Volume 247, number 2, 377-380 FEB 07068 April 1989

Purification and characterization of Ca^{2+}/calmodulin-dependent actin-binding proteins from squid retina

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Received 20 February 1989

Ca^{2+}/calmodulin (CaM)-dependent actin-binding proteins (CABPs) of 92, 105, 120 and 135 kDa were purified from squid retina. These proteins were eluted from the CaM affinity column in a Ca^{2+}-dependent manner, and binding of the CABPs to F-actin was regulated by Ca^{2+}/CaM. Electron microscopic observations employing the low-angle rotary shadowing technique showed the CABP molecules to have granular shapes similar to the granular proteins associated with actin filaments in squid rhabdomeral microvilli. We have previously reported that these actin filaments are fragmented upon exposure to light [(1988) J. Cell Biol. 106, 1151-1160]. Since the intracellular Ca^{2+} concentrations of the invertebrate retina are elevated during the light illumination, these results indicate that the CABPs are directly associated with the actin filament in the microvilli of the squid photoreceptors. We therefore suggest that the CABPs may regulate the light-induced structural changes of the microvillar cytoskeleton.

Calmodulin; Actin-binding protein; Calmodulin-binding protein; (Photoreceptor microvillus, Squid retina)

1. INTRODUCTION

Squid rhabdomeral microvilli, which include the photoreceptor protein of rhodopsin in the plasma membrane, normally contain a central and longitudinal actin-based microfilament [2]. We have recently studied the cytoskeleton in dark-adapted and light-illuminated squid microvilli by electron microscopy combined with rapid freezing using liquid helium [1]. The surfaces of the actin filaments in dark-adapted microvilli were regularly associated with granular structures cross-linked to the rhodopsin-bearing plasma membrane through slender strands. Furthermore, upon exposure to light the granular components detached from the actin filaments, which then appeared to be fragmented and/or depolymerized. The retinal microvilli contain calmodulin (CaM) [6] and the intracellular Ca^{2+} concentration is elevated in light-illuminated invertebrate photoreceptor cells [3-5]. Taken together, these observations suggest that Ca^{2+} and Ca^{2+}-binding proteins might regulate the light-induced structural changes of squid microvilli.

These previous studies have motivated us to isolate and characterize the cytoplasmic proteins of squid rhabdomeral microvilli which can bind to Ca^{2+}, CaM and actin filaments.

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2. MATERIALS AND METHODS

2.1. *Materials*

Squid (*Doryteuthis bleekeri*) were kept alive in a small circular closed-system aquarium [7]. Eyes from freshly killed squid were immediately immersed in liquid N_2 and stored until use. The retinal tissue was isolated by dissection, and for each purification experiment 7.5 g tissue was homogenized in 7.5 ml buffer A [2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.5]. The homogenate was centrifuged at $100000 \times g$ for 60 min at 4°C. The supernatant (~10 ml) was used as a retinal extract. Its protein concentration was about 20 mg/ml.

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00145793/89/$3.50

Actin was isolated from rabbit skeletal muscle acetone powder according to Spudich and Watt [8]. Porcine brain CaM was isolated using a modification of a previously described method [11], and was thereafter coupled to CNBr-activated Sepharose 4B (Pharmacia). Ca^{2+}-dependent cyclic nucleotide 3′:5′-phosphodiesterase was prepared according to [10].

2.2. *Purification of squid retinal proteins*

Retinal Ca^{2+} and actin-binding proteins were purified from the squid extract using TSK gel-DEAE 5pw (Toyo-Soda) in a high-pressure liquid chromatography (HPLC) system (Beckman), followed by separation using a phenyl-Sepharose column or CaM-affinity chromatography. The purity of proteins was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as in [11]. Protein concentrations were determined according to [12].

2.3. *Sedimentation assay*

Interaction of CABPs with F-actin was analyzed by sedimentation assay. The reaction mixture of CABPs and F-actin was incubated for 60 min at 25°C in a solution of 100 mM NaCl, 1 mM $Mg(CH_3COO)_2$, 1 mM ATP and 10 mM Mes buffer (pH 6.5), in a final volume of 350 μl. The mixture was then centrifuged at $100000 \times g$ for 30 min at 25°C, and proteins recovered in the pellet or supernatant were analyzed by SDS-PAGE.

2.4. *Electron microscopic observation*

The molecular shapes of CABPs were studied by rotary shadowing electron microscopy, according to [13]. The CABP preparation (100 μg/ml) in 0.6 M NaCl, 50% glycerol, Mes buffer (pH 6.5) was sprayed onto freshly cleaved mica. We obtained negative images of the molecular shapes of the droplets on the mica using a Hitachi 11-DS electron microscope, following the procedures described [14].

3. RESULTS

3.1. *Presence of calmodulin and actin in squid retina*

Calmodulin has previously been purified from squid optic lobe [15,16]. The presence of CaM in the squid retina was experimentally verified, as follows: (i) 1 ml retinal extract (containing ~20 mg retinal proteins) was heated to 80°C, and after 3 min rapidly cooled on ice. Heat-stable proteins were collected in the supernatant after centrifugation at $100000 \times g$ for 30 min at 4°C. The supernatant was applied to a phenyl-Sepharose column in the presence of 1 mM Ca^{2+}. About 120 μg Ca^{2+}-binding proteins were eluted from the column in the absence of Ca^{2+} (presence of 3 mM EGTA). (ii) The electrophoretic mobility of the Ca^{2+}-binding protein was dependent on the presence and absence of Ca^{2+}. Both the mobility and its change accompanied by the presence and absence of Ca^{2+} revealed by SDS-PAGE were almost the same as those of porcine brain CaM with a molecular mass of 18 kDa (fig.1A). (iii) The squid Ca^{2+}-binding protein stimulated the Ca^{2+}-dependent phosphodiesterase activity in a Ca^{2+}-dependent manner, similarly to the porcine brain CaM (fig.1B).

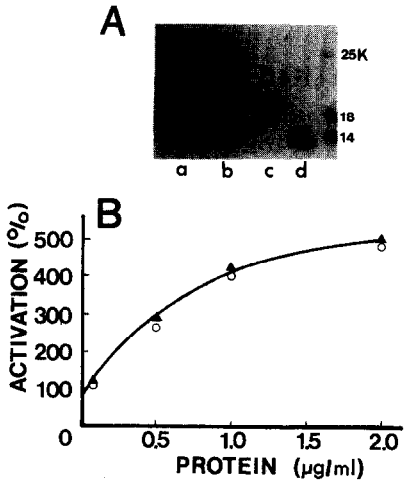

Fig.1. Verification of presence of calmodulin in squid retina. (A) Ca^{2+}-dependent change in electrophoretic mobility of the squid Ca^{2+}-binding protein from retina (lanes a,b) and of porcine brain CaM (lanes c,d), on 12.5% SDS-polyacrylamide gel. Applied samples contained 1 mM EGTA (lanes a,c) or 0.5 mM Ca^{2+} (lanes b,d). The extreme right lane illustrates standard peptides for molecular mass markers. (B) Dependence of activation of Ca^{2+}-dependent cyclic nucleotide 3′:5′-phosphodiesterase upon protein [squid retinal Ca^{2+}-binding protein (○) or porcine brain CaM (△)] concentration. The protein was incubated with a 0.5 μM cAMP containing reaction solution at 30°C for 15 min and the activity was assayed.

The retinal extract was applied on a DNase I affinity column [17] and then eluted with buffer A containing 6 M urea. About 450 μg actin was obtained from 1 ml retinal extract.

3.2. *Purification and characterization of Ca^{2+}/calmodulin-dependent actin-binding proteins (CABPs)*

To isolate CaM-binding proteins, the squid retinal extract was applied to the TSK gel-DEAE 5pw column using the Beckman HPLC system. The flow rate was usually 1 ml/min. The flow-through fraction in buffer A containing 1 mM $CaCl_2$ was applied to the CaM-affinity column. CaM-binding proteins were eluted with buffer A

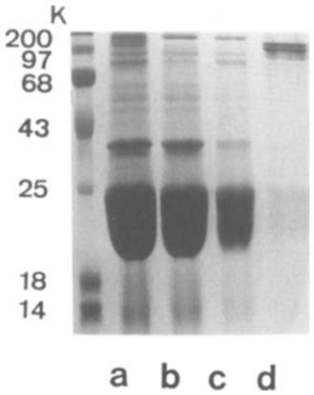

Fig.2. Purification of Ca^{2+}/calmodulin-binding proteins from DEAE flow-through fraction obtained from the squid retinal extract. The compositions were determined by 12.5% SDS-PAGE. The DEAE flow-through fraction (lane a) was applied to CaM-Sepharose equilibrated with buffer A containing 1 mM $CaCl_2$ and 100 mM NaCl. The column was then washed with the same solution (buffer A containing 1 mM $CaCl_2$ and 100 mM NaCl). The compositions of the proteins thus eluted are shown in lanes b,c. The peptide compositions of the proteins eluted with buffer A containing 3 mM EGTA and 100 mM NaCl are obtained in lane d, illustrating CaM-binding proteins.

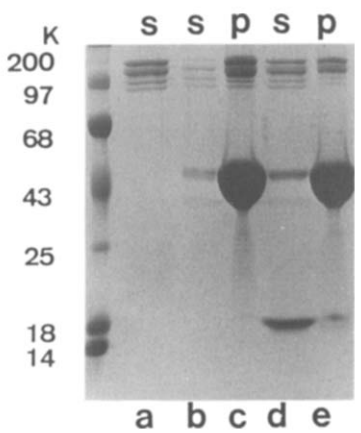

Fig.3. Polypeptide compositions of proteins obtained after sedimentation assay for reaction mixtures among the calmodulin-binding proteins, calmodulin and F-actin. The CaM-binding proteins (30 μg) were incubated with F-actin (100 μg) and CaM (20 μg) in the presence and absence of 1 mM $CaCl_2$. Sedimentation assay was performed by centrifugation at 100000 × g for 30 min at 25°C. Lanes: a, supernatant of mixture of CaM-binding proteins (without F-actin and CaM); b,c, supernatant and pellet of mixture of CaM-binding protein with F-actin (without CaM), respectively; d,e, supernatant and pellet of mixture of CaM-binding proteins, CaM and F-actin in the presence of 1 mM $CaCl_2$, respectively. 10% SDS-PAGE was used for the composition assay. The extreme left lane shows peptides for standard molecular masses.

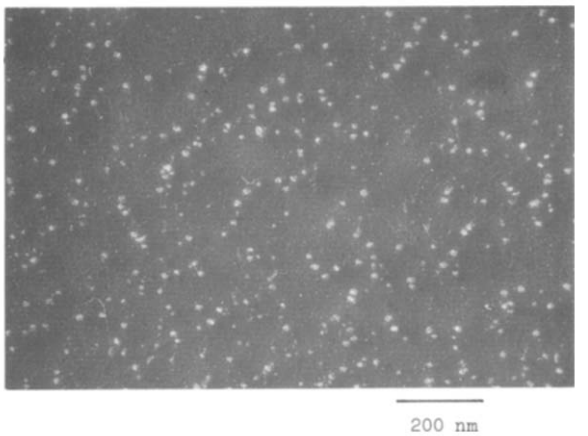

Fig.4. Molecular morphology of the Ca^{2+}/calmodulin-dependent actin-binding proteins (CABPs) in 0.6 M NaCl solution in the rotary-shadowed preparation. The CABP molecules appear with a monomeric granular shape of 25 nm diameter.

containing 3 mM EGTA. Four different CaM-binding proteins were thus obtained, their molecular masses estimated by SDS-PAGE being 135, 120, 105 and 92 kDa, respectively (fig.2). About 60 μg proteins were recovered from 1 ml squid retinal extract.

Interaction of the CaM-binding proteins with F-actin was studied via the sedimentation assay (fig.3). The proteins cosedimented with F-actin. In the presence of both Ca^{2+} and CaM, the binding ability of the proteins to F-actin was inhibited. However, the binding ability was not influenced by CaM in the absence of Ca^{2+} (not shown). Therefore, the CaM-binding proteins could bind to F-actin, and the F-actin-binding ability was inhibited by CaM in a Ca^{2+}-dependent manner. These proteins are thus Ca^{2+}/CaM-dependent actin-binding proteins (CABPs).

The morphology of the isolated CABP molecules was studied by low-angle rotary shadowing electron microscopy. All of the CABP molecules displayed a monomeric granular shape (fig.4). Regarding this shape, the CABP molecules resemble the granular actin-binding protein regularly arrayed along the helix of a single actin filament in the center of the squid rhabdomeral microvillus [1].

4. DISCUSSION

We have isolated the Ca^{2+}/CaM-dependent

actin-binding proteins (CABPs) from squid retina. The binding of CABPs to F-actin was inhibited by CaM in a Ca^{2+}-dependent manner. The squid retinal extract (20 mg/ml) contained $\sim 0.5\ \mu M$ CABPs, $\sim 10\ \mu M$ actin and 6–7 μM CaM. The molecular shape of CABP resembled the granular protein associated with actin filament which we have previously observed [1]. These findings suggest that CABPs may be associated with actin filaments in squid rhabdomeral microvilli, possibly regulating the light-induced structural changes of the microvillar cytoskeleton in a Ca^{2+}-dependent manner.

It should be noted that two other F-actin binding polypeptides of 200 and 145 kDa were included in the squid retinal extract (Arai et al., in preparation). The binding of these proteins to F-actin was not regulated by CaM in a Ca^{2+}-dependent manner. Saibil [2] suggested that the 145 kDa protein was involved in the actin-based cytoskeleton. According to our preliminary study, CABPs did not cause gelation, bundling or fragmentation of F-actin, or polymerization inhibition of G-actin. Caldesmon, which also binds to F-actin in a Ca^{2+}/CaM dependent manner, is known to have a modulatory function. This protein gives rise to Ca^{2+} sensitivity for the binding of other actin-binding proteins, such as myosin, which bind to F-actin without Ca^{2+} dependency [18]. These facts indicate that the CABPs may also be modulatory proteins producing Ca^{2+} sensitivity for the binding of the 200 and 145 kDa proteins. The detailed characterization and elucidation of the function of these proteins await future studies.

Acknowledgements: We express our sincere thanks to Drs Sachiko and Shoichiro Tsukita, Tokyo Metropolitan Institute of Medical Science, for experimental guidance and kind help in rotary shadowing electron microscopy.

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