtubules and microfilaments are involved in this response [4] but there is little direct evidence of a relationship between membrane-bound enzyme and cytoskeletal protein [5]; in erythrocyte plasma membrane, spectrin is linked to ankyrin which is tightly bound to Band III, the anion transport system [6]. However, colchicine or vinblastine, drugs perturbing microtubules, have been reported to modify adenylate cyclase sensitivity to isoproterenol and prostaglandin [7] and to prevent cAMP-mediated or β-adrenergic increase in ornithine decarboxylase activity [8]. Regulation of membrane-bound enzyme activities can also be mediated by proteins: as an example, the stimulatory action of Ca^{2+} is conferred on brain adenylate cyclase [9] and $(Ca^{2+} + Mg^{2+})$-ATPase by calmodulin [10].

Previous studies in our laboratory have shown

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that membrane proteins can significantly affect $(Na^+ + K^+)$-ATPase sensitivity to ouabain. The treatment by EDTA of plasma membranes purified from murine plasmacytoma cells (MF_2S) leads to a significant increase in the sensitivity of $(Na^+ + K^+)$-ATPase to ouabain without affecting its specific activity [11]. This alteration is associated with the solubilization of inner face plasma membrane proteins [12]. The recovery of the original resistance to ouabain requires Ca^{2+} specifically and is associated with a binding of the solubilized proteins to the membranes [13].

This paper describes the purification of a protein involved in the regulation of ouabain sensitivity of $(Na^+ + K^+)$-ATPase from MF_2S plasma membranes. The purified protein belongs to the microfilament system. It is different from tropomyosin which had been previously shown to exhibit the same effect on $(Na^+ + K^+)$-ATPase [14] but shares several characteristics with a previously identified actin regulatory protein, β-actinin [15,16].

Materials and Methods

Murine protein preparation from plasmacytoma cells

The MF_2S cells, derived from the MOPC 173 murine plasmacytoma, grown as ascitics were used as a source of membranes. Plasma membrane purification was achieved on a discontinuous sucrose gradient as previously described [17]. Purified membranes were treated with EDTA in a pH 6.8 buffer comprising 1 mM EDTA/30 mM imidazole chloride/250 mM sucrose according to Lelievre et al. [11]. The EDTA-treated membranes were resuspended in 60% glycerol (at least 5 mg/ml), stored at −20°C and used for the enzymatic assays within 2 weeks.

Supernatants from the EDTA treatments were then dialysed against a 50 mM Tris-HCl buffer, pH 6.8, and concentrated on a UM 10 Amicon membrane.

5–10 mg proteins from the EDTA-treated membrane supernatants were submitted to preparative polyacrylamide gel electrophoresis (Ultraphor preparative electrophoresis apparatus Colora) without detergent or dissociating agents, as previously reported [18]. Briefly, the stacking gel was made of 3% acrylamide, pH 6.8, and the running gel of two successive layers of 7.5 and 12% respectively, pH 8.8. The power was maintained at 10 W until the Bromophenol blue reached the running gel and was then increased to 15 W and maintained constant for 32 h. The flow rate of the eluting buffer was 50 ml/h.

Preparation of muscle proteins

Actin and tropomyosin from rabbit skeletal white muscle were prepared according to Spudich and Watt [19] and to Hartshorne and Mueller [20], respectively.

β-Actinin was prepared as follows, using as starting material chicken skeletal muscle. All the steps were carried out at +4°C. Skeletal muscle from freshly killed chicken was minced in a meat grinder and homogenized with a polytron (Sorvall) at full speed for 5 min in 3 vol. of neutral distilled water and centrifuged for 30 min at $17000 \times g$ in an MSE high-speed centrifuge. The supernatant was dialyzed overnight against a 10 mM acetate buffer, pH 5.5. The dialysate was centrifuged for 30 min at $17000 \times g$ and the supernatant was applied to a DEAE-52 cellulose (Whatmann) column (4 × 20 cm) equilibrated with the same buffer. The non-retarded proteins (crude β-actinin) were concentrated on a UM 10 Amicon membrane. 100 mg were dialyzed against a 10 mM Tris-HCl buffer, pH 6.8, and centrifuged prior to chromatography on a Sephadex G-100 (Pharmacia) column (80 × 1.5 cm) equilibrated with the same buffer. A double peak was eluted as shown in Fig. 1. The β-actinin recovered in the ascending part of the first peak (dashed area) was pooled, concentrated and dialyzed against a 2 mM sodium phosphate buffer, pH 6.8. 10 mg proteins were applied to a 5 ml column of hydroxyapatite Ultrogel (HA Ultrogel, Pharmindustrie) equilibrated with the same buffer. Retarded proteins were eluted by a linear gradient of sodium phosphate from 2 to 300 mM. β-Actinin was eluted as a single peak (Fig. 2) near 14 mM and pure β-actinin was recovered from fractions 20–33 (dashed area).

Biochemical and biological analysis

Protein concentration. Soluble and membrane proteins were respectively estimated according to Bradford [21] and Lowry et al. [22].

Gel electrophoresis. Electrophoresis was per-

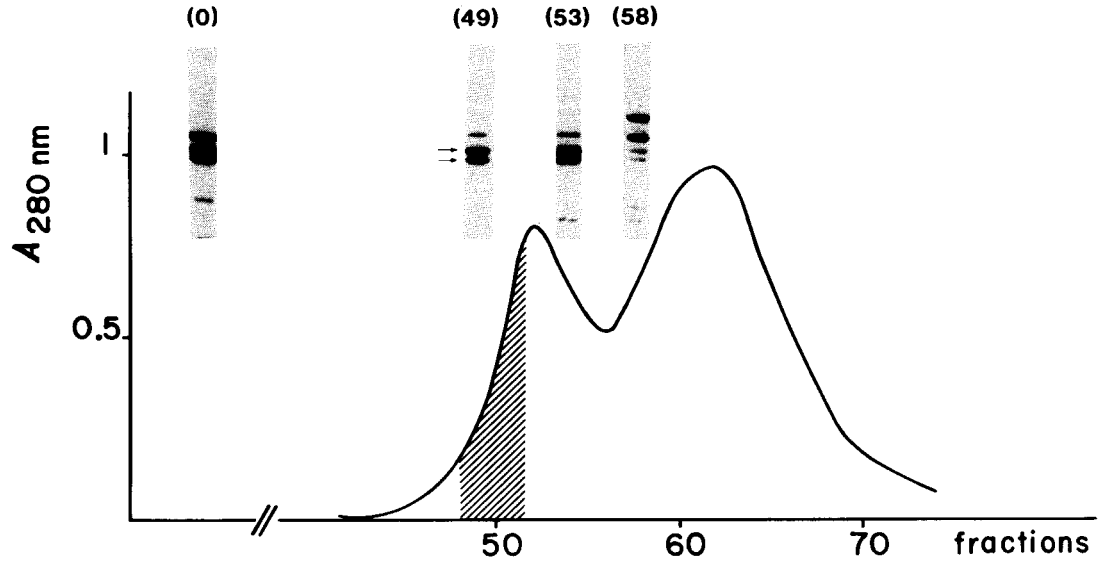

Fig. 1. (Top.) Sephadex G-100 chromatography of crude β-actinin chicken muscle aqueous extract. The elution solution contained 10 mM Tris-HCl buffer (pH 6.8); its flow rate was 10 ml/h at 4°C. Fraction volume: 1.5 ml. Absorbance at 280 nm (———). The numbers in parenthesis above the electrophoresis patterns correspond to the fraction numbers, except for O (original sample). The two arrows correspond to the two polypeptide chains of β-actinin. The hatched area corresponds to the fractions pooled and further purified.

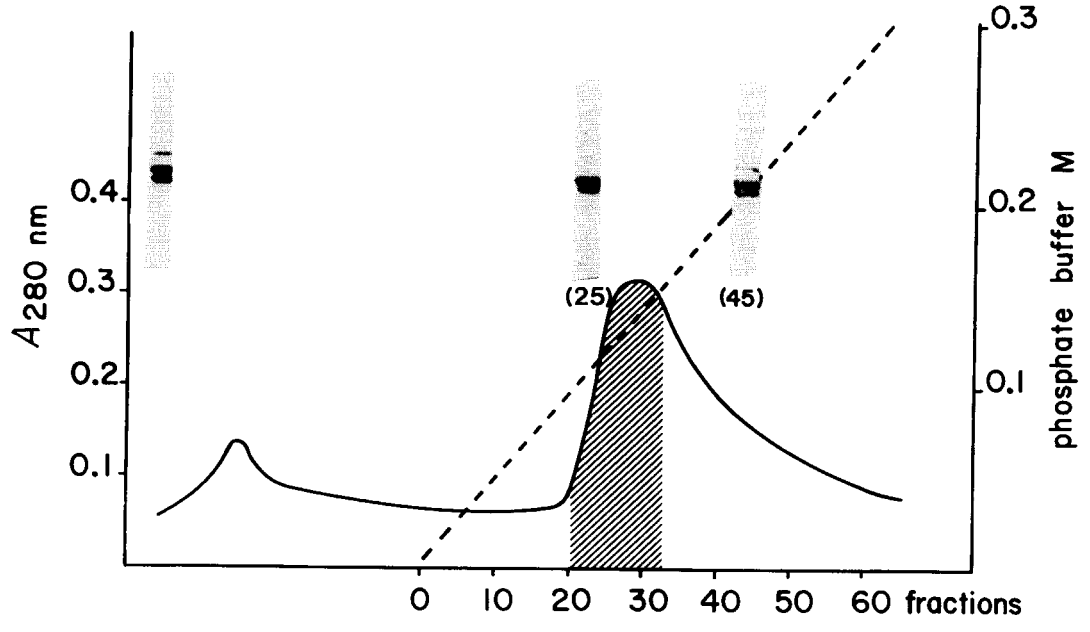

Fig. 2. (Bottom.) Hydroxyapatite Ultrogel chromatography of the enriched β-actinin fraction prepared on Sephadex G-100. The elution solution contained 2 mM sodium phosphate buffer (pH 6.8). The fraction volume was 1.0 ml. The continuous line (———) represents the absorbance at 280 nm and the dotted line (---) the linear phosphate gradient from 2 mM to 300 mM. The numbers under the electrophoresis patterns correspond to the fraction numbers after the beginning of the PO_4 gradient. Insert at left shows the electrophoresis pattern corresponding to the starting material.

formed using a polyacrylamide slab gel in the presence of sodium dodecyl sulfate (SDS) according to Leammli [23]. Running gels (14 × 14 cm) were prepared from 10% acrylamide and stacking gels from 5% acrylamide. The gels were stained with 1% Coomassie brillant blue R 250, in 30% methanol/10% acetic acid and then destained in 30% methanol/10% acetic acid. All proteins were checked for purity on SDS-polyacrylamide gel electrophoresis. Apparent molecular weights of the murine protein and of the two chicken β-actinin chains were estimated from SDS polyacrylamide gel electrophoresis after reduction by β-mercaptoethanol. The following proteins were used as markers: phosphorylase *b* (M_r 94000) bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), and carbonic anhydrase (M_r 30000).

Amino acid composition. Composition of the murine protein was determined after total acid hydrolysis (18 h at 110°C in 5M HCl) in a Technicon automatic analyzer.

($Na^+ + K^+$)-ATPase activity. The assay for the sensitivity of ($Na^+ + K^+$)-ATPase to ouabain was carried out at 37°C as previously described, in the absence and presence of ouabain (10^{-7} M to 10^{-3} M) [11,24]. EDTA-treated membranes (150 μg) were preincubated for 30–90 min at 4°C with 0.1 mM $CaCl_2$ with either 15 μg dialysed EDTA-treated membrane supernatant or 0.7–10 μg of the 32 kDa murine protein or chicken β-actinin. The reaction was initiated by the addition of prewarmed ATP.

DNAase I inhibition assay. The rate of polymerization of G-actin to F-actin with or without chicken β-actinin was determined using a DNAase I inhibition assay based on the inhibition of DNAase I by the monomeric form of actin [25]. The hydrolysis of DNA was followed by measuring the hyperchromicity at 260 nm, using a Philips-Unicam SPI 800 spectrophotometer. The amount of G-actin, at zero time, was chosen in order to obtain a 75% inhibition of the DNAase I activity.

Immunologic analysis

Antiserum against the murine protein was produced in rabbit by a single immunization with 50 μg of the purified protein using the immunization scheme described by Vaitukaitis et al. [26].

Antiserum against chicken β-actinin was raised by intramuscular injections of 1 mg crude β-actinin homogenized in complete Freund's adjuvant. Injections were made at weekly intervals for 3 weeks. Antisera against actin and tropomyosin were prepared as respectively reported [27,28].

The animals were bled weekly over a period of 2 months and the appearance of antibodies was followed using an ELISA micromethod [27]. The hydrolysis of *para*-nitrophenyl phosphate (substrate of alkaline phosphatase) was registered at 405 nm after 25 min at 37°C (Multiskan Titertek).

Antibody purification was carried out using immunoadsorbant columns of pure murine protein and pure β-actinin. These proteins were immobilized on CNBr-activated Sepharose 4-B (Pharmacia) [29]. A 40% $(NH_4)_2SO_4$-precipitated fraction of each antiserum was applied to its respective immunoadsorbant. Specific antibodies were eluted with glycine-HCl buffer, pH 2.8, and immediately neutralized with 1 M Tris-HCl buffer, pH 8.

Specific and cross reactions of antigens with purified antibodies were studied using an immunoperoxidase method on thin-layer SDS slab gel electrophoresis as described by Van Raamsdonck et al. [30]. The slab gels were run as described above, frozen at −30°C and cut into slices of 50 μm thickness (Kryomat 1700, Leitz). Goat anti-rabbit immunoglobulins labeled with peroxidase were purchased from Miles. The enzymatic reaction of peroxidase was stopped with distilled water after 10 min incubation with a diaminobenzidine solution.

Results

To demonstrate the similarity between β-actinin and our murine protein, we purified the β-actinin from chicken muscle and studied its properties.

Purification and properties of chicken β-actinin

The purification procedure used here yields 70 mg pure β-actinin per kg muscle, which is about 3-times that of Maruyama's procedure for rabbit β-actinin [16]. This yield can be explained by (i) water extraction [31], (ii) the use of an acidic buffer for the DEAE-52 cellulose column which did not retain the protein and (iii) the use of HA Ultrogel chromatography of the β-actinin to remove contaminating actin.

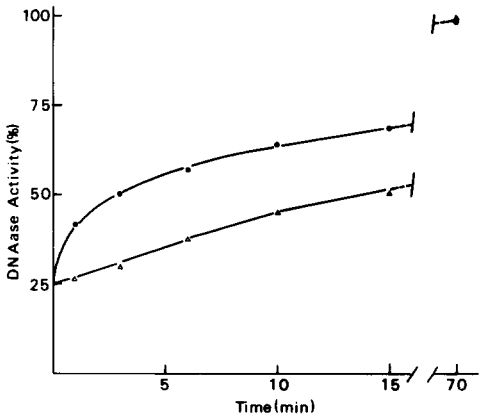

Fig. 3. Effect of β-actinin from chicken muscle on the rate of transformation G-actin to F-actin. Polymerization of G-actin (0.1 mg/ml in 2 mM Tris-HCl (pH 8.0)/1 mM dithiothreitol/0.2 mM ATP/0.2 mM $CaCl_2$) to F-actin was induced by 50 mM KCl and 2 mM $MgCl_2$. Aliquots of 10 μl were taken every 2 min and mixed with 10 μl DNAase I (beef pancreas – DN 100, Sigma: 0.1 mg/ml in 50 mM Tris-HCl (pH 7.5)/0.01 mM phenylmethylsulfonyl fluoride/0.5 mM $CaCl_2$) and 1 ml DNA substrate (calf thymus – Type 1, Sigma: 40 μg/ml in 0.1 M Tris-HCl (pH 7.5)/4 mM $MgSO_4$/1.8 mM $CaCl_2$) for no more than 10 s (△———△). Samples of G-actin (0.1 mg/ml)+β-actinin (0.02 mg/ml) were incubated under the same conditions (●———●). 100% DNAase I activity was measured in the absence of G-actin.

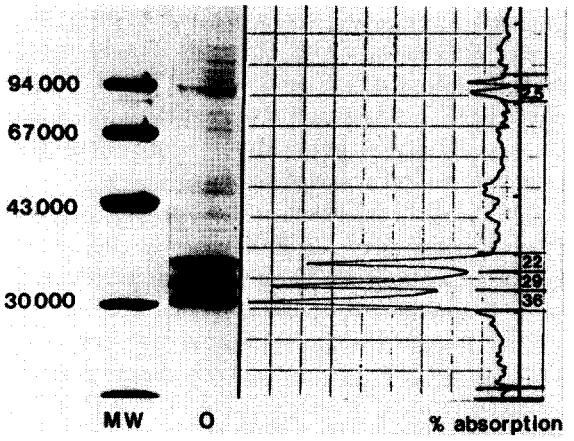

Fig. 4. Scanning by SDS-polyacrylamide gel electrophoresis of EDTA-removed membrane proteins. From left to right: protein markers and their respective molecular weights; Coomassie blue staining of EDTA-removed membrane proteins (O); scanning of (O) at 570 nm (Scanner DCD 16 – Gelman); respective percentage of the main proteins of (O) to the total amounts of protein.

As described for rabbit β-actinin [32] the pure protein aggregated easily and had to be used within a few days for biological tests.

As shown on SDS-polyacrylamide gel electrophoresis, the protein was composed of two polypeptide chains with apparent molecular weights of 34000 and 37000, respectively.

As reported for other β-actinins [32,33] the purified chicken β-actinin was able to accelerate the initial rate of polymerization of G-actin to F-actin without change in the final yield of polymerization. Fig. 3 shows a typical time course of actin polymerization with or without β-actinin. As measured by the DNAase I inhibition assay, the recovery of the enzyme activity reflected the polymerization of G-actin to F-actin. The initial recovery was clearly enhanced in the presence of β-actinin. Within the first min of polymerization, addition of β-actinin increased 6-fold the recovery of DNAase I activity. By birefringence measurement, identical results were obtained with rabbit muscle β-actinin by Maruyama et al. [16].

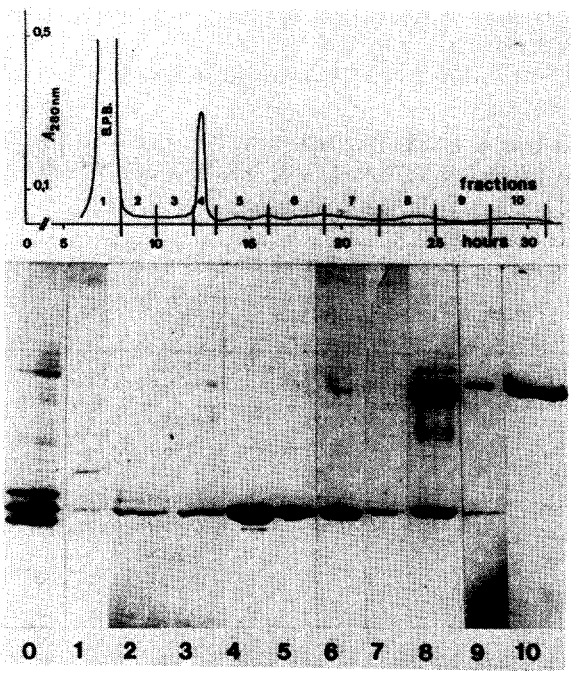

Fig. 5. Analysis of the preparative electrophoresis. Upper panel: Elution profile at 280 nm of the preparative electrophoresis. The lower numbers indicate the duration of the run in hours, the upper numbers the samples obtained by pooling eluted fractions. The first peak is due to the elution of Bromophenol blue (B.P.B.). Lower panel: SDS-polyacrylamide gel electrophoresis analysis of the EDTA-solubilized proteins (O) and of the ten samples obtained during the 32-h run.

Purification and properties of the murine protein

(a) Purification. From 10^9 cells, 30–40 mg of purified plasma membranes were recovered, from which EDTA treatment removed 5–10% of the proteins, finally yielding about 1 mg of protein after dialysis and concentration.

SDS gel electrophoresis of these proteins showed three main bands of M_r 30000, 32000 and 34000, representing about 36, 29 and 22%, respectively, of the total amount of protein (Fig. 4).

Each preparative electrophoresis was done with 7–10 mg of EDTA-solubilized membrane proteins and after elution, fractions were dialyzed against 50 mM Tris-HCl buffer (pH 6.8) and concentrated on a UM 10 Amicon membrane. The 32 kDa protein was eluted from fractions 1–9. However, the eluate absorption curve (top, Fig. 5) showed only one significant peak containing the 32 kDa protein (fraction 4). The final recovery of the highly pure 32 kDa protein was about 1% of the total protein submitted to electrophoresis. The 30 kDa and 34 kDa proteins were not eluted after 32 h of electrophoresis. They could either be retained on the gel or eluted as an aggregated 68 kDa protein not dissociable by SDS (fractions 8–10 in Fig. 5).

As for β-actinin, the pure protein aggregated easily, even with glycerol, β-mercaptoethanol or urea, and had to be used within a few days after purification.

(b) Biochemical properties. The murine protein had an apparent molecular weight of 32000 as judged by SDS-polyacrylamide gel electrophoresis analysis.

Table I depicts the high homology between the amino acid compositions of the murine protein and the β-actinins from rabbit skeletal muscle and plasmodium [16,34]. Note that these proteins contain proline; however, the murine protein contains less glutamic and rather more glycine residues than the β-actinins already described.

The exess of acidic over basic residues clearly indicated that these three proteins are acidic (Table I). Nevertheless, the 32 kDa murine protein

TABLE I

AMINO ACID COMPOSITION OF THE 32 kDa MURINE PROTEIN IN COMPARISON TO THAT OF RABBIT MUSCLE β ACTININ [16] AND β-ACTININ FROM PLASMODIUM (SLIME MOLD) [34]

Values are residues per 1000 residues.

	Rabbit muscle β-actinin			32 kDa murine protein [a]	Plasmodium β-actinin
	B_I	B_{II}	Total		
Asp	149	128	144	93	97
Thr	39	57	49	67.5	50
Ser	77	45	63	78	45
Glu	112	146	138	100	138
Pro	46	48	44	31.7	54
Gly	76	55	59	133.5	84
Ala	55	82	68	77.5	96
Val	62	64	63	63.1	62
Met	9	19	12	15.4	29
Ile	56	67	62	49	52
Leu	117	74	95	108.4	80
Tyr	35	33	33	29.5	28
Phe	23	45	33	37.1	41
His	17	23	19	15.2	20
Lys	73	73	73	60.7	84
Arg	55	41	45	42	40
$\frac{Asp+Glu}{Arg+Lys+His}$	1.8	2	2	1.64	1.62

[a] Mean of four different experiments.

and β-actinin from plasmodium may be less acidic than rabbit muscle β-actinin.

Biological activity of purified murine protein and comparison with purified chicken β-actinin

The capacity of the murine protein and chicken β-actinin to restore the resistance to ouabain of EDTA-treated membranes was examined (see Materials and Methods).

The $(Na^+ + K^+)$-ATPase activity of the native plasma membranes exhibited an one-step dose-response curve to ouabain and was half-maximally inhibited by 120 ± 20 μM ouabain (Fig. 6A). The EDTA treatment led to a two-step dose-response curve (Fig. 6B) with a plateau associated with half the activity between 1 and 50 μM ouabain. It has been shown that this EDTA treatment solubilizes proteins located at the internal face of the plasma membrane [12]. Indeed, only inside-out vesicles, which represented about 50% of the membranes, showed modified ouabain-binding sites and gave rise to proteins active in the restoration of the initial sensitivity of the enzyme to its inhibitor. When either Ca^{2+} alone, the purified murine protein or the purified chicken β-actinin was added to EDTA-treated plasma membranes without Ca^{2+}, no change occurred (Fig. 6C); however, if the protein (either purified murine protein or purified chicken β-actinin) and Ca^{2+} were added, the initial resistance of the $(Na^+ + K^+)$-ATPase recovered fully (Fig. 6D).

The restoration of the original sensitivity of the

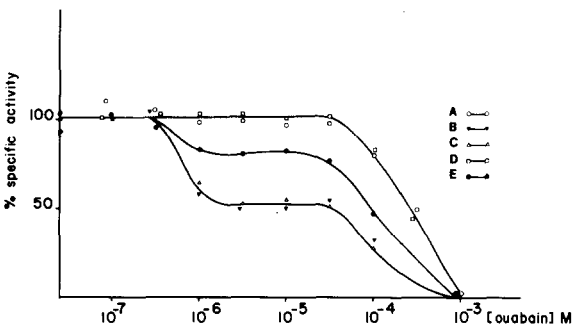

Fig. 6. Dose-response curve of $(Na^+ + K^+)$-ATPase sensitivity to ouabain. Specific activity as percentage of the total, versus ouabain concentration (logarithmic scale) in: (A) native plasma membranes; (B) EDTA-treated membranes; (C) EDTA-treated membranes (150 μg) + Ca^{2+} alone (0.2 mM $CaCl_2$) or with the 32 kDa murine protein or chicken β-actinin (0.7–10 μg); (D) 0.2 mM $CaCl_2$ + EDTA-treated membranes (150 μg) + the 32 kDa murine protein or chicken β-actinin (0.7–10 μg); (E) 0.2 mM $CaCl_2$ + EDTA-treated membranes (150 μg) + the 32 kDa murine protein (0.4 μg).

$(Na^+ + K^+)$-ATPase is a stepwise procedure. As full recovery was obtained with 0.7–10 μg of the purified murine protein, an intermediate plateau was observed with 0.1–0.7 μg for a standard amount of EDTA-treated plasma membrane (150 μg) (Fig. 6E).

Immunological studies

ELISA and slab gel electrophoresis tests were used for their high sensitivity even with nonprecipitating antibodies.

As shown in Table II, the anti-murine protein

TABLE II

ANTIBODY EVALUATION IN THE DIFFERENT ANTISERA TESTED AGAINST PURE ANTIGENS BY THE ELISA TECHNIQUE

Values give the colorimetric absorption at 405 nm. (a) Specific antigen antibody reaction – antiserum dilution of 1/1000 except for anti-murine protein (1/100). (b) Cross-reaction between β-actinin and anti-murine protein and vice versa. Antiserum dilution of 1/100. (c) Controls – antiserum dilutions of 1/20. n.d., non-detectable.

Purified antigen (1 μg)	Crude antisera			
	anti-murine protein	anti-β actinin	anti-tropomyosin	anti-actin
Murine protein	0.7 (a)	0.4 (b)	0.08 (c)	0.02 (c)
Chicken muscle β-actinin.	0.6 (b)	0.4 (a)	0.02 (c)	0.03 (c)
Tropomyosin	n.d. (c)	n.d. (c)	1 (a)	–
Actin	n.d. (c)	n.d. (c)	–	1.2 (a)

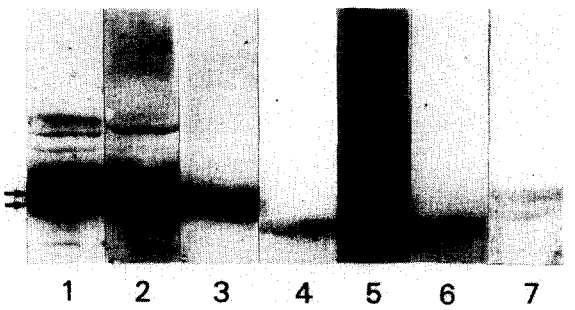

Fig. 7. Electrophoresis and thin-layer SDS slab gel electrophoresis of chicken muscle β-actinin (1,2,3 and 7) and EDTA-removed membrane proteins (4,5 and 6) in 10% polyacrylamide gel. 1 and 5, 1-mm-thick gel slice stained with Coomassie blue (50 μg protein); 2–4, 50 μm-thick gel section incubated with (2) anti-crude chicken β-actinin (serum dilution 1/20) (3 and 4) specific anti-chicken β-actinin antibodies (solution of 0.1 mg/ml). 6 and 7: 50-μm-thick gel sections incubated with specific antibodies to the 32 kDa murine protein (solution of 0.1 mg/ml). The two arrows indicated β_I and β_{II} subunits of chicken β-actinin.

antiserum reacted as well with the purified 32 kDa protein as with the chicken β-actinin: the same serum dilution gave about the same absorbance with both antigens. In contrast, the reaction of the anti-β-actinin antiserum is higher with its specific antigen than with the murine protein: the same absorbance was obtained with dilutions of 1/1000 and 1/100, respectively. None of these antisera recognized actin or tropomyosin (see Table II).

Purified specific antibodies against the murine protein and chicken β-actinin were tested against crude chicken β-actinin and murine EDTA-treated membrane supernatant, respectively, by slab gel electrophoresis method as shown in Fig. 7.

On lanes 1 and 5, respectively, the Coomassie staining of crude β-actinin and EDTA-treated membrane supernatant can be seen. While unpurified anti-β-actinin reacted against several components of the crude β-actinin (lane 2), purified antibodies against β-actinin recognized only the two bands of this protein (lane 3) and also the 32 kDa protein of EDTA-treated membrane proteins (lane 4). Conversely, purified specific antibodies against the murine protein recognized the 32 kDa band (lane 6) and also the two bands of the chicken β-actinin (lane 7).

As a control, anti-tropomyosin and anti-actin antibodies did not react either against β-actinin or against the 32 kDa murine protein (data not shown).

From these results it can be concluded that the murine protein and chicken β-actinin share common antigenic determinants.

Discussion

In this paper, a single protein, removed by EDTA treatment from murine plasmacytoma MF_2S plasma membranes has been shown to alter the sensitivity of $(Na^+ + K^+)$-ATPase to ouabain.

The murine protein and β-actinin are similar by their respective biochemical, immunological and biological properties.

(i) Biochemicql properties. Both proteins are acidic and their amino acid composition is very similar. It is noteworthy that rabbit and chicken β-actinins are heterodimers with two bands (M_r 34000 and 37000) upon SDS-polyacrylamide gel electrophoresis [16], while plasmodium β-actinin is an homodimer with one band (M_r 43000) on electrophoresis [34]. The migration upon electrophoresis as a single band (M_r 32000) and the estimated molecular weight (60000–70000) on a velocity sucrose density gradient (data not shown) suggests that the murine protein is a homo-dimer.

Identical observations of differences in amino acid composition, isoelectric point and molecular weight were found for other microfilamental proteins such as actins or tropomyosins from different organs or species [35,37].

(ii) Immunological reactivity. By two immunological tests, the murine protein and chicken β-actinin were shown to share common antigenic sites: antiserum raised against the murine protein recognized its specific antigen and both polypeptide chains from chicken β-actinin. Immune serum raised against chicken β-actinin recognized chicken β-actinin and the 32 kDa protein present in the supernatants from EDTA treatment on SDS gels. (Fig. 7). Cross-immune reactivity has indeed been described between homologous cytoskeletal proteins from different species [38–40].

(iii) Biological effect. Both the murine protein and chicken β-actinin have the same effect in altering the ouabain sensitivity of $(Na^+ + K^+)$-ATPase in EDTA-treated plasma membranes from

MF_2S cells. Furthermore, both proteins required Ca^{2+} to be effective.

The fact that chicken β-actinin and murine protein display a similar biological property strongly suggests that these molecules are highly conserved. This remarkable feature is shared by other proteins of the cytoskeleton [41–43].

However, some questions have to be raised following this work and we would like to discuss some of them.

The biological effect on the ouabain sensitivity of the $(Na^+ + K^+)$-ATPase was exhibited by β-actinin-like murine protein and chicken β-actinin, as well as by tropomyosin [14]. Although murine protein and tropomyosin showed similar molecular weight they differed by: (i) their amino acid composition – it is well known that tropomyosin does not contain proline and has a very high content of glutamic acid (more than 30%) [35], while the murine protein contains proline and only 10% of glutamic acid (Table I); (ii) their immunological reactivity (Table II) which excluded the presence of any common antigenic determinants between both proteins. As a similar amount (about 1 μg) of either the murine protein or tropomyosin is required to restore the initial sensitivity of EDTA-treated membrane, the hypothesis that tropomyosin might be contaminated by β-actinin, as mentioned by Maruyama [16], can be ruled out. The acidic properties of both proteins cannot explain their biological effect, as another acidic protein, troponin C, was not able to restore the enzyme sensitivity to ouabain [14].

As not only the purified protein but also Ca^{2+} is required in this effect, this might be due to the necessity of a Ca^{2+}-binding protein bound to membrane such as calmodulin. Indeed Lelievre et al. [44] showed that drastic EDTA treatment of purified plasma membranes also removed calmodulin. After such treatment, Ca^{2+}, calmodulin and tropomyosin were necessary to restore the ouabain resistance of the enzyme. In erythrocytes it has been already demonstrated that EDTA treatment of purified membranes removed calmodulin, a protein activator of the $(Ca^{2+} + Mg^{2+})$-ATPase activity [45].

Therefore it is unlikely that β-actinin-like protein or tropomyosin are directly and specifically interacting with the $(Na^+ + K^+)$-ATPase, as they both are known to bind to actin which is present in the plasma membrane (data not shown). Both are also known not to bind Ca^{2+}, which is required at the same time to restore the initial sensitivity of the $(Na^+ + K^+)$-ATPase ouabain receptor site.

In conclusion, the current dogma that the cytoskeleton controls some membrane bound enzymes is strongly supported by the data reported here. A pure cytoskeletal protein (from murine plasmacytoma cell) identical to β-actinin is able to modify, from the inner face of the membrane, the conformation of the external binding site of ouabain on the $(Na^+ + K^+)$ transport system.

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