Characterization of Calmodulin and Calmodulin Isotypes from Sea Urchin Gametes*

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Procedures were developed for the purification of calmodulin from the eggs and from the sperm of the sea urchin Strongyllocentrotus purpuratus and Arbacia punctulata. Two forms of calmodulin were isolated from A. punctulata eggs, one from each of the other three sources. All five calmodulins are similar to vertebrate calmodulin as judged by their activation of bovine brain cyclic nucleotide phosphodiesterase, calcium-dependent interaction with troponin I and with chlorpromazine, increased anode mobility on sodium dodecyl sulfate gels in the presence of calcium, cross-reactivity with anti-vertebrate calmodulin antibodies and amino acid compositions including the presence of a single residue of trimethyllysine.

The two forms of calmodulin from A. punctulata eggs, calmodulin A and calmodulin B, can be distinguished from one another on the basis of DEAE-cellulose chromatography, amino acid analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence or absence of calcium and competitive radioimmunoassays. There is no evidence that either form is a proteolytic product of the other.

Calmodulin is the prototype of calcium-modulated proteins. It is present in the cytosol of all (or most) cells of all (or most) eukaryotes (see review by Klee et al., 1980; New York Academy of Sciences, Vol. 356, 1980). Unlike other calcium-modulated proteins such as the myosin light chains, troponin Cs, and parvalbumins, neither tissue-specific nor species-specific isotypes of calmodulin have been found. Although differences in the properties of calmodulin from vertebrates, plants, or invertebrates have been reported, only a single calmodulin from one another on the basis of DEAE-cellulose chromatography, amino acid analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence or absence of calcium and competitive radioimmunoassays. There is no evidence that either form is a proteolytic product of the other.

Calmodulin has evolved very slowly. Of 148 residues, there are at most only seven differences in amino acid sequence between calmodulin from bovine brain (Watterson et al., 1980b) and that from the marine invertebrate, Renilla reinhardii (Jamieson et al., 1980). This is consistent with the fact that in contrast to the specialized roles of other calcium-modulated proteins, calmodulin interacts in a calcium-dependent and -specific manner with at least 15 different proteins.

We are studying the distribution and the regulatory functions of calmodulin during fertilization and early development.

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‡ Portions of this paper (including “Materials and Methods,” some data for “Results,” Figs. 1 and 2, and analysis of some of the data) have been submitted for publication in “Results.”
Calmodulin from Sea Urchin Gametes

RESULTS

Purification of Calmodulin from S. purpuratus Eggs

Shedding of eggs from S. purpuratus was induced by injecting 0.5 M or 0.5 M KCl into the ovary cavity via a cannula. The seawater was replaced with seawater-free sea water (Marine Biological Laboratory, Piscataway, N.J.) saturated with 0.1 M NaCl, using a Marline blender (B. Braun, Melsungen, West Germany) and stirred at 4°C for 15 min. The supernatant was adjusted to a pH of 7.0, stirred and centrifuged as above. The resulting pellet was resuspended in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1 mM CaCl₂, and dialyzed overnight against two changes of the same buffer. The dialysate was clarified by centrifugation in a Microfuge at 15,000 × g for 15 min. The clarified supernatant was then fractionated on a column of troponin I-Sepharose 4B as described above. The resulting protein was resuspended in about 1 ml of an appropriate buffer and applied to a 2-10% deoxycholate gradient (Pharmacia, Uppsala, Sweden). The void volume, which contained calmodulin, was collected and used accordingly.

Purification of Calmodulin from S. purpuratus or A. punctulata Sperm

Trypsin from S. purpuratus or from A. punctulata were mixed and used at pH 8.0 until they were not clear, homogenized, and resuspended in calcium-free seawater and filtered through cheesecloth to remove the bulk of the cellular tissue. The filtrate was centrifuged at 15,000 × g for 30 min to pellet the sperm. The resulting pellet was homogenized in two volumes of 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1 mM CaCl₂, and dialyzed against 4°C. The homogenate was frozen by dropping addition to liquid nitrogen, thawed at room temperature and then dialyzed against 4°C for six hours at 70 °C without a biophase buffer to lyse the sperm heads. The freeze-thaw, centrifugation procedure was repeated. The supernatant was centrifuged at 15,000 × g for 45 min to remove cellular debris. The supernatant was made 5% (v/v) in ethanol and treated exactly as described for the purification of calmodulin from S. purpuratus eggs. Alternatively, calmodulin could be purified from sperm using the procedure described previously in Burgues et al. (1989).

Purification of Calmodulin from A. punctulata or S. purpuratus Sperm

The procedure described by Head et al. (1979) for isolating calmodulin from A. punctulata eggs was used with modifications. A. punctulata eggs were obtained by injecting 0.5 M or 0.5 M KCl into the ovary cavity of the female. The shed eggs were washed in artificial sea water then stirred gently for 30 min in artificial sea water, adjusted to pH 5.0 with 0.2 M HCl, in order to lyse the yolk sac contents. The pH of the sea water was increased to 8.0 with NaOH and the eggs were pelleted by centrifugation at 200 × g for one min. The eggs were suspended in seawater and homogenized in calcium-free seawater and filtered through cheesecloth to remove the bulk of the cellular tissue. The filtrate was centrifuged at 15,000 × g for 30 min to pellet the sperm. The resulting pellet was homogenized in two volumes of 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1 mM CaCl₂, and dialyzed against 4°C. The homogenate was frozen by dropping addition to liquid nitrogen, thawed at room temperature and then dialyzed against 4°C for five hours at 70°C without a biophase buffer to lyse the sperm heads. The freeze-thaw, centrifugation procedure was repeated. The supernatant was centrifuged at 15,000 × g for 45 min to remove cellular debris. The supernatant was made 5% (v/v) in ethanol and treated exactly as described for the purification of calmodulin from S. purpuratus eggs. Alternatively, calmodulin could be purified from sperm using the procedure described previously in Burgues et al. (1989).

RESULTS

Purification of Calmodulin from S. purpuratus Eggs—Purification schemes for calmodulin from S. purpuratus eggs similar to that one described by Head et al. (1979) or that described under "Materials and Methods" of this paper for A. punctulata eggs were used initially. However, neither procedure was satisfactory because a large amount of lipid or lipoprotein material comes to the surface during centrifugation following the 70% saturated ammonium sulfate step or the 70% saturated ammonium sulfate, pH 4.1, step in lieu of a protein pellet. It is believed this occurred as a result of the increase in the density of the solution as the ammonium sulfate concentration was increased from 50% to 70% saturation. The ethanol precipitation procedure described by Charbonneau and Cormier (1979) was tried because the density of the protein solution decreases as the concentration of precipitant, ethanol, is increased. This procedure proved to be satisfactory for S. purpuratus eggs. The 0 to 50% ethanol cut yields a large pellet, which was discarded. The 50 to 80% ethanol fraction yields a small white pellet, which contains calmodulin and other proteins.

The electrophoretic pattern of the fractions eluted from CAPP-Sepharose by EGTA is shown in Fig. 1a. The major component of these fractions is calmodulin; however, there are several contaminants of apparent lower molecular weight as well as a contaminant of apparent molecular weight 42,000 which co-migrates with S. purpuratus egg actin. These contaminants can be removed by chromatography on troponin I-Sepharose as shown in Fig. 2, a and b. As can be seen in these figures, the purified calmodulin migrates as a single band on SDS gels with an apparent molecular weight of approximately 20,000 to 21,000 in the presence of 0.1 mM EDTA and an apparent molecular weight of approximately 14,000 to 15,000 in the presence of 0.1 mM CaCl₂.
purpuratus sperm using a modification of the procedure of Head et al. (1979) was reported previously (Burgess et al., 1980). More recently, the procedure described under "Materials and Methods" to isolate calmodulin from S. purpuratus or from A. punctulata sperm has been used. Fig. 1b shows the electrophoretic pattern of the proteins from the 50 to 80% ethanol precipitate of S. purpuratus sperm that are eluted from CAPP-Sepharose by EGTA. The major protein present is calmodulin. Several higher and lower apparent molecular weight contaminants are present; they are removed by chromatography on troponin I-Sepharose (Fig. 2, a and b). The sperm calmodulin migrates as a single band on SDS gels with an apparent molecular weight of 20,000 to 21,000 in the presence of 0.1 mM EDTA or with an apparent molecular weight of 14,000 to 15,000 in the presence of 0.1 mM CaCl2. Similar results were obtained using A. punctulata sperm (Fig. 2, a and b, "Discussion").

Purification of Calmodulin A and Calmodulin B from A. punctulata Eggs—The electrophoretic pattern of selected fractions from the first DE-52 column is shown in Fig. 3a. When these fractions were subjected to electrophoresis in the presence of SDS a large calcium-dependent change in the electrophoretic mobility of two proteins was observed; apparent molecular weights in the presence of 0.1 mM EDTA of 20,000 to 21,000 (labeled CM-A) and 16,500 to 17,500 (labeled CM-B) see Figs. 3a and 4, a and b. When the fractions containing these two proteins were applied to a troponin I-Sepharose column, both proteins were eluted with EGTA (Fig. 4, a and b). The results obtained from the first DE-52 column indicated that the two proteins could be separated from one another on DE-52 using a more shallow salt gradient. The results of one such experiment are shown in Fig. 3b. Some fractions contained only calmodulin A, others only calmodulin B or both proteins. The fractions that contained both proteins were rechromatographed on the same column in order to obtain additional pure calmodulin A and pure calmodulin B.

The electrophoretic mobilities of calmodulin A and of calmodulin B in the presence of 0.1 mM EDTA are compared with those of protozoan calmodulin from *Tetrahymena pyriformis*, vertebrate calmodulin, and spinach leaf calmodulin in Fig. 4c. Calmodulin A co-migrates with the vertebrate calmodulin as do the other urchin calmodulins, whereas the electrophoretic mobility of calmodulin B is intermediate to that observed for the protozoan or the plant calmodulins.

In one preparation soybean trypsin inhibitor, phenylmethylsulfonyl fluoride and p-tosyl-L-arginine methyl ester were included to inhibit proteolysis. There was no detectable difference in the yield of the A or of the B form of calmodulin in this preparation.

There is a variable amount of non-protein material associated with the final lyophilized solid from all of the urchin calmodulins we have purified. This material is present even after exhaustive dialysis and high speed centrifugation, yet can be removed from the protein by passing the sample over a PD-10 (Pharmacia) desalting column equilibrated with 10
phosphate buffer (pH 7.4) containing 10 mM ammonium bicarbonate. The excluded volume contains pure protein, the included volume contains a variable amount of UV absorbing material. This result is similar to that first reported by Watterson et al. (1980a) during the purification of spinach leaf calmodulin.

**Phosphodiesterase Stimulatory Activity**—The ability of the urchin calmodulins to stimulate bovine brain cyclic nucleotide phosphodiesterase was assayed as described under "Materials and Methods." The stimulation of the enzyme by these proteins including both the A and the B form of calmodulin from *A. punctulata* eggs is both qualitatively and quantitatively similar to the stimulation by bovine brain calmodulin (data not shown).

**Radioimmunoassays**—The results of a competition radioimmunoassay are shown in Fig. 5. Calmodulin from *S. purpuratus* eggs or sperm, calmodulin from *A. punctulata* sperm, and calmodulin B from *A. punctulata* eggs are similar in their ability to compete with chicken gizzard calmodulin in the assays. Calmodulin A from *A. punctulata* eggs also competes for the antiserum; however, 10 to 20 times more protein is required for 50% competition.

**Chloropromazine Binding of Calmodulin A and Calmodulin B from A. punctulata Eggs**—As discussed under "Materials and Methods," the CAPP-Sepharose chromatography step was not used in the purification of calmodulin A and B from *A. punctulata* eggs. After the troponin I-Sepharose column step the mixture of the two proteins was applied to a CAPP-Sepharose column in order to determine whether this step might be used to separate the two proteins. Both proteins bind to CAPP-Sepharose in a calcium-dependent manner even in the presence of 0.5 M NaCl.

**Amino Acid Analysis**—The amino acid compositions of the five urchin calmodulins that were isolated are given in Table I along with the published compositions of calmodulins from other sources.

Several of the features of the amino acid composition of vertebrate calmodulin are shared by all five urchin calmodulins including: a high aspartic acid and glutamic acid content, the absence of tryptophan and cysteine, and the presence of a single residue of trimethyllysine. There are several differences in the compositions as can be seen in the table.

The two most important points to make from the composition data are 1) that the amino acid compositions of the urchin calmodulins are all very similar to the compositions of bovine brain calmodulin and of *Renilla reniformis* calmodulin and 2) that the amino acid compositions of the A and of the B forms of calmodulin from *A. punctulata* eggs does not indicate that one is a breakdown product of the other.

**DISCUSSION**

Calmodulin from the eggs and the sperm of the sea urchins, *S. purpuratus* and *A. punctulata*, was isolated and characterized. *A. punctulata* eggs contain what appears to be two different calmodulins. Only one calmodulin was identified in each of the other three sources. All five calmodulins are similar to vertebrate calmodulin as judged by their activation of bovine brain cyclic nucleotide phosphodiesterase, calcium-dependent interaction with troponin I and with chloropromazine, increased anode mobility on SDS gels in the presence of calcium, cross-reactivity with anti-vertebrate calmodulin antibodies and amino acid compositions. In addition, calmodulin.

![Fig. 5. Comparison of urchin calmodulins and vertebrate calmodulin by competition radioimmunoassay. Various concentrations of calmodulin from chicken gizzard (○), *S. purpuratus* sperm (□), *S. purpuratus* eggs (●), *A. punctulata* sperm (○), *A. punctulata* egg A (△), and *A. punctulata* egg B (△) were mixed with anti-calmodulin serum. A fixed amount of 125I-labeled chicken gizzard calmodulin was added. The degree of competition is expressed as a per cent of the counts per min bound in the absence of competing antigen (see "Materials and Methods").](http://www.jbc.org/)

**Table I**

<table>
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<th>Residue</th>
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<th><em>R. reniformis</em></th>
<th><em>S. purpuratus</em> sperm</th>
<th><em>S. purpuratus</em> eggs</th>
<th><em>A. punctulata</em> sperm</th>
<th><em>A. punctulata</em> eggs A</th>
<th><em>A. punctulata</em> eggs B</th>
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*By sequence: bovine brain (Watterson et al., 1980b), *R. reniformis* (Jamieson et al., 1980).
from *S. purpuratus* eggs increases the calcium sensitivity of microtubules purified from bovine brain by cycles of temperature-dependent polymerization and depolymerization to the same extent as does bovine brain calmodulin. 

These results on the properties of urchin calmodulins are consistent with those of Jamieson et al. (1980), Seamon and Moore (1980), Yazawa et al. (1980), and others who showed only minor differences between the properties of calmodulin isolated from marine invertebrates and those of vertebrate calmodulin.

The procedures described under “Materials and Methods” for the isolation of calmodulin from the different sources should be useful, particularly to those who have encountered difficulties using ammonium sulfate fractionation to isolate calmodulin from *S. purpuratus* eggs and to those interested in studying the functional role of the two forms of calmodulin in *A. punctulata* eggs.

Jamieson et al. (1980) reported the isolation and characterization of a calmodulin-like protein from *Chlamydomonas reinhardtii*. Unlike the second form of calmodulin we isolated from *A. punctulata* eggs, the proteins characterized by these two groups can be distinguished from calmodulin on the basis of cyclic nucleotide phosphodiesterase activation as well as by amino acid analysis or electrophoretic mobility on SDS-gels.

The two forms of calmodulin from *A. punctulata* eggs exhibit similar tropolin I binding, chloropromazine binding, and cyclic nucleotide phosphodiesterase activation. Both contain 1 eq of trimethyllysine.

The two proteins can be distinguished by DEAE-cellulose chromatography, amino acid analysis, ultraviolet absorption spectra, and SDS-polyacrylamide gel electrophoresis in the presence or the absence of calcium although both proteins undergo a large mobility shift. One form, calmodulin A, co-migrates with vertebrate calmodulin and the other urchin calmodulins in the presence of 0.1 mM EDTA (apparent molecular weight 20,000 to 21,000) whereas the other form, calmodulin B, migrates with an apparent molecular weight closer to that of plant or protozoan calmodulin (17,000 to 18,000) under the same conditions. The two forms migrate as a tight doublet with an apparent molecular weight similar to that of vertebrate and plant calmodulin (14,000 to 15,000) when electrophoresis is conducted on SDS gels in the presence of 0.1 mM CaCl₂. The electrophoretic mobility of calmodulin on SDS gels is itself anomalous. For example, Van Eldik et al. (1980a) showed that spinach calmodulin does not co-migrate with vertebrate calmodulin on SDS gels in the presence of EDTA (apparent molecular weight difference 1000 to 2000), yet amino acid sequence analysis reveals little or no difference between spinach and vertebrate calmodulin with respect to the number of amino acid residues (Iverson et al., 1981).

In addition, calmodulin A can be distinguished from calmodulin B by competition radioimmunoassays using anti-vertebrate calmodulin antibodies. Both proteins compete quantitatively for the antisem; however, 10 to 20 times more calmodulin A than calmodulin B or the other urchin calmodulins is required for 50% competition.

The antibodies used in these experiments have been characterized by Van Eldik and Watterson (1981). They showed that all of the immunoreactivity of vertebrate calmodulin to these antibodies is contained in a peptide consisting of residues 127 to 144, the calcium-binding loop of the fourth domain. The reactivity with this particular antiserum can be affected by amino acid sequence differences in this region (Fok et al., 1981). For example, one of the differences between the amino acid sequences of *R. reinformis* and spinach calmodulin and the sequence of bovine brain calmodulin occurs at residue 143, glutamine in bovine brain, lysine in *R. reinformis* and spinach (Jamieson et al., 1980, and Iversen et al., 1981). The reactivity of spinach calmodulin with these antibodies is similar to the reactivity observed with calmodulin A from *A. punctulata* eggs.

Further studies are necessary to determine the differences between the A and the B forms of calmodulin that we isolate from *A. punctulata* eggs. As many as six putative calmodulin-binding proteins of apparent molecular weights 33,000 to 150,000 in various fractions of unfertilized *A. punctulata* eggs have been identified using the 125I-calmodulin gel overlay technique of Carlin et al. (1980). This procedure is being used to determine whether we can detect a difference in the number, type, compartmentalization, or affinity of proteins that bind to calmodulin A and those that bind to calmodulin B.

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