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Purification and characterization of a Ca^{2+}-binding 450-kDa protein (MCBP-450) in the plasma membrane-enriched fraction from a molluscan smooth muscle

Tomoyo Yamanobe [a] and Haruo Sugi [b]

[a] *Central Laboratory of Analytical Biochemistry and* [b] *Department of Physiology, School of Medicine, Teikyo University, Itabashi-ku, Tokyo (Japan)*

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In accordance with physiological and electronmicroscopic evidence that, in the anterior byssal retractor muscle (ABRM) of a common mussel *Mytilus edulis*, Ca^{2+} activating the contractile system is accumulated at the inner surface of the plasma membrane and at the membrane of sarcoplasmic reticulum (Ebashi, S. and Endo, M. (1968) Prog. Biophys. Mol. Biol. 18., 123–183; Suzuki, S. and Sugi, H. (1982) in The role of calcium in biological systems, Vol. I (Anghileri, L.J. and Tuffet-Anghileri, A.M., eds.), pp. 201–207, CRC Press, Boca Raton), we have found a high-molecular-mass (450 kDa) Ca^{2+}-binding protein (MCBP-450) in the membrane fractions of the ABRM by ^{45}Ca autoradiography of proteins transferred to nitrocellulose membrane (Rüegg, J.C. (1971) Physiol. Rev. 51, 201–248). MCBP-450, purified to electrophoretic homogeneity, exhibited Ca^{2+}-dependent changes in mobility, tryptophan fluorescence, UV absorption and CD spectrum, indicating its Ca^{2+}-dependent conformational changes. MCBP-450 has a high content of aspartic and glutamic acid (23.8%) and a high content of basic residues (27%). It has a high capacity Ca^{2+}-binding site, which binds about 38 mol of Ca^{2+} per mol with an adissociation constant of 10^4 M^{-1}, and a low-capacity Ca^{2+}-binding site, which binds about 7 mol of Ca^{2+} per mol with an association constant of 10^5 M^{-1}. These characteristics of MCBP-450 are consistent with the view that it is actually involved in regulating the contraction-relaxation cycle in the ABRM.

Introduction

It has been well-established that the contraction–relaxation cycle in vertebrate skeletal muscle is regulated by the release of Ca^{2+} from, and its uptake by, the sarcoplasmic reticulum (SR) [1]. In various kinds of vertebrate and invertebrate smooth muscles, the source of Ca^{2+} activating the contractile system (activator Ca) still remains to be investigated, though some intracellular structures such as the SR and the mitochondria are also known to accumulate Ca^{2+} [2].

The anterior byssal retractor muscle (ABRM) of a common mussel *Mytilus edulis* exhibits extremely prolonged contraction with little energy expenditure. The above type of contraction is called the catch state, which has been studied intensively by a number of investigators [3,4]. Sugi and Yamaguchi [5] presented evidence that the ABRM contains intracellularly stored activator Ca in the amount enough to fully activate its contractile system. In accordance with this, Atsumi and Sugi [6] have demonstrated that abundant pyroantimonate precipitate containing Ca is localized at the inner surface of the plasma membrane as well as in the SR of pyroantimonate-treated ABRM fibers, and that the precipitate is released into the myoplasm during contraction [6]. Sugi and his co-workers further applied the pyroantimonate method to other types of vertebrate and invertebrate smooth muscles, and showed that the inner surface of the plasma membrane can accumulate activator Ca, which is released into the myoplasm to cause contraction (guinea-pig taenia coli, [7]; *Dolabella* body wall muscle, [8]; Sea cucumber retractor muscle; [9]). These results suggest that there are Ca^{2+}-binding proteins at the inner surface of the plasma membrane in various smooth muscles to play a role in the contraction-relaxation cycle. As a matter of fact, Mimura et al. [10] have recently shown that the

Correspondence to: H. Sugi, Department of Physiology, School of Medicine, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173, Japan.

plasma membrane-enriched fraction from *Mytilus* ABRM contains Ca^{2+}-binding as well as Ca-pumping proteins.

The present work was undertaken to isolate and characterize Ca^{2+}-binding proteins in the plasma membrane-enriched fraction from *Mytilus* ABRM. It will be shown that a high-molecular-mass (450 kDa) Ca^{2+}-binding protein (MCBP-450), which shows Ca^{2+}-dependent conformational changes, is present in the plasma-membrane-enriched fraction as well as the SR membrane-enriched fraction from the ABRM. Characterization of MCBP-450, purified to electrophoretic homogeneity, indicates that MCBP-450 may be involved in the regulation of mechanical activity in the ABRM. A preliminary account of this work has already been published [11].

Materials and Methods

Preparation of membrane fractions. Specimens of *Mytilus edulis* were obtained at the Misaki Marine Biological Station, and kept in aerated seawater at 15°C until use. The crude microsomal fraction was prepared from the ABRM by the method of Mimura et al. [10,12], suspended in 0.25 M sucrose solution, and layered over a discontinuous sucrose gradient consisting of consecutive layers of 0.88, 1.03, 1.41 and 1.88 M sucrose solutions (8 ml for each sucrose concentration) containing 10 mM Tris-HCl buffer (pH 6.8). The gradient tube was centrifuged at $100\,000 \times g$ for 90 min to obtain subfractions F1, F2, F3 and F4 [10]. In the present study, we focused attention to F1 which exhibits the highest Na^+/K^+-ATPase and 5′-nucleotidase activities and was regarded as the plasma-membrane-enriched fraction and F3 which shows the highest (Ca^{2+}, Mg^{2+})-ATPase activity and was regarded as the SR-membrane-enriched fraction [10].

Polyacrylamide gel electrophoresis. Samples were solubilized by heating at 100°C for 5 min in 1% SDS, 1% β-mercaptoethanol, 15% glycerol, 10 mM Tris-HCl buffer (pH 6.8). SDS-PAGE was performed using the discontinuous buffer system of Laemmli [13] in gels containing 5–15% polyacrylamide. The gel was stained with Coomassie brilliant blue R-250 [14] or with silver nitrate.[15].

Electrophoretic transfer of proteins to nitrocellulose membrane. This was performed by the method of Towbin et al. [16]. After SDS-PAGE, the gel was placed on a nitrocellulose membrane (Schleicher and Schuell, BA83/1, pore size 0.2 μm), and covered with chemical sponge sheets. Electrophoretic transfer of proteins from the gel to the nitrocellulose membrane was performed with an electrophoretic blotting apparatus (Atto, AE 3280) at 10 V/cm for 20 h at 4°C.

Labelling of Ca^{2+}-binding proteins with ^{45}Ca. The nitrocellulose membrane, with proteins transferred to it, was washed with a solution containing 60 mM KCl, 5 mM $MgCl_2$ and 10 mM Pipes buffer (pH 6.8), and then incubated in the same solution with 1 mCi/l of ^{45}Ca for 10 min at 25°C by the method of Maruyama et al. [17]. The membrane was rinsed with distilled water and dried for autoradiography.

^{45}Ca autoradiography. Autoradiographs of ^{45}Ca-labelled proteins on the nitrocellulose membrane were obtained by exposure of the dried membrane to Kodak XAR-5 X-ray film for 1–7 days. The exposed X-ray films were subjected to direct densitometry with a densitometer (Joyce-Leobel, chromoscan 3). After the above procedure, the proteins on the nitrocellulose membrane were then stained with Amido black 10B, (0.1% in 50% methanol and 10% acetic acid) to estimate their respective concentrations. The nitrocellulose membrane was then destained with 10% acetic acid, and also subjected to direct densitometry with the densitometer. Densitometer tracings of the Amido-black-stained samples in the low-molecular-mass region did not change appreciably if the time of electrophoretic blotting was decreased from 20 to 6–1 h, indicating that the loss of low-molecular-mass proteins during the blotting was small.

Purification of MCBP-450. MCBP-450 (abbreviation of a new Ca^{2+}-binding protein, see Results) was purified to homogeneity with the following steps.

Step 1. The plasma membrane-enriched fraction containing 10–20 mg protein was first suspended in 7–15 ml of 50 mM n-heptyl-β-D-thioglucoside, 1 mM DTT and 50 mM Tris-HCl buffer (pH 7.5), stirred for 12 h at 0°C, and then the solution was centrifuged at $100\,000 \times g$ for 60 min at 4°C to remove insoluble materials.

Step 2. The solubilized fraction (10 ml) was applied to a 2.6×90 cm Sephacryl S-300 column (Pharmacia) equilibrated with 5 mM n-heptyl-β-D-thioglucoside, 2 mM β-mercaptoethanol and 20 mM Tris-HCl buffer (pH 7.5). Fractions of 10 ml were collected at a flow rate of 60 ml/h while monitoring their absorbance at 280 nm. Fractions No. 17–20 containing high-molecular-mass proteins were pooled.

Step 3. The pooled fractions were further applied to a Bond Elut C_{18} column (column size 3 ml, Analytichem) equilibrated with 5 mM n-heptyl-β-D-thioglucoside, 2 mM β-mercaptoethanol and 20 mM Tris-HCl buffer (pH 7.5), and the first 40 ml of fractions were pooled for purification of MCBP-450.

Step 4. MCBP-450 was purified by high-performance liquid chromatography (HPLC, Pharmacia). The fractions were applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with 2 mM n-heptyl-β-D-thioglucoside, 2 mM β-mercaptoethanol and 20 mM Tris-HCl buffer (pH 7.5). Fractions of 1 ml were collected at a flow rate of 1 ml/min. After discarding the first 10–20 ml of fractions which did not contain

MCBP-450, a linear NaCl gradient was applied, in which NaCl concentration increased from 0 to 1 M. Fraction 29 containing MCBP-450 was dialyzed against 10 mM Pipes buffer (pH 6.8) at 4°C for 10 h, Ca^{2+} attached to the protein being removed with a Ca^{2+}-chelating resin (Bio-Rad, Chelex 100).

When high concentrations of MCBP-450 samples were required for characterization of MCBP-450, fraction 29 was again applied to another Mono Q column with a linear NaCl gradient; fraction 34 containing MCBP-450 was also dialyzed and treated with the Ca^{2+}-chelating resin.

Chromatofocussing. MCBP-450 (fraction 29) was dialyzed against 5 mM n-heptyl-β-D-thioglucoside, 25 mM bis-Tris-iminodiacetic acid buffer (pH 7.1), and applied to a Mono P HR 5/20 column (Pharmacia) equilibrated with 5 mM n-heptyl-β-D-thioglucoside, 25 mM bis-Tris-iminodiacetic acid buffer (pH 7.1). Elution (pH 7.1–4.0) was performed with Polybuffer 74 (diluted 10-times with distilled water, pH 4.0 by iminodiacetic acid) containing 5 mM n-heptyl-β-D-thioglucoside, while elution (< pH 4.0) was achieved by adding 70% acetic acid at the late stage of the Polybuffer elution. The column effluent was monitored at 280 nm and the pH change was monitored by a flowthrough pH electrode.

Spectroscopic measurements. Tryptophan fluorescence was measured with a fluorescence spectrophotometer (Aminco, SPF 500) using 285 nm emission–340 nm excitation pair. Ultraviolet (UV) absorption was measured with a double-beam spectrophotometer (Hitachi, U3200). Ca^{2+} titration was done in 2 ml of 60 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA and 10 mM Pipes buffer (pH 6.8) [18]. The free Ca^{2+} concentration was calculated by the equation of Harafuji and Ogawa [19]. Circular dichroism (CD) spectra were measured with a circular dichrometer (JASCO, J20).

Ca^{2+}-binding assay. Ca^{2+}binding properties of MCBP-450 were studied by the method of Kawasaki et al. [20] with slight modification. Ca-EGTA buffers used were prepared from stock solutions of 50 mM Ca-EGTA and 100 mM EGTA to give the desired free Ca^{2+} concentrations. The Ca-EGTA buffers contained 100 mM KCl, 8 mM $MgCl_2$, 10 mM Pipes buffer (pH 6.8) and 25 mCi/ml of $^{45}CaCl_2$. Protein samples were suspended in the radioactive Ca-EGTA buffer solutions (1 ml for each), and were shaken for 20 h at 4°C. Then each radioactive Ca-EGTA buffer solution (400 μl) was pipetted into each cylindrical well (diameter 3 mm) of a microfiltration apparatus (Atto, AE6190), with which the solution was filtered with a 9 × 12 cm nitrocellulose membrane filter (Schleicher and Schuell, pore size 0.2 μm) under mildly reduced pressure using a water aspirator. Each well was then washed with 400 μl of 10 mM Pipes buffer (pH 6.8). The proteins were spotted on the nitrocellulose membrane filter as circles of 3 mm diameter corresponding to the wells. Each protein spot was isolated from the membrane filter with a punch, and put into a vial containing scintillation liquid. The amount of Ca^{2+} retained in each protein spot was estimated by counting ^{45}Ca radioactivity with a liquid scintillation spectrometer (Packard, Tri-carb 2250CA). The blank values were determined by filtration without samples. Bovine brain calmodulin and bovine serum albumin were treated in the same way and spotted on the same nitrocellulose membrane filter for reference.

Amino-acid analysis. Samples of MCBP-450 were hydrolyzed with 6 M HCl at 110°C for 20 h [21]. Amino acids were separated by HPLC (Shimazu, LC-4A), and

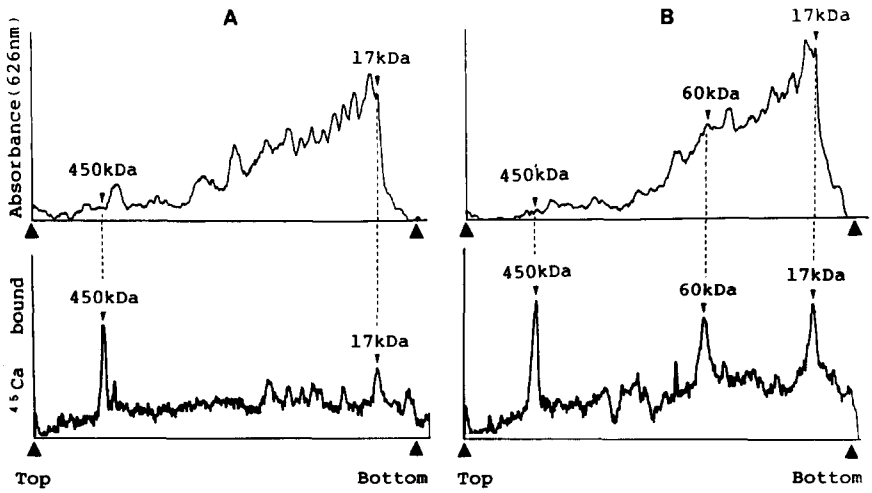

Fig. 1. Detection of Ca^{2+}-binding proteins in the plasma membrane-enriched (A) and the SR membrane-enriched (B) fractions of *Mytilus* ABRM. The upper records are densitometer tracings of proteins, transferred to nitrocellulose membrane after SDS-PAGE and stained with Amido black, while the lower records are densitometer tracings of ^{45}Ca autoradiographs obtained from the same samples.

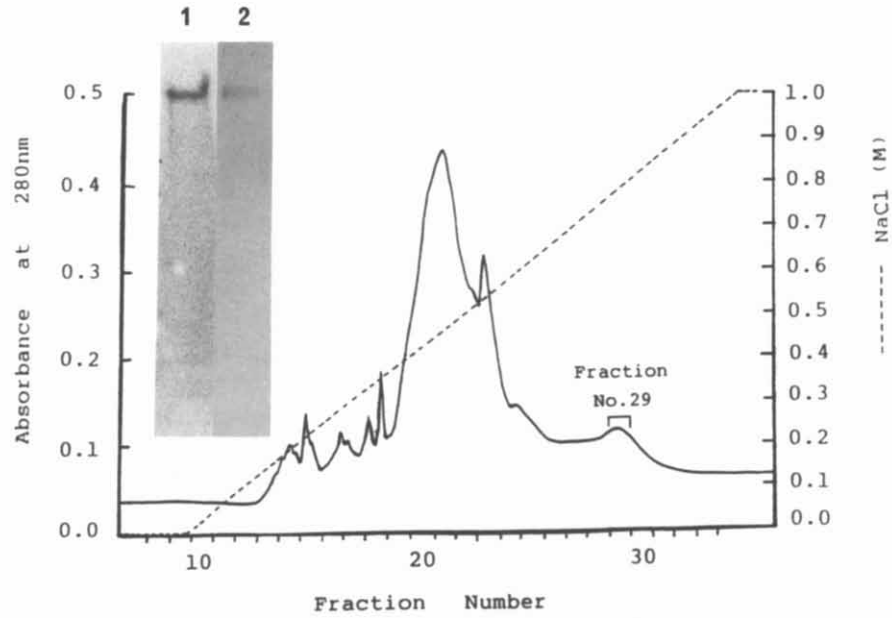

Fig. 2. Mono Q chromatography of the sample (about 10 mg) from purification step 3 (see Materials and Methods). The elution was carried out with a linear NaCl gradient from 0 to 1 M. Bracket indicates fraction 29 where MCBP-450 is localized. Inset 1, ^{45}Ca autoradiograph of fraction 29 after SDS-PAGE, which shows a distinct radioactive band corresponding to 450 kDa MCBP-450. Inset 2 shows fraction 29 stained with Amido black.

were detected with a fluorescence spectrometer (Shimazu, RF-530) by the method of Benson and Hare [22].

The amount of sugars contained in MCBP-450 was also estimated by the phenol-sulfunic acid method [23].

Reagents and other methods. $^{45}CaCl_2$ was obtained from New England Nuclear Corporation. Stains-all was purchased from Sigma. All reagents were of analytical grade. Molecular mass markers were purchased from Boehringer-Mannheim and Pharmacia; RNA polymerase (165, 155 and 39 kDa), thyroglobulin (669 and 330 kDa), ferritin (440, 220 and 18.5 kDa), catalase (232 and 60 kDa), phosphorylase *b* (94 kDa), serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (24.5 kDa) and cytochrome *c* (12.5 kDa).

Protein concentrations were determined by the method of Lowry et al. [24] and by the method of Smith et al. [25]. The latter method was used to measure low concentrations of proteins.

Results

Detection of Ca^{2+}-binding proteins in Mytilus ABRM membrane fractions

Proteins in the membrane fractions, prepared from *Mytilus* ABRM by the method of Mimura et al. [10], were separated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with ^{45}Ca as described in Materials and Methods. Typical densitometer tracings of the ^{45}Ca autoradiographs of proteins in the plasma membrane-enriched and the SR-membrane-enriched fractions [10] are shown in Fig. 1, together with those of Amido-black-stained protein bands prepared from the same samples after autoradiography. In the autoradiographs, the plasma membrane-enriched fraction exhibited two distinct radioactive bands corresponding to 450-kDa and 17-kDa Ca^{2+}-binding proteins, while the SR membrane-enriched fraction showed three distinct bands corresponding to 450-kDa, 60-kDa and 17-kDa Ca^{2+}-binding proteins. In the present study, we focused attention to the 450-kDa Ca^{2+}-binding protein (MCBP-450) present in both the PM and SR fractions.

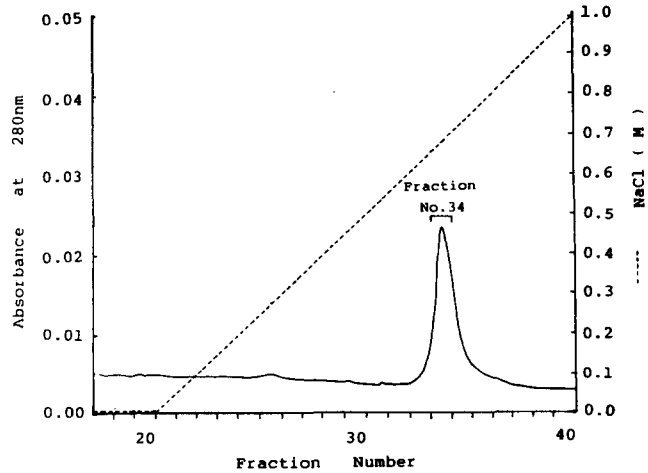

Fig. 3. Mono Q chromatogtaphy of fraction 29 (about 35 μg). The elution was carried with the same NaCl gradient. Note that no peaks are present, except for a single symmetrical peak.

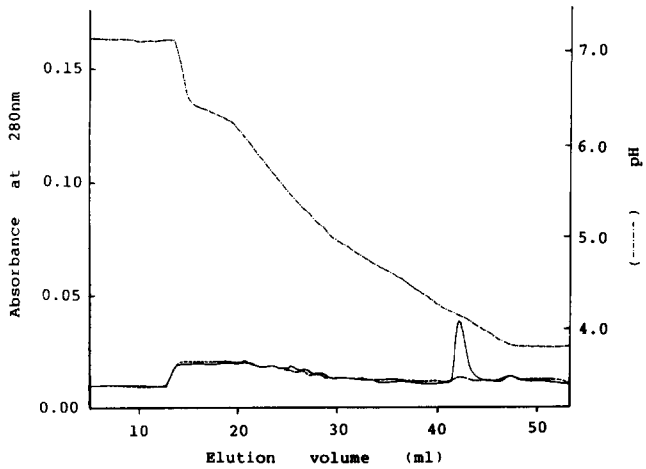

Fig. 4. Elution pattern obtained on chromatofocussing of fraction 29 (solid line) and of buffer without fraction 29 (broken line).

Purification of MCBP-450

An example of chromatograms on a Mono Q column is shown in Fig. 2. A distinct radioactive band was only present in fraction 29 (inset 1), indicating the localization of MCBP-450 in the same fraction. Densitometer tracing of both the radioactive protein band and the same protein sample stained with Amido black (inset 2) exhibited a sharp single peak, indicating that the purified MCBP-450 (fraction 29) was >95% homogeneous. When fraction 29 was again applied to another Mono Q column, the chromatogram only showed a single symmetrical peak (fraction 34, Fig. 3). In addition, chromatofocussing of fraction 29 also exhibited an elution pattern with a single isoelectrical peak at pH 4.2 (Fig. 4). These results may also be taken to support the high MCBP-450 purity of fraction 29.

TABLE I

Yields of MCBP-450 from the plasma membrane-enriched fraction

	Total protein (mg)	Yield (%)
Plasma membrane-enriched fraction	15.1	100
Solubilized fraction in Step 1	7.6	50
Fraction 17–20 in Step 2	2.3	15
Fraction 34 in Step 4	0.008	0.05

Fig. 5 shows SDS-PAGE of MCBP-450 containing samples at various steps of purification. In the case of Coomassie brilliant blue staining (lane 1–5), the bands corresponding to MCBP-450 were observable in lanes 1–3, and became invisible in lanes 4–6. In the case of silver nitrate staining (lanes 1′–6′), the MCBP-450 bands were observable in all lanes. In both cases, the other bands eventually disappeared in lanes 5 and 5′ and 6 and 6′, reflecting the purification of MCBP-450; the disappearance of the MCBP-450 band in lanes 5 and 6 may result simply from that Coomassie brilliant blue staining is not sensitive to small amounts of MCBP-450.

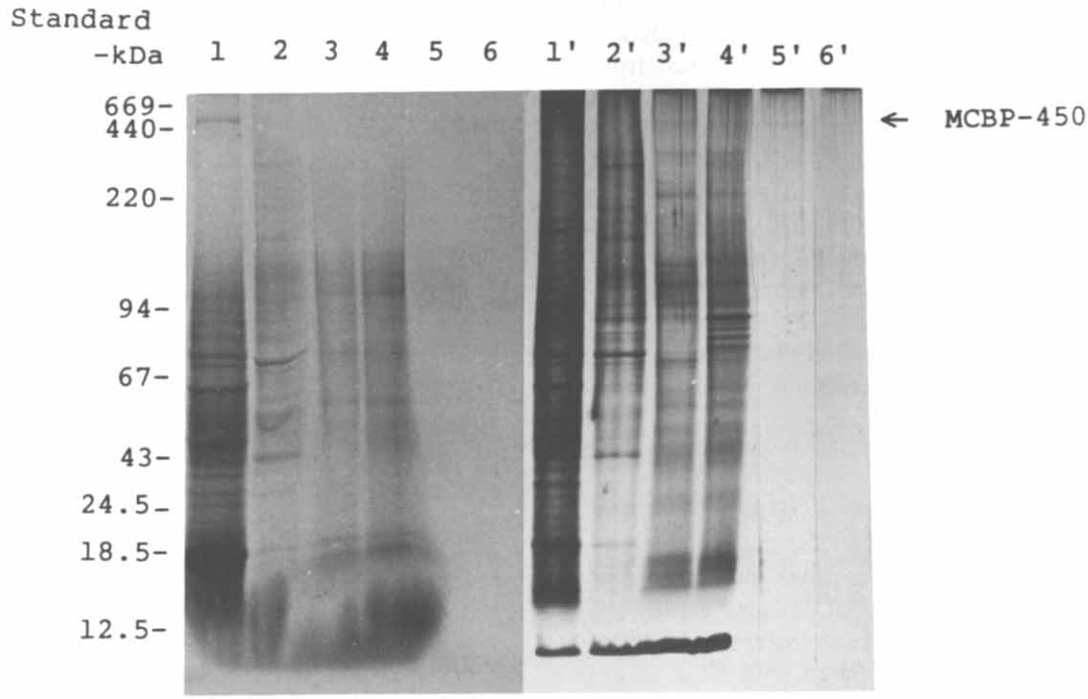

Fig. 5. Coomassie-brilliant-blue-stained (lanes 1–6) and silver nitrate stained (lanes 1′–6′) SDS-PAGE (5–15%) of samples at different steps of MCBP-450 purification. 1 and 1′, crude microsomal fraction of the ABRM; 2 and 2′, plasma membrane-enriched fraction; 3 and 3′, fractions 17–20 in Step 2; 4 and 4′, fractions in Step 3; 5 and 5′, fraction 29 in Step 4; 6 and 6′, fraction 34 in Step 4.

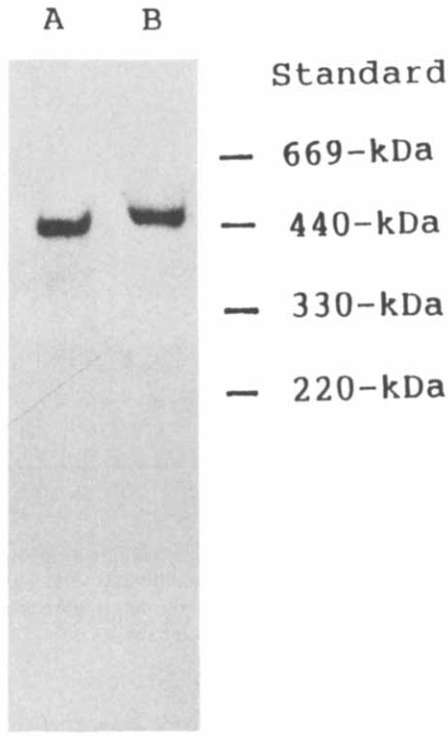

Fig. 6. Ca^{2+}-dependent change in mobility of MCBP-450. A, SDS-PAGE of MCBP-450 (10 μg) in the presence of Ca^{2+} (1 mM $CaCl_2$). B, SDS-PAGE of MCBP-450 (10 μg) in the absence of Ca^{2+} (2 mM EGTA). Note that MCBP-450 shows faster mobility in the presence of Ca^{2+} than in its absence.

Yields of MCBP-450 in different purification steps are summarized in Table I. MCBP-450 in fraction 34 in Step 4 was purified to a final yield of 0.05% from the plasma membrane-enriched fraction. Considering the yield of the plasma membrane-enriched fraction from ABRM, about 75 μg of MCBP-450 was obtained from 100 g wet mass of the ABRM. The following characterization of MCBP-450 was performed using the samples of fraction 34 obtained in step 4.

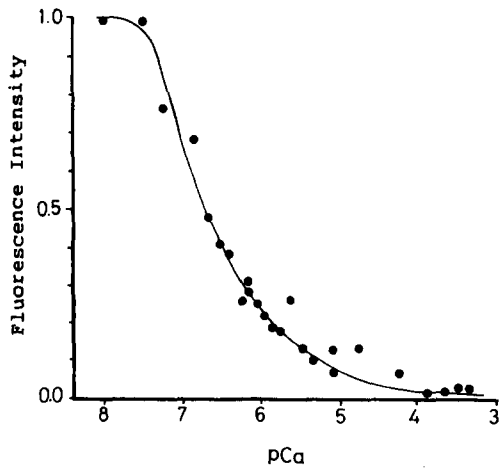

Fig. 7. Dependence of tryptophan fluorescence of MCBP-450 on pCa. Fluorescence intensity is expressed relative to the maximum value at pCa 8. MCBP-450 (0.7 mg/ml) in a solution containing 60 mM KCl, 2 mM $MgCl_2$ and 10 mM Pipes (pH 6.8).

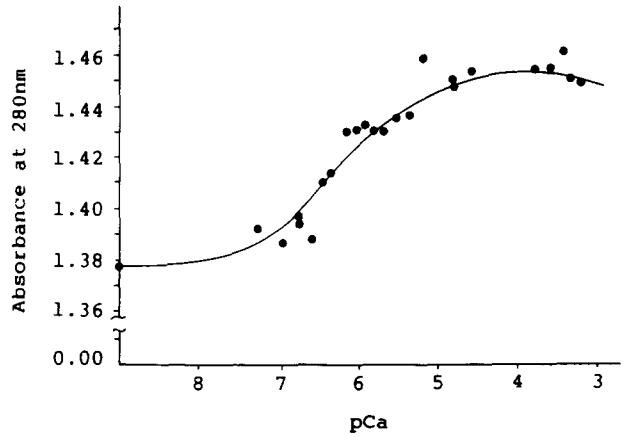

Fig. 8. Dependence of UV absorption (at 280 nm) of MCBP-450 on pCa. Measurements were made in a solution containing of MCBP-450 (0.7 mg/ml), 60 mM KCl, 2 mM $MgCl_2$ and 10 mM Pipes (pH 6.8).

Ca^{2+}-dependent change in mobility of MCBP-450

Fig. 6 shows ^{45}Ca autoradiographs of MCBP-450 transferred to nitrocellulose membrane after SDS-PAGE in the absence (2 mM EGTA) and presence of Ca^{2+} (1 mM $CaCl_2$). MCBP-450 had different mobilities in the absence and presence of Ca^{2+}, as has been the case for calmodulin [12,26]. MCBP-450 exhibited the mobility corresponding to 450 kDa without Ca^{2+} and the mobility corresponding to 430 kDa with Ca^{2+}.

Ca^{2+}-dependent changes in spectroscopic properties of MCBP-450

To obtain information about Ca^{2+}-dependent conformational changes of MCBP-450, its tryptophan fluo-

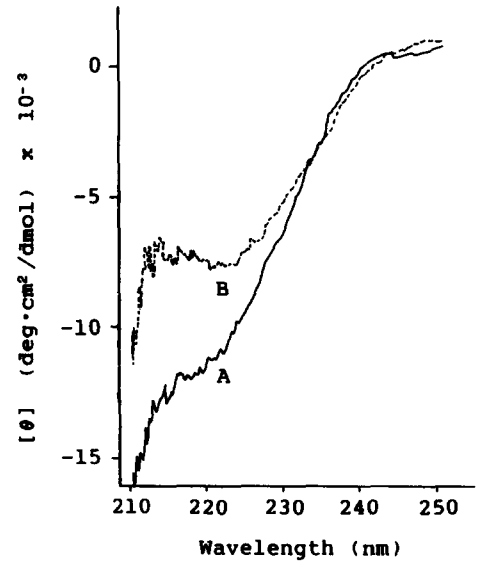

Fig. 9. Ca^{2+}-dependent change in CD spectrum of MCBP-450. A, CD spectrum of MCBP-450 (0.25 mg/ml) at pCa 3. B, CD spectrum of MCBP-450 (0.25 mg/ml) at pCa 7.

rescence, UV absorption and CD spectra were measured. The decrease of tryptophan fluorescence with decreasing pCa from 9 to 3 was half-maximal near pCa 7 (Fig. 7), while the increase of UV absorbance (at 280 nm) with decreasing pCa from 9 to 3 was the half-maximal around pCa 6.5 (Fig. 8). The far-UV CD spectrum of MCBP-450 at pCa 3 was different from that at pCa 7 (Fig. 9). The mean residue ellipticity at 222 nm was -8250 deg cm^2/dmol at pCa 7, and $-11\,550$ deg cm^2/dmol at pCa 3, indicating that the α-helix content of MCBP-450 is 33% at pCa 3 [27].

Ca^{2+}-binding properties of MCBP-450

Fig. 10 shows the mode of Ca^{2+}-binding to MCBP-450. The molecular mass of MCBP-450 is assumed to be 450 kDa. At pCa 3, MCBP-450 bound about 38 mol of Ca^{2+} per mol of MCBP-450, indicating the presence of a high-capacity Ca^{2+}-binding site. In addition, there was a low-capacity Ca^{2+}-binding site, which bound about 7 mol of Ca^{2+} per mol of MCBP-450. The Scatchard plot (inset) showed that Ca^{2+} bound to the low-capacity and high-capacity sites with association constants of $6 \cdot 10^5$ M^{-1} and $4 \cdot 10^4$ M^{-1}, respectively.

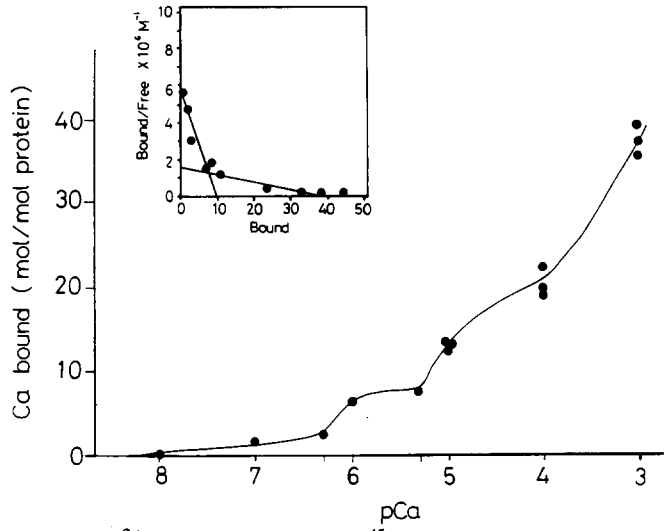

Fig. 10. Ca^{2+} binding to MCBP-450. ^{45}Ca binding to MCBP-450 was measured as described in Materials and Methods. The inset shows a Scatchard plot of Ca^{2+} binding data, indicating a low-capacity and a high-capacity binding site.

Amino-acid composition

The amino-acid composition of MCBP-450 is shown in Table II together with those of ABRM and scallop calmodulin [12,28], rabbit calsequestrin [29] and bovine calregulin [30]. The characteristic features of MCBP-450 are that it shows a high content of aspartic and glutamic acid (23.8% in mol%) and a high content of basic residues (lysine, histidine and arginine, 27%), indicating a high proportion of charged residues. Though a high content of aspartic and glutamic acid is also seen in *Mytilus* and scallop calmodulin, rabbit calsequestrin and bovine calregulin, none of them con-

TABLE II

Amino-acid composition of MCBP-450 and other Ca^{2+}-binding proteins

All values are mol%.

Amino acid [e]	*Mytilus* ABRM		Scallop calmodulin [b]	Rabbit calsequestrin [c]	Bovine calregulin [d]
	MCBP-450	Calmodulin [a]			
Aspartic acid	6.2	5.0	15.6	18.9	16.5
Threonine	4.5	7.3	8.2	2.8	3.7
Serine	12.5	6.3	3.4	3.6	3.7
Glutamic acid	17.6	15.0	17.7	18.4	17.7
Proline	0.0	3.0	1.4	5.1	6.4
Glycine	12.6	8.1	7.5	3.8	6.8
Alanine	5.8	7.9	6.8	6.6	4.6
Valine	3.4	6.0	4.8	6.9	4.2
Cysteine	0.0	0.0	0.0	0.8	0.5
Methionine	0.1	3.4	6.1	1.5	0.9
Isoleucine	2.6	4.8	5.4	5.1	4.4
Leucine	3.9	7.1	6.1	8.9	4.4
Tyrosine	1.3	1.0	0.77	1.5	3.5
Phenylalanine	2.5	5.3	6.1	6.1	4.6
Lysine	18.8	6.2	5.4	6.6	9.5
Histidine	3.4	0.8	0.7	1.8	2.0
Arginine	4.8	2.6	4.1	1.8	2.4

[a] From Mimura et al. [12].
[b] From Takagi et al. [28].
[c] From MacLennan and Wong [29].
[d] From Waisman et al. [30].
[e] Tryptophan was not determined.

tain a high content of basic residues. The sugar content of MCBP-450 as determined by the phenol-sulfunic acid method was about 9%.

Discussion

In agreement with the experimental results that *Mytilus* ABRM contains intracellulary stored activator Ca enough to fully activate the contractile system [5] and that activator Ca is localized at the inner surface of the plasma membrane, as well as in the SR of the ABRM [6], we have found a high-molecular-mass (450 kDa) Ca^{2+}-binding protein MCBP-450 in the plasma membrane-enriched and the SR membrane-enriched fractions of the ABRM by the method of detecting Ca^{2+}-binding proteins with ^{45}Ca autoradiography of proteins transferred to nitrocellulose membrane [17] (Fig. 1). In addition to MCBP-450, the plasma membrane-enriched fraction contained 17-kDa protein, while the SR membrane-enriched fraction contained 60-kDa and 17-kDa proteins. The 17-kDa protein may correspond to calmodulin which is also present in the ABRM [10]. On the other hand, the 60-kDa protein is likely to be calsequestrin which is present in the SR lumen of vertebrate skeletal muscle [29], since the apparent molecular mass of calsequestrin from various animals is reported to be around 60 kDa in the gel system used in the present study [31].

MCBP-450 and calsequestrin have some properties in common. They show a high content of aspartic and glutamic acid (Table II) and a high Ca^{2+}-binding capacity; about 38 mol of Ca^{2+} per mol of MCBP-450 (Fig. 10) and 40–50 mol Ca^{2+} per mol of calsequestrin [29]. The above analogy may be taken to indicate that, as with calsequestrin in the SR lumen of skeletal muscle, MCBP-450 serves as storage sites for activator Ca in the ABRM. In the intact ABRM, MCBP-450 found in the SR membrane-enriched fraction may be mainly present in the SR lumen to serve as Ca^{2+} reservoir, whereas MCBP-450 found in the plasma-membrane-enriched fraction to serve another kind of Ca^{2+} reservoir in the ABRM fibres, as evidenced by the Ca^{2+} accumulation at the inner surface of the plasma membrane demonstrated electron microscopically [6]. It seems possible that the Ca^{2+}-binding capacity of MCBP-450 is influenced by changes in potential difference across the plasma membrane; if membrane depolarization effectively reduced the Ca^{2+}-binding capacity of MCBP-450 at the plasma membrane, then it provides a mechanism of direct regulation of intracellular Ca^{2+} concentration by membrane potential changes. This interesting possibility should be studied in future.

MCBP-450, purified to electrophoretic homogeneity (Fig. 5), showed Ca^{2+}-dependent conformational changes as indicated by the Ca^{2+}-dependent change in mobility (Fig. 6), change in tryptophan fluorescence (Fig. 7), change in UV absorbance (Fig. 8) and change in CD spectrum (Fig. 9). The result that the dependence of tryptophan fluorescence and UV absorbance on pCa is most prominent around pCa 7 (Figs. 7 and 8) is also consistent with the view that MCBP-450 is actually involved in the regulation of contraction-relaxation cycle in the ABRM.

It would be of interest to detect Ca^{2+}-binding proteins in the plasma membrane-enriched fraction of other kinds of smooth muscles, since the accumulation of Ca^{2+} at the inner surface of the plasma membrane has been demonstrated in many kinds of vertebrate and invertebrate smooth muscle [7–9].

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