CALCIUM-BINDING MODULATOR PROTEIN FROM THE UNFERTILIZED
EGG OF THE SEA URCHIN ARBACIA PUNCTULATA

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ABSTRACT
We have purified and partly characterized a calcium-binding protein from the
unfertilized egg of the sea urchin Arbacia punctulata. This protein closely
resembles the calcium-binding modulator protein of bovine brain in its molecular
weight, electrophoretic mobility, amino acid analysis, and peptide map. It
activates bovine brain phosphodiesterase in the presence of calcium but has no
effect on the phosphodiesterase of the Arbacia egg.

Densitometric scanning of acrylamide gels of Arbacia egg homogenates shows
the modulator protein to represent 0.1% of the total protein of the egg.

At 10^-4 M free calcium, the protein binds four calcium ions per 17,000-dalton
molecule.

We have used a column of rabbit skeletal muscle troponin-I covalently coupled
to Sepharose 4B as an affinity column to selectively purify the Arbacia egg
calcium-binding protein. This column has also been used to purify bovine brain
modulator protein and may prove of general use in isolating similar proteins from
other sources. The technique may be particularly helpful when only small
quantities of starting material are available.

KEY WORDS sea urchin · calcium-binding
modulator protein · phosphodiesterase ·
affinity chromatography

The calcium-binding modulator protein was ini-
tially identified as an activator of cAMP phospho-
diesterase in mammalian brain (5, 21). It has since
been isolated from a number of vertebrate tissues
(6, 9, 10, 17, 24, 25, 37, 42), from vertebrate
and invertebrate sperm (20), and from extracts of
the earthworm Lumbricus terrestris (40). Its pres-
ence has also been indirectly implicated in sea
urchin eggs (20) and a number of other vertebrate
(33) and invertebrate (39) tissues on the basis of
the ability of boiled tissue extracts to activate
brain (33) and heart (39) cAMP phosphodiester-
ases. In addition to activating cAMP phosphodi-
esterase, the modulator protein may also have a
role in a number of other calcium-regulated pro-
cesses (4, 9, 13, 19, 28, 45).

The apparent ubiquity of the modulator protein
and its evidently highly conserved structure indi-
cate that its function or functions are of funda-
mental importance in the calcium regulation of events
within the cell.

In the egg of the Arbacia and other species,
large fluxes in free calcium levels have been
observed after fertilization (2, 8, 29, 31, 32, 35),
and it has been suggested that this intracellular
release of calcium may be the universal factor
promoting the activation of metabolism in the egg
(34, 35). The role of the modulator protein in
regulating the processes occurring during and after
activation of the egg may therefore be of particu-
lar significance in the control of cell growth,
division, and differentiation. We have turned to
the egg of the sea urchin Arbacia punctulata to
investigate the properties and function or func-
tions of the modulator protein in this cell and to
determine any developmental changes in these
functions. In the present study, we describe the identification, purification, and partial characterization of the modulator protein from this source.

MATERIALS AND METHODS

Arbacia eggs were obtained by injecting 0.5 M KCl into the coelomic cavity of the female. Collected eggs were washed in seawater, and the pH was adjusted to 5.0 with 0.1 M HCl to remove the gelatinous coat. After 2 min, the eggs were spun down at 200 g for 30 s. The eggs were resuspended twice in seawater and stored at -20°C.

Preparation of Phosphodiesterase and Calcium-Binding Modulator Protein from Unfertilized Arbacia Eggs

All procedures were carried out at 4°C unless otherwise indicated. Eggs were homogenized in 3 vol of 20 mM Tris-HCl, 1 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.8, in a Sorvall omnimix blender (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.) until disrupted. The homogenate was then spun at 100,000 g for 30 min. The supernate was retained and the pellet was re-extracted in 3 vol of the same buffer and centrifuged again at 100,000 g for 30 min. The supernates were pooled, and an equal volume of saturated ammonium sulfate solution was slowly added with continuous stirring. Precipitating material was removed by centrifuging at 17,000 g for 30 min. The precipitate was discarded, and the supernate was adjusted to 70% saturation by slow addition with stirring of 13.5 g of solid ammonium sulfate/100 ml of solution. The material was allowed to stand for a further 30 min after addition of the ammonium sulfate and then spun at 17,000 g for 30 min. The precipitate was redissolved in a minimum of 20 mM Tris-HCl, 1 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.8, dialyzed against the same buffer (2 × 6 liters) for 24 h, and subsequently spun at 50,000 g for 30 min. The clarified solution was then applied to a 2.5 × 7.5 cm column of DE-52 cellulose equilibrated in the same buffer for 12 h. The solution was clarified by centrifuging at 50,000 g for 30 min, and the supernate was then applied to a 2.5 × 7.5 cm column of rabbit skeletal muscle troponin-I covalently coupled to Sepharose 4B (2 mg troponin-I/g packed Sepharose) (18) equilibrated in the same buffer. Unbound material was washed through with 500 ml of buffer, and the modulator protein eluted at 0.3 M total NaCl and was similarly dialyzed and freeze-dried.

Affinity Chromatographic Purification of Arbacia Egg Modulator Protein and Bovine Brain Modulator Protein

Arbacia egg modulator protein and the bovine brain protein obtained from salt gradient elution of DE-52 cellulose columns (see above) were purified to homogeneity using the same affinity chromatographic procedure. The crude freeze-dried protein was dissolved to ~1 mg/ml in 20 mM Tris-HCl, 1 mM CaCl₂, 15 mM 2-mercaptoethanol, pH 7.8, and dialyzed against 6 liters of the same buffer for 12 h. The solution was clarified by centrifuging at 50,000 g for 30 min, and the supernate was then applied to a 2.5 × 7.5 cm column of rabbit skeletal muscle troponin-I covalently coupled to Sepharose 4B (2 mg troponin-I/g packed Sepharose) (18) equilibrated in the same buffer. Unbound material was washed through with 500 ml of buffer, and the modulator protein eluted with 20 mM Tris-HCl, 5 mM ethylene glycol-bis(β-aminoethyl ether)N,N',N'-tetraacetate (EGTA), 15 mM 2-mercaptoethanol, pH 7.8. The column flow rate was maintained at 100 ml/h. The eluate was monitored for the presence of the modulator protein by alkaline gel electrophoresis of samples of the fractions collected. Under the conditions described, some modulator protein usually also appeared in the unbound material; this could be partly recovered by recycling the column with a calcium buffer wash and passing the unbound material through again. Diminishing returns usually made more than two passes through the column impractical. Fractions containing the protein were pooled, dialyzed against 6 liters of 2 mM Tris-HCl, 15 mM 2-mercaptoethanol for 12 h, and then freeze-dried.

Electrophoresis

Alkaline gel electrophoresis in the presence of 8 M urea was performed using 8% acrylamide gel slabs for
qualitative studies and gel cylinders for quantitative studies. A continuous buffer system of 25 mM Tris-80 mM glycine, pH 8.6, was employed (15). Samples electrophoresed in the presence of calcium contained 0.1 mM CaCl₂, those in the absence of free calcium contained 5 mM EGTA. For qualitative studies, the gel slabs were stained in naphthol blue-black (0.6% wt/vol, 5:3:1 water:methanol:acetic acid) for 30 min and destained electrophoretically in 7% acetic acid. For quantitative studies, the gel cylinders were stained in fast green (Fisher FCF, Fisher Scientific Co., Pittsburgh, Pa.) (0.25% wt/vol, 5:5:1 methanol:water:acetic acid) for 12 h at room temperature and destained in 7% acetic acid for 24 h at 50°C in a Bio-Rad 170 diffusion destainer (Bio-Rad Laboratories, Richmond, Calif.). The gels were scanned at 650 nm in a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) using a linear transport adaptor and a Beckman DU monochromator (Beckman Instruments, Inc., Fullerton, Calif.).

Sodium dodecyl sulfate gel electrophoresis was carried out on 10 or 15% acrylamide gels (as indicated) using a continuous buffer system of 0.1 M sodium phosphate, pH 7.0 (43). Gels were stained in Coomassie Brilliant Blue R (0.25% wt/vol, 5:4:1 methanol:water:acetic acid) and diffusion destained in 10% methanol, 10% acetic acid.

**Calcium Binding**

Calcium binding to the Arbacia egg modulator protein at 10⁻⁸ M calcium was determined by equilibrium dialysis. The protein sample (0.1 mg/ml) was first dialyzed for 12 h against 10 mM imidazole, 100 mM NaCl, 1 mM EGTA, pH 7.0, then 12 h against the same buffer but containing 0.1 mM EGTA and, finally, for 48 h against 500 vol of 10 mM imidazole, 100 mM NaCl, 0.1 mM *CaCl₂* (3.2 μCi/mol). At the end of this time, protein and radioactivity determinations were made.

**Peptide Mapping**

Samples of freeze-dried modulator protein were dissolved in 0.1 M NH₄HCO₃, 0.1 mM EGTA to give 1 mg protein/ml and dialyzed against the same solution for 12 h at 37°C. The digest was subsequently dried under a stream of N₂ and redissolved in water to give 10 mg/ml protein. 5 μl of the redisolved digest was applied to a silica gel G plate (0.25 mm thickness, 20 × 20 cm) and subjected to thin-layer chromatography in n-propanol-30% NH₃OH (7:3 vol/vol), and thin-layer electrophoresis, in pyridine:acetic acid:water (1:10:439 vol/vol) pH 3.5, as described by Stephens (36). Spots were visualized by spraying with 0.025% fluorescamine in acetone (10 ml/plate) and stabilized by spraying with 5% triethylamine in acetone (10 ml/plate). The map was photographed under UV light using a UV and yellow (K2) filter with a Polaroid 250 Land camera and type 665 film. The negative was rephotographed and the resulting transparency was printed on high contrast paper (36).

**Phosphodiesterase Measurement**

Phosphodiesterase activity was measured using the method of Boudreau and Drummond (3). For determining activity at differing calcium ion concentrations, 1 mM EGTA was included in the incubation mixture and calcium was added to give the required concentration of free calcium ions. Free calcium levels were calculated assuming a Kd for Ca-EGTA of 2 × 10⁻⁸ at pH 7.5.

**Protein Determination**

Protein was measured by total N determination using microesslerization, assuming that protein samples contain 16% N. In the presence of nitrogen-containing buffers or reagents, protein was estimated using the method of Lowry et al. (26) with a solution of bovine serum albumin as a standard. For quantitation of the modulator protein in Arbacia eggs using densitometric gel scanning, the total protein content of the homogenized whole egg samples applied to the gel was determined. 5 M urea homogenates of whole Arbacia eggs were dialyzed against 2 × 1,000 vol of 8 M urea to remove free amino acids. Aliquots of the dialysate (also used for gel samples) were diluted by known amounts (>100 × vol/vol) in 0.1 M NaOH, keeping protein in solution while reducing the urea concentration below 0.5% (below this level, urea does not interfere with the Folin reaction [26]). The protein concentrations of the diluted samples were determined by the method of Lowry et al. (26). The protein value determined by this method was taken as a measure of the total protein content of the egg.

**RESULTS**

In common with the modulator proteins from bovine brain (1, 11) and chicken gizzard (17), the Arbacia egg modulator shows calcium-dependent mobility changes on alkaline urea gel electrophoresis. In urea extracts of the whole egg, the modulator interacts with one or more other proteins in the presence of calcium to form a protein complex. On electrophoresis, this complex migrates slowly and no separate modulator band is evident (Fig. 1 b). In the presence of EGTA, however, the complex dissociates and the modulator is apparent as a distinct rapidly migrating band (Fig. 1 a). That these interactions occur even in the presence of high urea concentrations suggests a remarkable structural stability in the modulator protein and in the site to which

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it binds. This stability is similar to that observed in the structurally related troponin-C subunit of vertebrate striated muscle which undergoes similar calcium-dependent mobility changes on alkaline urea gel electrophoresis (15). In vertebrate striated muscle, the protein with which troponin-C interacts in the presence of calcium is the troponin-I subunit. This interaction is stable in up to 8 M urea, providing calcium is present to $10^{-4}$ M (15).

The structural similarity of modulator proteins to troponin-C (38) enables them to mimic troponin-C in binding to troponin-I in the presence of calcium (1, 11, 17). We have employed this modulator-troponin-I interaction in an affinity chromatographic purification of the modulator protein from the egg of the Arbacia and have found the technique to be applicable to the preparation of modulator from bovine brain, and chicken gizzard (17). A preliminary partial purification of the modulator by ammonium sulfate fractionation and diethylaminoethyl (DEAE) cellulose chromatography separates the modulator from the mass of the other proteins in the tissue and particularly from those with which it would otherwise interact in the presence of calcium. The partially purified modulator preparation is then passed over the troponin-I-Sepharose matrix (18) in the presence of calcium. The modulator protein now binds to the troponin-I without interference from other interacting proteins. Proteins not binding to the troponin-I are washed through, and the modulator is subsequently dissociated from the troponin-I and eluted by reducing the free calcium ion concentration with 5 mM EGTA.

The modulator protein prepared from the unfertilized Arbacia egg by this method is single banded on SDS gel electrophoresis (Fig. 2C), having an apparent mol wt of 17,000 daltons. The Arbacia egg protein is indistinguishable from bovine brain modulator on SDS or alkaline gel electrophoresis. It is also indistinguishable from rabbit skeletal muscle troponin-C on the alkaline gel system but migrates slightly faster than troponin-C on 15% acrylamide SDS gels (Fig. 2B).

Purified Arbacia egg modulator protein undergoes a small calcium-dependent mobility change when electrophoresed on its own on the alkaline gel system (Fig. 1 c and d). A similar change is observed with pure troponin-C from rabbit muscle (Fig. 1 e and f) and this appears to reflect the calcium-induced conformational change in the protein such that in the presence of calcium the protein has a smaller Stokes radius and therefore migrates slightly more rapidly (15).

The quantity of the modulator protein present in the Arbacia egg was determined by densitometry.
metric scanning of alkaline urea gels of samples of whole *Arbacia* egg homogenate containing known amounts of protein. Gels were run in the presence of EGTA at a range of sample sizes. Because available quantities of pure *Arbacia* egg modulator are limited, a dye binding study with known amounts of the pure *Arbacia* protein has not yet been made. We have therefore assumed, in our calculations, that the dye-binding constant for the *Arbacia* egg modulator is the same as that of the chemically similar rabbit striated muscle troponin-C. The modulator protein was found to represent 0.1 (±0.02)% of the total protein of the egg.

Amino acid analysis and peptide mapping of tryptic peptides of the *Arbacia* egg modulator show it to bear a very close resemblance to the modulator of bovine brain (Table I and Fig. 3).

Preliminary calcium-binding studies carried out at 10^{-4} M calcium show that *Arbacia* egg modulator binds 3.8 ± 0.11 mol of calcium per mole of protein at this free calcium ion concentration, assuming a mol wt for the modulator of 17,000. This is similar to the value for the binding of calcium to bovine brain modulator (44).

**DISCUSSION**

The troponin-I-Sepharose 4B affinity column was originally developed as a means of purifying troponin-C from vertebrate muscle sources (18). It was subsequently used for the purification of "troponin-C-like" proteins from a number of other tissues (12, 14, 16), although more recent studies have indicated that these troponin-C-like proteins correspond to modulator proteins (11, 17). The present study has utilized the troponin-I affinity column to purify modulator protein from the unfertilized egg of the *Arbacia* and, for comparative purposes, from bovine brain. Identifying a protein purified on the affinity column as a troponin-C or a modulator protein depends on a number of properties of the protein. Unambiguous identification of a troponin-C is not simple, because its functional property, namely to combine with troponin-I, troponin-T, and tropomyosin to sensitize actomyosin ATPase activity to calcium, is mimicked by modulator protein (1, 8). In the present study, we have designated the *Arbacia* egg protein as a modulator because it activates bovine brain cAMP phosphodiesterase, which troponin-C does very poorly (10), and otherwise resembles the brain modulator protein in its electrophoretic mobility, calcium binding, and peptide map. The presence of a single residue of trimethyllysine is apparently also a characteristic of modulator proteins; however, we have not yet made a determination of the trimethyllysine content of the *Arbacia* protein.

Complications with the use of the troponin-I affinity column technique will arise when tissues are studied which contain significant amounts of both modulator and troponin-C. Under these circumstances, competition will occur for binding

**TABLE I**

| Amino Acid Analysis | Bovine brain modulator protein |
|---------------------|-------------------------------|
| *Arbacia* egg Ca-binding protein | *Arbacia* egg Ca-binding protein |
| Asx | 22.5 | 23 |
| Thr | 11 | 12 |
| Ser | 9.1 | 4 |
| Glx | 25.8 | 27 |
| Pro | 3.5 | 2 |
| Gly | 16.8 | 11 |
| Ala | 12.5 | 11 |
| Cys | 0 | 0 |
| Val | 8.5 | 7 |
| Met | 7.4 | 9 |
| Ile | 7.6 | 8 |
| Leu | 9.6 | 9 |
| Tyr | 1.4 | 2 |
| Phe | 7.8 | 8 |
| Lys | 6.8 | 8§ |
| His | 1.5 | 1 |
| Arg | 5.7 | 6 |

* Moles/mole calculated assuming a mol wt of 17,000. Average of two analyses.
† From sequence data (38).
§ Includes 1 mol of trimethyllysine.
to the troponin-I, and the nature of the material bound will depend on the relative proportions of the troponin-C and modulator present and on their relative affinities for troponin-I.

Our peptide mapping studies indicate that the modulator protein purified from the Arbacia egg resembles more closely the bovine brain modulator than does the protein isolated by Waisman et al. (40) from the earthworm Lumbricus terrestris. Although the modulators from the two invertebrates appear to have certain differences, in calcium binding as well as peptide structure, they share the common feature that both of them are able to activate bovine brain cAMP phosphodiesterase but are unable to activate the cAMP phosphodiesterase of the tissues from which they were derived. This observation has also been made in studies of the modulator and phosphodiesterase of the electric organs of the electric eel (6, 7) and of the torpedo fish (unpublished observation). It is possible that the phosphodiesterases of these tissues have, in some way, been rendered insensitive to the modulator by the preparative techniques used. However, in the case of the Arbacia egg this seems unlikely because, in accordance with the results of Nath and Rebhun (30), we find that the cAMP phosphodiesterase of the tissues from which they were derived is not activated by the modulator.
phosphodiesterase activity of Arbacia eggs, even of gently disrupted fresh eggs, is calcium-insensitive under conditions where endogenous modulator would be expected to render the enzymic activity calcium-sensitive. The functional role of the modulator in the unfertilized Arbacia egg at least, therefore appears unlikely to be one of regulating phosphodiesterase activity. In several other tissues, modulator proteins have been found to be able to regulate a number of processes other than phosphodiesterase; these processes include adenylate cyclase activity (4), myosin light chain kinase activity (9, 45), membrane (Mg$$^{2+}$$-Ca$$^{2+}$$) ATPase (13, 19), and tubulin polymerization (28). The modulator protein is also able to substitute for tropinin-C in troponin-tropomyosin regulation of actomyosin ATPase (1, 10). (Both actin [22] and myosin [27] are present in the Arbacia egg.) It is evident that many of these are possible functions for the modulator in the Arbacia egg. Determining which proteins in the egg will interact with the modulator may provide valuable information on the modulator’s functional role in this tissue. The ability of one or more proteins to bind to the modulator on alkaline gel electrophoresis in the presence of calcium suggests that these may be the target proteins for the modulator in this cell. The characterization of these proteins, which may correspond to modulator-binding proteins of other tissues (23, 41), and a study of their interaction with the modulator will provide further insight into the modulator’s functional role in the calcium regulation of events occurring during and after fertilization.

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