Purification and characterization of calmodulin from sea urchin spermatozoa


Departments of Pharmacology and Physiology and The Howard Hughes Medical Institute Laboratory at Vanderbilt University, Nashville, Tennessee 37232, U.S.A.

Summary. Calmodulin was purified to apparent homogeneity from sea urchin spermatozoa by heat-treatment at 85°C, ammonium sulphate precipitation at pH 4.2, DEAE-Sephacel chromatography and gel filtration on Sephadex G-100. Approximately 8.3 μg calmodulin were recovered per 10^10 sperm cells. The sperm calmodulin had an apparent molecular weight of 17 800. The purified calmodulin activated calmodulin-deficient phosphodiesterase from pig coronary arteries, with half-maximal activation occurring at approximately 40 ng calmodulin/ml. Trifluoperazine also inhibited the sperm calmodulin activity. These results demonstrate that calmodulin is present in high amounts in sea urchin spermatozoa, and that it is essentially the same as the calmodulin isolated from various other tissues.

Introduction

Many of the physiological actions of calcium are now known to be mediated by a low molecular weight protein called calmodulin. A number of Ca^{2+}-sensitive enzymes, including cyclic nucleotide phosphodiesterases (Cheung, 1970; Kakiuchi & Yamazaki, 1970), adenylate cyclase (Brostrom, Huang, Breckenridge & Wolff, 1975), myosin light chain kinase (Dabrowska, Sherry, Aromatorio & Hartshorne, 1978), phosphorylase kinase (Cohen et al., 1978), and the Mg^{2+}-Ca^{2+} dependent ATPase of the erythrocyte (Jarrett & Penniston, 1977; Gopinath & Vincenzi, 1977), are regulated by interaction with the Ca^{2+}-calmodulin complex rather than by Ca^{2+} directly. Brooks & Siegel (1973) first demonstrated that bovine epididymal spermatozoa contained calmodulin, but the identification of the protein was based on Ca^{2+}-binding properties and not on a physiological effect of the molecule. Wells & Garbers (1976) later showed that cyclic nucleotide phosphodiesterases of sea urchin spermatozoa were not activated by sperm calmodulin, but that a phosphodiesterase from pig coronary arteries could be activated. Jones, Bradford, McRorie & Cormier (1978) have suggested that calmodulin is localized exclusively in the acrosomal area of rabbit spermatozoa. Since the acrosome reaction is known to be a Ca^{2+}-dependent event in both invertebrate and vertebrate spermatozoa (Dan, 1954; Yanagimachi & Usui, 1974), the localization of calmodulin within the acrosomal area may indicate an important functional role for the protein in the acrosome reaction.

In studies reported here, calmodulin was purified to homogeneity from sea urchin spermatozoa and its properties were compared to those of the protein from other tissues.
Materials and Methods

Sea urchins (*Strongylocentrotus purpuratus*) were purchased from Pacific BioMarine (Venice, California). Tritiated cyclic GMP was from Amersham. Triethanolamine, dithiothreitol and unlabelled cyclic GMP were from Sigma. Biophore precast polyacrylamide gels were purchased from Bio-Rad.

Analytical polyacrylamide gel electrophoresis

Preparations containing calmodulin were applied to 12% polyacrylamide gels pre-electrophoresed in the presence of a buffer containing 25 mM-glycine at pH 8.4. After application of the sample, electrophoresis (5 mA/tube) continued until the tracker dye, bromophenol blue, migrated within 10 mm of the end of the gel. Gels were removed and stained with Coomassie blue R-250. Under protein denaturing conditions, calmodulin preparations were heated at 100°C for 10 min in the presence of 1-0% sodium dodecyl sulphate (SDS), 5% glycerol, 0-002% bromophenol blue, 800 mM-Tris–acetate at pH 6.4 and 140 mM-dithiothreitol. Samples were then applied to 12% polyacrylamide gels that had been pre-electrophoresed in the presence of 0-1% SDS and 0-21 mM-Tris–acetate at pH 6.4. Electrophoresis was at 5 mA/tube.

Phosphodiesterase assay

Cyclic nucleotide phosphodiesterase activity was estimated as described by Wells & Garbers (1976) using 1 μM-cyclic [3H]GMP as the substrate. Calmodulin-deficient phosphodiesterase from pig coronary arteries was prepared by the method of Wells, Baird, Wu & Hardman (1975). This form of the phosphodiesterase is activated 3–4-fold by addition of the Ca2+-calmodulin complex. In general the phosphodiesterase assay mixture contained 50 mM-Tris buffer, pH 7-6, 10 mM-Mg2+, 0.1 mM-Ca2+, 1 x 104 d.p.m. of cyclic [3H]GMP, and 1 μM-cyclic GMP. Assays were at 30°C for 30 min.

Amino acid analysis

Samples containing approximately 200 μg freeze-dried calmodulin were hydrolysed for 25 h at 110°C in 6 N-HCl under vacuum. The sample was then applied to a Beckman 121 amino acid analyser equipped with a Durrum DC-6A ion exchange column.

Results

Purification of calmodulin. Sea urchin spermatozoa, 1.82 kg wet weight representing approximately 1.5 x 1014 cells, were suspended in 4 litres of a solution containing 25 mM-triethanolamine + 5 mM-dithiothreitol and were homogenized with an Ultraturrax homogenizer. The broken cells were then centrifuged at 11 000 g for 12 h at 2°C, and the resulting supernatant fluid was saved. Solid (NH4)2SO4 was added to the supernatant fluid to 50% saturation at 4°C. After mixing for 30 min, the suspension was centrifuged at 19 200 g for 12 h. The supernatant fluid was saved and adjusted to pH 4-2 with HCl, and the resulting suspension was centrifuged at 19 200 g for 2 h. The pellets were resuspended in 750 ml 25 mM-triethanolamine buffer at pH 7-6 and heated at 85°C for 10 min. The precipitate was removed by centrifugation at 19 200 g for 2 h, and the supernatant fluid was then dialysed against 25 mM-triethanolamine buffer at pH 7-6. The dialysed solution containing the calmodulin was then added to a DEAE-Sephacel column (5 x 16 cm) equilibrated with 25 mM-triethanolamine buffer at pH 7-6. The column was washed with 750 ml triethanolamine buffer after addition of the sample volume.
Calmodulin was eluted with a linear (NH₄)₂SO₄ gradient (300 ml 25 mM-triethanolamine (pH 7-6) in the mixing tank, and 300 ml 25 mM-triethanolamine (pH 7-6) and 0-5 mM-(NH₄)₂SO₄ in the reservoir tank). Calmodulin was eluted between 0-3 and 0-4 mM-(NH₄)₂SO₄. The fractions containing calmodulin activity were pooled and dialysed extensively against 25 mM-triethanolamine buffer at pH 7-6. After dialysis the solution containing the calmodulin was freeze dried and the resultant dried material was then redissolved in 60 ml of a solution containing 25 mM-triethanolamine at pH 7-6, 1 mM-EGTA and 0-1 M-NaCl. Aliquots (5 ml) of the calmodulin solution were applied to a Sephadex G-100 column (2-6 × 34 cm) and the fractions (3-5 ml each) containing calmodulin were routinely pooled, dialysed extensively against Millipore H₂O and freeze dried. The freeze-dried calmodulin was then stored desiccated at 4°C.

**Yield of calmodulin.** The total amount of purified calmodulin recovered was 125 mg. This represents a recovery of 8-3 μg calmodulin from 10¹⁰ cells; this value is about 8 times higher than that estimated by Jones et al. (1978). The actual amount of calmodulin in the sperm cell is probably higher than this because recoveries during purification were not estimated.

**Purity.** The final preparation of calmodulin was homogeneous based on analytical gel electrophoresis under both native and denaturing conditions. A single protein-staining band was observed using 7-5 or 12% polyacrylamide gels under native conditions, and a single band was also observed on 12% gels run in the presence of SDS (Text-fig. 1). Single bands were even observed after overloading gels with 75 μg added calmodulin.

**Molecular weight.** The apparent molecular weight of the purified calmodulin was 17 800 as estimated by SDS-polyacrylamide gel electrophoresis. The marker protein standards used were α-lactalbumin, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin and bovine serum albumin.

**Absorption spectra.** The spectrum of the purified calmodulin was similar to spectra reported for troponin C and for calmodulin from other tissues (Watterson, Harrelson, Keller, Sharief & Vanaman, 1976). The fine structure of the spectra between 250–280 nm also resembled previously reported spectra (not shown).

![Text-fig. 1. SDS-polyacrylamide (12% gels) electrophoresis of purified sperm calmodulin. The gels were electrophoresed under identical conditions (see text) but contained (a) 75, (b) 37-5 and (c) 7-5 μg added calmodulin, respectively.](https://example.com/textfig1.png)
**Amino acid analysis.** The amino acid composition of sperm calmodulin (Table 1), was similar to that of calmodulin from brain (Watterson et al., 1979), sea urchin eggs (Head, Mader & Kaminer, 1979) and other tissues (Grand, Perry & Weeks, 1979). The molecule was relatively acidic with only small amounts of aromatic amino acids.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mole (%)</th>
<th>Residues/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>15.65 ± 0.54</td>
<td>22</td>
</tr>
<tr>
<td>Thr</td>
<td>8.19 ± 0.14</td>
<td>11</td>
</tr>
<tr>
<td>Ser</td>
<td>4.75 ± 0.05</td>
<td>7</td>
</tr>
<tr>
<td>Glu</td>
<td>18.42 ± 0.42</td>
<td>25</td>
</tr>
<tr>
<td>Pro</td>
<td>4.08 ± 0.50</td>
<td>6</td>
</tr>
<tr>
<td>Gly</td>
<td>8.85 ± 0.06</td>
<td>12</td>
</tr>
<tr>
<td>Ala</td>
<td>8.08 ± 0.11</td>
<td>11</td>
</tr>
<tr>
<td>Val</td>
<td>4.46 ± 0.11</td>
<td>6</td>
</tr>
<tr>
<td>Met</td>
<td>2.62 ± 0.06</td>
<td>4</td>
</tr>
<tr>
<td>Ile</td>
<td>4.28 ± 0.11</td>
<td>6</td>
</tr>
<tr>
<td>Leu</td>
<td>5.73 ± 0.09</td>
<td>8</td>
</tr>
<tr>
<td>Tyr</td>
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</tr>
<tr>
<td>Phe</td>
<td>4.85 ± 0.13</td>
<td>7</td>
</tr>
<tr>
<td>His</td>
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</tr>
<tr>
<td>Lys</td>
<td>5.52 ± 0.28</td>
<td>8</td>
</tr>
<tr>
<td>Arg</td>
<td>3.34 ± 0.07</td>
<td>5</td>
</tr>
</tbody>
</table>

The values are mean ± s.e.m. for 3 analyses.
* Tryptophan and trimethyllysine were not estimated.
† Based on a molecular weight of 17 800.

Text-fig. 2. Activation of calmodulin-deficient phosphodiesterase from coronary arteries by the purified sperm calmodulin. Calmodulin was added to the calmodulin-deficient coronary artery phosphodiesterase assay mixture (O) at the concentrations shown: 7.5 μM-trifluoperazine was included in some of the assay mixtures (Δ). The phosphodiesterase was activated approximately 3.5-fold when maximally stimulated by added calmodulin.
**Phosphodiesterase activation.** The amount of purified calmodulin required to activate a calmodulin-deficient phosphodiesterase from pig coronary arteries is shown in Text-fig. 2. Approximately 40 ng calmodulin/ml were required for half-maximal activation of the coronary artery, calmodulin-deficient phosphodiesterase. Trifluoperazine (7·5 μM), a known inhibitor of calmodulin in other tissues (Levin & Weiss, 1976), altered the concentration-response curve such that the amount required for half-maximal enzyme activation was 160 ng calmodulin/ml.

**Discussion**

The calmodulin purified from sea urchin spermatozoa appears to be very similar to the protein isolated from various other tissues based on its molecular weight, u.v. spectra and amino acid composition (Watterson et al., 1976). The sea urchin sperm calmodulin is also very similar to the calmodulin purified from sea urchin eggs (Head et al., 1979). Although the function of calmodulin in spermatozoa remains unknown, the relatively high amounts suggest that this protein may serve as an important mediator of calcium effects in spermatozoa.

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**References**


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