Identification of Sea Urchin Sperm Adenylate Cyclase

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Abstract. Calmodulin (CaM) affinity chromatography of a detergent extract of sea urchin sperm yielded ~20 major proteins. One of these proteins, of M_r 190,000, was purified and used to immunize rabbits. After absorption with living sperm, the serum reacted monospecifically on one- and two-dimensional Western immunoblots with the M_r 190,000 protein. The anti-190-kD serum inhibited 94% of the adenylate cyclase (AC) activity of the CaM eluate. An immunoaffinity column removed 95% of the AC activity, and the purified (but inactive) M_r 190,000 protein was eluted from the column. The antiserum also inhibited 28% of the activity of bovine brain CaM-sensitive AC and 90% of the activity of horse sperm CaM-sensitive AC. These data support the hypothesis that the M_r 190,000 protein is sea urchin sperm AC. Although this AC bound to CaM, it was not possible to demonstrate directly a Ca^{2+} or CaM sensitivity. However, two CaM antagonists, calmidazolium and chlorpromazine, both inhibited AC activity, and the inhibition was released by added CaM, suggesting the possibility of regulation of this AC by CaM. Indirect immunofluorescence showed the M_r 190,000 protein to be highly concentrated on only the proximal half of the sea urchin sperm flagellum. This asymmetric localization of AC may be important to its function in flagellar motility. This is the first report of the identification of an AC from animal spermatozoa.

ANIMAL spermatozoa undergo profound physiological changes between the time of their release from the male reproductive tract and their fusion with eggs. Some of these changes, or "activations," are the initiation of motility, capacitation, and the acrosome reaction. A large literature exists on the regulation of the initiation of sperm flagellar motility by Ca^{2+}, cAMP, and cAMP-dependent protein kinase (A-kinase; for reviews, see references 1, 3, 8, 11, 21, 22). In summary, these data support the hypothesis that increases in cellular Ca^{2+} activate sperm adenylate cyclase, resulting in increased concentrations of cAMP. This in turn activates A-kinase, which phosphorylates axonemal proteins. These phosphorylation events are necessary for the initiation and maintenance of flagellar motility. Sea urchin sperm are a model cell for studying the mechanism of flagellar motility. It is important to the ultimate elucidation of the underlying mechanism to identify, localize, and characterize the enzymes and their protein substrates that function in the process.

Previous work (reviewed in reference 3) has shown that sea urchin spermatozoa contain considerable adenylate cyclase (AC)[1] activity. This activity requires Mn-ATP and exhibits a 50-fold activation when living sperm are treated with soluble sea urchin egg jelly. This activation is dependent on extracellular Ca^{2+} and is blocked by the calmodulin (CaM) antagonist trifluoroperazine. Temporally correlated with the egg jelly–induced activation of AC is the occurrence of the acrosome reaction. However, no direct experimental evidence exists linking the activation of AC with the acrosome reaction (3).

Further study of the sea urchin sperm AC and the role of its activation in the fertilization process would be facilitated by its identification as a protein. Procedures exist for isolating sea urchin sperm plasma membranes (reviewed in reference 27). One such procedure using N_2 cavitation (30) yields membrane vesicles of *Strongylocentrotus purpuratus* sperm in which a M_r 190,000 protein becomes phosphorylated upon addition of γ-^{32}P-ATP (Bookbinder, L. H., G. W. Moy, and V. D. Vacquier, manuscript in preparation). In this paper we show that this protein binds to calmodulin-agarose and is eluted by EGTA. A monospecific rabbit antiserum was raised to the M_r 190,000 protein. The antiserum inhibits sea urchin and horse sperm AC. An immunoaffinity column using this antiserum depletes detergent extracts of AC activity. Immunofluorescent localization shows the M_r 190,000 protein to be highly concentrated in only the proximal half of the flagellar length, with only slight staining of both the distal half of the flagellum and sperm head. The concentration of spermatozoal AC in the proximal half of the sea urchin sperm flagellum may be related to crucial steps in the motility process.

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1. *Abbreviations used in this paper*: AC, adenylate cyclase; CaM, calmodulin; CaM-El, sperm proteins eluted from calmodulin-agarose by EGTA.

Materials and Methods

Calmodulin Affinity Chromatography

Sperm of the sea urchin *S. purpuratus* was spawned into seawater by injection of animals with 0.5 M KCl. After a 10–20-fold dilution in filtered seawater the pigmented coelomocytes were pelleted by centrifugation for 10 min at 1,000 *g* (4°C). The sperm cell suspension was poured into a clean tube and the cells pelleted by centrifugation for 20 min at 3,000 *g*. The sperm pellet was resuspended in 10 vol buffer A (10 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EGTA, 5 mM PMSF, and 1 mM benzamidine HCl) for 30 min (4°C). The sperm suspension was then centrifuged 20 min at 10,000 *g* and the pellet resuspended in 10 vol buffer A containing 2% (wt/vol) Brij-78. The detergent treated cell suspension was stirred gently for 1 h (4°C) and then centrifuged 1 h at 40,000 *g*. 1 M $CaCl_2$ was added to 3 mM final concentration and the extract passed five times through a 20-ml bed of CaM-agarose, which was equilibrated in buffer B (10 mM Hepes pH 7.5, 150 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 2 mM $CaCl_2$, and 10 mM NaN_3). The column was washed with 10 vol buffer B containing 0.125% Brij-78 and the $CaCl_2$ reduced to 0.2 mM. Calmodulin binding proteins were eluted as a single peak in buffer B containing 0.125% Brij-78 and 2 mM EGTA. The A_{280} peak fractions were pooled, $CaCl_2$ was added to 2 mM, and the eluate was concentrated 10–20-fold with a microconcentrator (Centricon 30; Amicon Corp., Danvers, MA), mixed with an equal volume of glycerol and stored in 0.5 ml aliquots at −70°C. This material is hereafter denoted CaM-eluate (CaM-El). It retains full adenylate cyclase activity when stored at −70°C. Except where noted otherwise, chemicals were all of the highest reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

Electrophoresis and Western Immunoblots

Proteins were solubilized, denatured, and reduced in Laemmli sample buffer and separated by SDS-PAGE on 5% gels (7) which were then silver stained (12). Two-dimensional gels were run as described (10). Western transfers to nitrocellulose were performed using a Trans-Blot Cell (Bio-Rad Laboratories, Richmond, CA) at 24 mV for 16 h using Laemmli SDS-PAGE chamber buffer containing 20% methanol. After protein transfer, the nitrocellulose was blocked for 1 h in TBS containing 5% (wt/vol) nonfat dry milk. The nitrocellulose membrane was transferred to a dish containing TBS, plus 1 mg/ml BSA, and a 1:1,000 dilution of the unabsorbed or absorbed anti-190-kD serum or preimmune serum. The blot was incubated for 1 h with shaking (20 ml per 150 cm^2 nitrocellulose). The blot was washed twice (5 min) with 200 ml of buffer C (10 mM Tris-Base, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 0.1% SDS). The blot was then washed twice (5 min) in buffer C containing 500 mM NaCl, followed by two more washes in buffer C without the SDS. Proteins binding the anti-190-kD IgG were visualized using a Vectastain ABC, goat anti-rabbit IgG horseradish peroxidase kit (Vector Laboratories, Inc., Burlingame, CA), following the manufacturer's instructions. All incubations were carried out at room temperature.

Antibody Production and Serum Absorption

Stored CaM-El was diluted 1:1 with Laemmli sample buffer, and boiled 5 min. 100-μg samples of protein were separated by SDS-PAGE on 5% gels for 6 h at 60 mA. Proteins were visualized by negative staining using 2 M KCl and the M_r 190,000 band excised from the gels and electroeluted as described (28). Electroeluted protein was desalted, precipitated, washed, and concentrated (28), then checked for purity by SDS-PAGE and silver staining. Absorbed serum was prepared by sedimentation of blood cells by centrifugation at 10,000 *g* for 20 min followed by addition of 22 mg NaCl per ml of supernatant to increase the osmolarity to that of seawater (roughly 1050 mOsm). Concentrated sea urchin "dry sperm" (0.5 ml; 50 mg sperm protein) was mixed with 25 ml serum and placed on ice for 1 h with occasional stirring. The cells were pelleted by centrifugation at 3,000 *g* for 20 min. The supernatant was transferred to a clean tube and the absorption repeated. A third absorption with living sperm was done overnight on ice and the sperm removed by centrifugation at 10,000 *g* for 30 min. The resulting absorbed serum, denoted "anti-190 serum," was stored in 1-ml aliquots at −70°C.

Adenylate Cyclase Assay

Sea urchin sperm adenylate cyclase activity was assayed at 23°C as described (29), with the inclusion of 0.05% Brij-78. Concentrated CaM-El, 40,000 *g* supernatant, or sperm (diluted 1:50 in buffer B containing 2 mM calcium) were assayed. Bovine brain adenylate cyclase was assayed as described (32). Horse sperm adenylate cyclase was assayed in a thawed cell lysate as described (4). In all assays the reaction was linear with time and solely dependent on the quantity of sperm protein. Preparations of CaM-El ranged in AC activity between 35 and 100 nMol cAMP formed per minute per milligram protein. Cryogenically preserved domestic horse sperm was a gift of Dr. Barbara Durrant of the San Diego Zoo.

Immunoaffinity Chromatography

Anti-190 serum was covalently crosslinked to protein A–Sepharose (14). Concentrated CaM-El (50–100 μg) diluted 1:10 with buffer D (2 mM Hepes, pH 7.5, 10 mM NaCl, 10 mM KCl, 5 μM EDTA, 20 mM azide, and 12 mg/ml BSA), was incubated on ice for 10 min and then centrifuged 10,000 *g* for 10 min. The supernatant was passed 10 times through a 1-ml bed of the anti-190 Sepharose beads which had been previously equilibrated in buffer D. The beads were then washed with 20 vol of buffer D. The beads were eluted with buffer D containing 50 mM diethylamine, pH 11.5. The eluted 1-ml fractions were collected in tubes containing 0.2 ml of 500 mM Hepes, pH 6.0 to immediately neutralize the eluant. Total protein concentration of samples was determined by the Lowry et al. (9) method, or the BCA protocol (20). Elution of the purified M_r 190,000 protein shown in Fig. 6 was done in buffer D in the absence of BSA.

Immunofluorescence

Sea urchin semen (50 μl) was fixed by a 1:100 dilution in millipore-filtered seawater containing 3% paraformaldehyde and 0.1% glutaraldehyde for 1 h. The sperm were sedimented by centrifugation at 3,000 *g* for 10 min. The fixed sperm were washed five times by resuspension and sedimentation in 10 vol of TBS containing 100 mM glycine and 10 mM sodium azide. The sperm were permeabilized by resuspension in 10 vol TBS containing 0.5% Triton X-100, 10 mM azide and shaken for 1 h. The cells were pelleted by centrifugation, and resuspended in blocking buffer: TBS containing 5% nonfat dry milk, 1 mg/ml BSA, 10 mM azide, which had been previously cleared of milk particulates by centrifugation at 20,000 *g* for 20 min. After an overnight incubation the sperm were pelleted, resuspended in 0.5 ml of blocking buffer, and 100-μl aliquots transferred to glass tubes containing 100 μl of either preimmune or "anti-190 serum." The lowest final dilution of the serum was 1:32 in blocking buffer. The cells were incubated with the primary antiserum for 1 h at room temperature with shaking. The sperm were then washed five times with 3 ml of blocking buffer and resuspended after the final wash in 100 μl of blocking buffer. 100 μl of rhodamine-conjugated goat anti-rabbit IgG (Organon Teknika-Cappel, Durham, NC) diluted 1:200 in blocking buffer was added to the sperm, which were incubated 1 h at room temperature with shaking. The cells were then washed five times in 3 ml blocking buffer as above, and the final pellet resuspended in 250 μl of TBS containing 0.2% Triton X-100, and 10 mM azide for immunofluorescent microscopy.

Results

Production of Monospecific Antiserum to a M_r 190,000 CaM Binding Protein

Calmodulin-agarose affinity chromatography of supernatants of Brij-78 solubilized sperm membranes reveals >20 CaM binding proteins (Fig. 1, lane *C*). One protein of approximate M_r 190,000 is greatly enriched in the CaM-El as compared to whole sperm (Fig. 1, lane *A*), or total membrane lysate (lane *B*). Densitometry of Coomassie blue-stained gels indicates that it comprises roughly 10% of the total CaM-El protein (not shown). This protein was electroeluted from gels (Fig. 1, lane *D*) and used to immunize rabbits. Western immunoblots of the crude rabbit serum detected reactions with three sperm proteins of approximate M_r 50,000, 190,000, and 230,000 (Fig. 1, lane *E*). Extensive absorption of the immune serum with living sperm cells removes the reactions with the M_r 50,000 and 230,000 proteins and results in a serum reacting with only the M_r 190,000

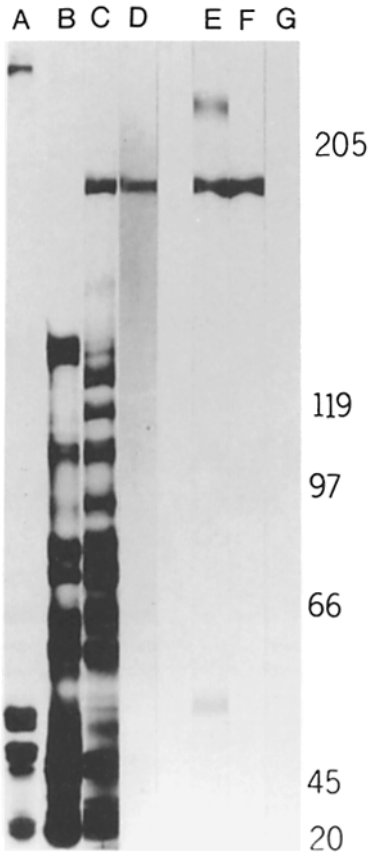

Figure 1. Production of a monospecific rabbit antiserum to the M_r 190,000 CaM-binding protein from sea urchin sperm. Lanes *A–D*, silver stained proteins; lanes *E*–G, Western immunoblots of SDS-PAGE gels. Lane *A*, whole sperm protein; lane *B*, proteins of sperm membranes remaining 40,000 *g* soluble in 2% Brij-78; lane *C*, sperm proteins eluted from CaM-agarose; lane *D*, 1 μg electro-eluted M_r 190,000 protein used as antigen; lane *E*, reaction of crude rabbit immune serum with proteins in lane *C*; lane *F*, reaction of the immune serum with proteins in lane *C* after absorption with living sperm; lane *G*, reaction of preimmune serum with the proteins in lane *C*. Protein load is 5 μg per lane. Relative molecular mass standards in thousands are marked on the right.

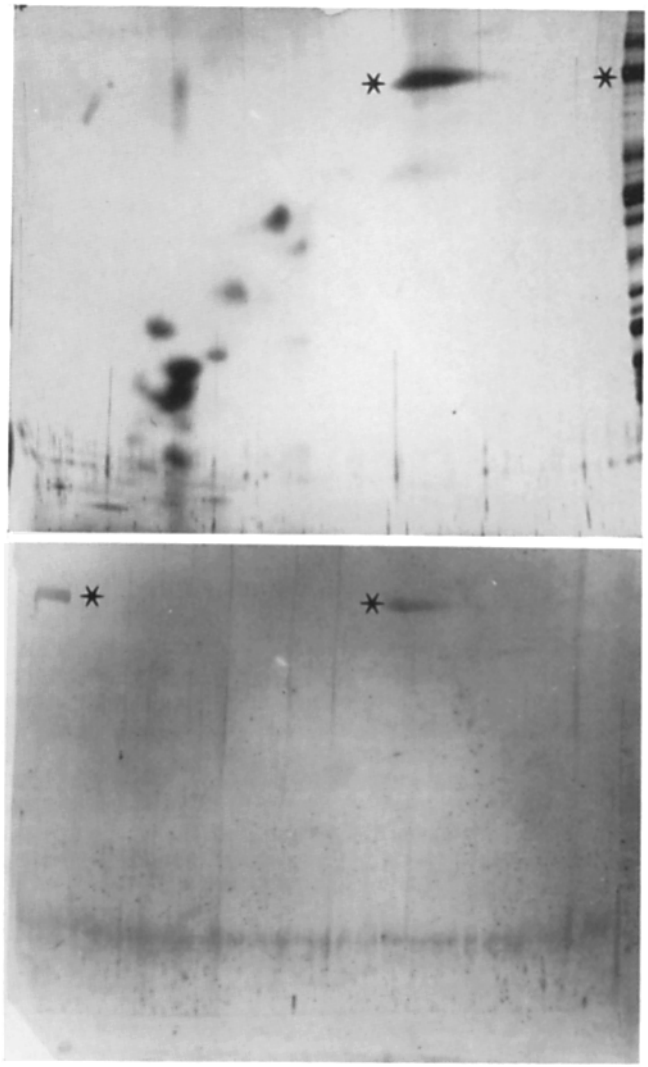

Figure 2. Two-dimensional polyacrylamide gel electrophoretic separation of proteins eluted from CaM-agarose (*top*, silver stain). Western immunoblot on nitrocellulose of a duplicate gel using the monospecific absorbed rabbit serum (*bottom*), shows a single reaction with the M_r 190,000 protein (*stars*). 7 μg protein per gel. One-dimensional SDS-PAGE separation of the CaM-El is on the right in top panel and the left in bottom panel.

protein (Fig. 1, lane *F*). Preimmune serum does not react with sperm membrane proteins (Fig. 1, lane *G*). Two-dimensional gel electrophoresis of the CaM-El followed by Western immunoblotting and reaction with the monospecific serum shows one reacting band at M_r 190,000 (Fig. 2, *top and bottom*). We conclude from the data in Fig. 1 and 2 that the sperm-absorbed antiserum reacts monospecifically with a M_r 190,000 CaM-binding protein of sperm.

Evidence that the M_r 190,000 Protein Is Adenylate Cyclase

AC specific activity in the 40,000 *g* supernatant of Brij-78 extracts of sea urchin sperm was 0.74 ± 0.23 nMol cAMP formed min^{-1} mg^{-1} protein (n = 3), which was essentially the same value as in the total sperm lysate. The AC specific activity of the CaM-El was 35.85 ± 4.7 (n = 6), which represents a 42-fold increase over the starting material.

The monospecific antiserum to the M_r 190,000 protein (anti-190 serum) inhibits up to 94% of the AC activity of the CaM-El (Fig. 3) and 90% of the AC activity of the 40,000 *g* supernatant of Brij-78 solubilized sperm. The anti-190 serum also inhibits 28% of the activity of bovine brain CaM-sensitive AC (data not shown) and up to 90% of the activity of horse sperm AC (Fig. 4).

95% of the AC activity of the sea urchin sperm CaM-El is removed after passage through a column of anti-190–Sepharose (Fig. 5). This loss in AC activity correlates with the disappearance of the M_r 190,000 band from the flow through fraction of the antibody column (Fig. 6, lanes *B* and *E*). Adenylate cyclase activity is measureable on washed anti-190–Sepharose. Pure M_r 190,000 protein was eluted from the antibody column (Fig. 6, lane *C*), however, without retention of AC activity. Elution with high and low pH, chaotropic re-

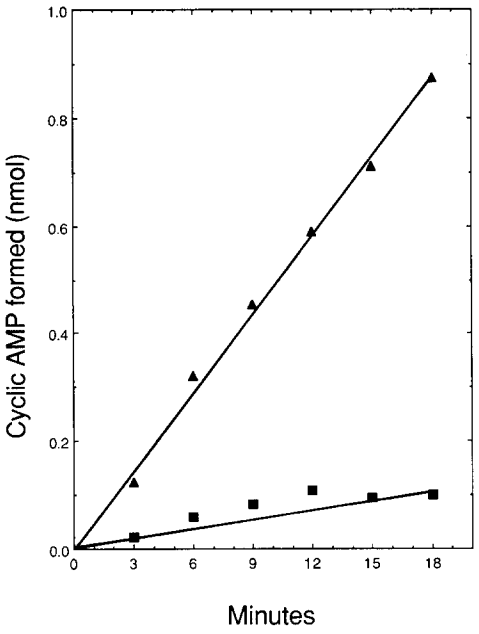

Figure 3. Inhibition of the sea urchin sperm adenylate cyclase activity in the activity in the CaM-El by anti-190 serum (■), or preimmune serum (▲). 5 μl CaM-El (5 μg protein) was added to 100 μl of anti-190 serum or preimmune serum, followed by addition of 240 μl of the AC reaction mixture (29) and 38 μl of 1% Brij-78. After 30 min on ice the mixtures were warmed to 23°C and time points taken at 3-min intervals. The same level of inhibition of AC was seen at 10 μl of anti-190 serum. Data are expressed as nanomoles of cAMP formed.

agents, and high salt fails to yield active AC when assayed in the presence or absence of Ca^{2+} and CaM.

The M_r 190,000 protein does not stain for carbohydrate, bind to wheat germ agglutinin, or concanavalin A-Seph-

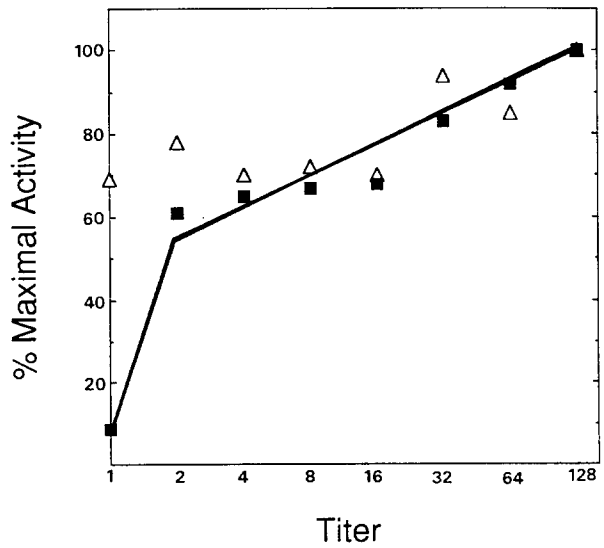

Figure 4. Inhibition of horse sperm AC activity by the anti-190 serum raised against the sea urchin sperm protein. Assay conditions were as described in reference 4. Preincubation of horse sperm protein with anti-190 serum or preimmune was as in Fig. 3. Each point is the average of three determinations. (■) Anti-190 serum; (△) preimmune serum.

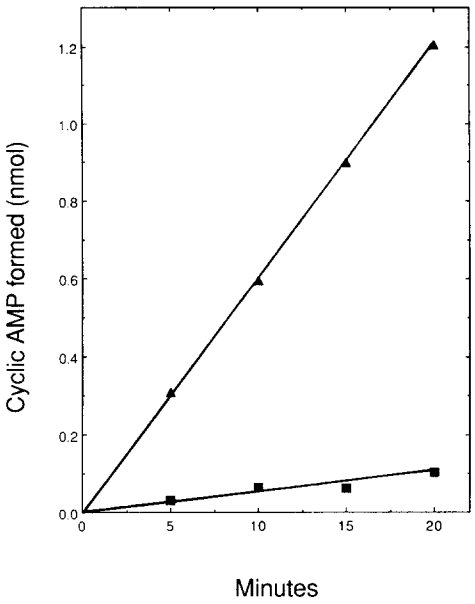

Figure 5. Depletion of AC activity after passage of the CaM-El over an anti-190-Sepharose column. 50 μg CaM-El was loaded on 1 ml of anti-190-Sepharose. The flow-through fraction was assayed for AC activity and a depletion of 94% was found. Total CaM-El (▲); flow through (■).

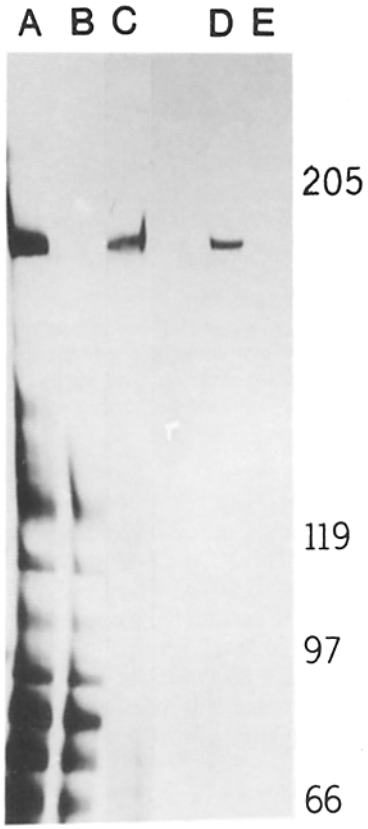

Figure 6. Purification of the M_r 190,000 protein by immunoaffinity chromatography. Lanes *A–C*, proteins stained with silver; lanes *D* and *E*, Western immunoblots of 5% SDS-PAGE gels. 50 μg CaM-El was loaded on an anti-190-Sepharose column (1 ml). Lanes *A* and *D*, CaM-El; lanes *B* and *E*, flow-through fraction from the column; lane *C*, pH 11.5 eluant from the column (2 μg). Lanes *A*, *B*, *D*, and *E* are 5 μg protein. Relative molecular mass standards in kilodaltons are on the right.

Table I. Effect of Calmodulin Antagonists on Adenylate Cyclase Activity in the CaM-El of Sea Urchin Sperm

Addition	Micromolar	Specific activity	Change
			%
No additions	0	106.56	0.00
Calmodulin	10	120.37	+12.96
Calmidazolium	200	5.87	−94.49
Chlorpromazine	156	8.59	−91.94
Trifluoroperazine	200	25.13	−76.41
W-5	100	75.4	−29.24
W-7	100	94.79	−11.04
W-12	100	81.98	−23.07
W-13	100	54.92	−48.46

4 μg of CaM-El was preincubated with the additives at the specified concentrations for 15 min at 4°C before initiation of the assay. Specific activity is nmol cAMP formed min^{-1} mg^{-1} protein.

arose, and is not labeled by procedures in which the M_r 210,000, 140,000, 80,000, and 60,000 sperm membrane proteins are radioiodinated (18). Also, the M_r 190,000 protein does not shift its relative molecular mass after digestion with *N*-glycanase or neuraminadase. The above data are evidence that the M_r 190,000 protein is sperm AC and that it is not exposed to the extracellular surface and is therefore most probably not a transmembrane protein.

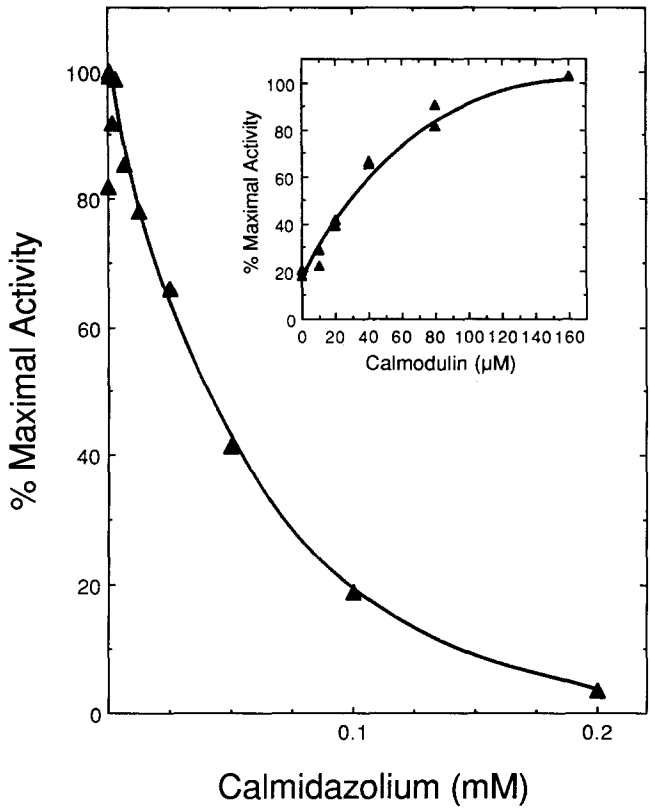

Figure 7. Inhibition of AC activity in the CaM-El by calmidazolium and restoration of activity by bovine brain CaM (*inset*). 2.5 μg CaM-El was incubated 15 min at 4°C with calmidazolium before initiation of the assay. Half-maximal activity was at 40 μM. For the restoration of activity (*inset*) CaM-El was incubated 15 min at 4°C with 100 μM calmidazolium before addition of CaM and 1 mM $CaCl_2$. After a 15-min incubation at 4°C the AC assay was initiated.

Is the M_r 190,000 Sea Urchin Sperm Adenylate Cyclase CaM Sensitive?

The fact that sea urchin sperm AC binds to CaM-agarose is not in itself evidence for the regulation of this AC by CaM. Others had attempted unsuccessfully to strip CaM from sea urchin sperm membrane preparations with EGTA and then to show an increase in AC by readdition of purified bovine brain or sea urchin sperm CaM (3). We were equally unsuccessful in attempts to reduce the AC activity of the CaM-El with EGTA and we did not find a consistent significant increase in AC activity after readdition of purified CaM or Ca^{2+} (Table I). However, these data are not conclusive evidence that the sea urchin sperm adenylate cyclase is not a CaM-sensitive enzyme. The AC activity in the CaM-El is inhibited to varying extents by several classical antagonists of CaM-mediated processes (Table I). Calmidazolium, chlorpromazine, and trifluroperazine are the most effective inhibitors of the CaM-El AC activity (Table I). The inhibition of AC activity by both calmidazolium (Fig. 7; ID_{50} ~40 μM) and chlorpromazine (Fig. 8; ID_{50} ~75 μM) are dose dependent. The inhibition by both these CaM antagonists can be released by addition of bovine brain CaM (Figs. 7 and 8, *insets*).

Immunolocalization

Sea urchin sperm were fixed, permeabilized with Triton X-100 and reacted with either preimmune or anti-190 serum. Indirect immunofluorescence using a rhodamine-conjugated goat anti-rabbit second antibody shows no reaction with

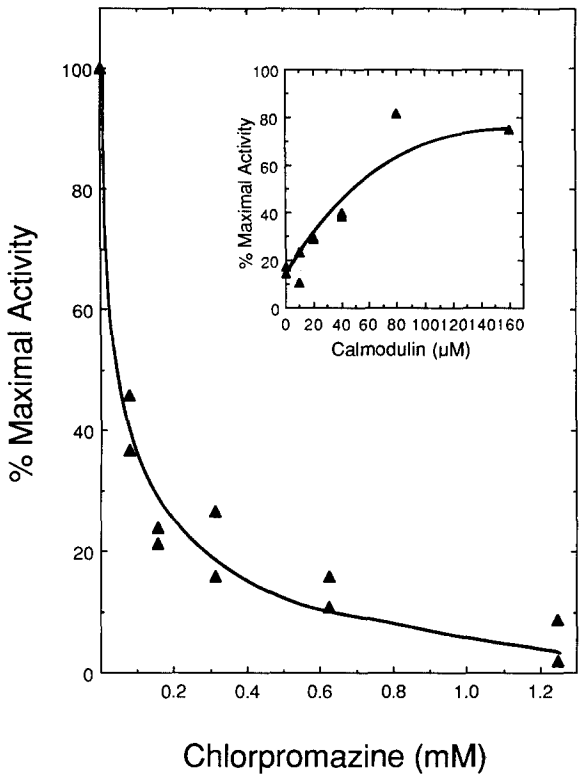

Figure 8. Inhibition of AC activity in the CaM-El by chlorpromazine and restoration of activity by CaM (*inset*). Conditions are as in Fig. 7. The half-maximal activity was at 70 μM. For the data in the inset preincubation was with 70 μM chlorpromazine.

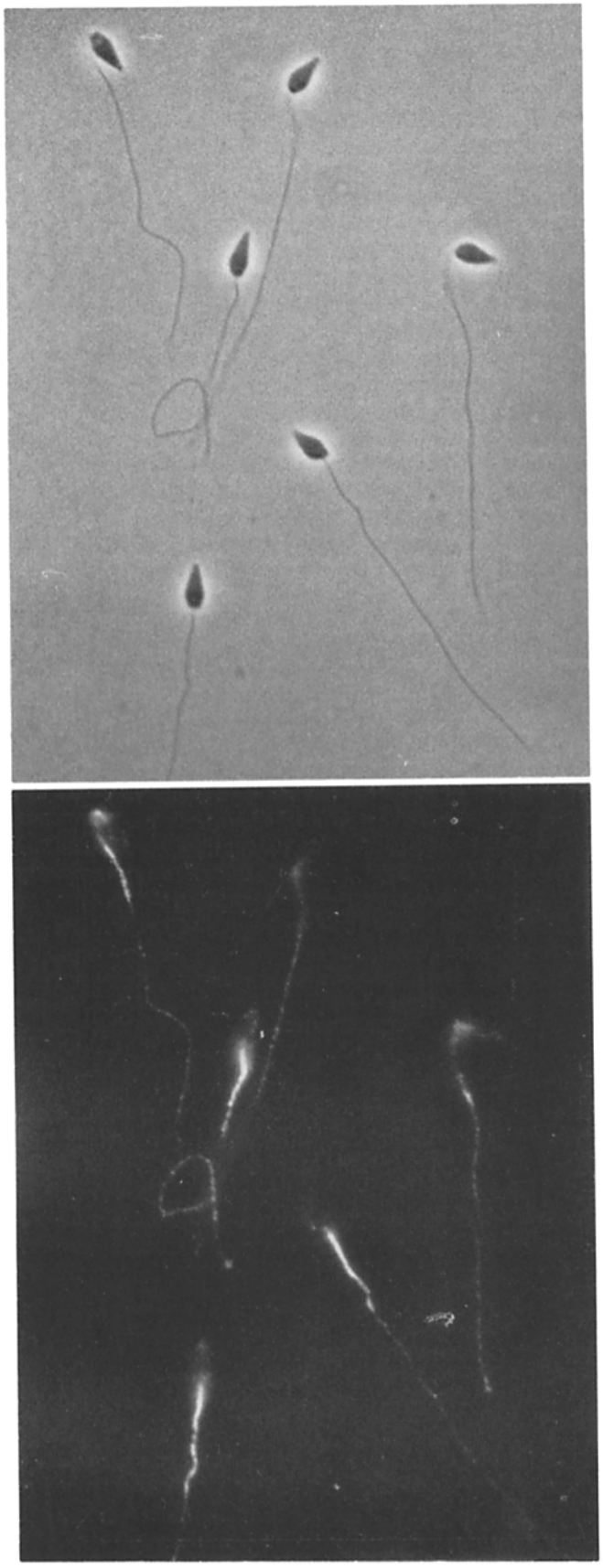

Figure 9. Indirect immunofluorescence of the reaction of anti-190 serum with sea urchin sperm. Preimmune serum did not react with the cells. At a 1:8 dilution of absorbed serum, fluorescence is concentrated on the proximal (anterior) portion of the flagellum. A much weaker reaction is seen on the posterior portion of the flagellum and sperm head. (*Top*) Phase contrast; (*bottom*) rhodamine fluorescence.

preimmune-treated cells. However, in the anti-190–treated cells, the fluorescence is intensely concentrated on the proximal one-half of the length of the flagellum (Fig. 9). There is only a weak reaction along the distal half of the flagellum and on the cell membrane of the sperm head.

Discussion

Our interest in the M_r 190,000 protein began with the finding that it becomes phosphorylated (Bookbinder, L. H., G. W. Moy, and V. D. Vacquier, manuscript submitted for publication) when [γ-^{32}P]-ATP is added to sperm membrane vesicles prepared by N_2 cavitation (30) and that it binds to CaM-agarose and is eluted by EGTA. In this paper we present evidence gained by the use of a monospecific polyclonal antiserum that this protein is at least one form of sea urchin sperm adenylate cyclase. Indirect immunofluorescence shows that this AC is concentrated on the proximal part of the sperm flagellum. This is the first report of the identification of an AC of animal spermatozoa. Unlike somatic cell AC, spermatozoal AC is not activated by forskolin (3), and there are no direct data supporting its activation by G-proteins (2). Spermatozoal AC is also unusual in showing a high Mn^{++}-supported activity (3).

Calmodulin-agarose affinity chromatography of a 40,000 *g* supernatant of Brij-78 extracted sperm yields a great number of major protein bands (Fig. 1). This eluted fraction, termed the CaM-El, is a rich source of AC activity. The great number of proteins in the CaM-El does not imply that they are all regulated by CaM. The binding of the protein to CaM merely depends on possession of a positively charged amphiphilic α-helix (16). The proteins in the CaM-El could be aggregates of many individual proteins only one of which binds CaM, since no attempt was made to alter the secondary structure of the proteins. After loading the preparation on CaM-agarose and washing with buffer B containing 200 μm $CaCl_2$, the agarose beads are themselves a rich source of AC activity, which can be subjected to various treatments as a solid state enzyme preparation. The fact that there is no apparent enrichment of AC specific activity in the whole cell lysate compared to the 40,000 *g* supernatant indicates much of the total AC activity sediments under these conditions. The AC activity of invertebrate sperm is fairly insoluble (5, 6, 18). In a previous paper from our laboratory (18) we showed that sea urchin sperm plasma membrane vesicles could be isolated by pH 9-induced lysis of the cells followed by differential sedimentation. There is only one protein of M_r 190,000 in this membrane isolate. Incubation of these membrane vesicles in 3% Triton X-100, 3% deoxycholate, or 3% SDS at 23°C for 60 min with stirring, followed by 170,000 *g* centrifugation (1–4 h) results in the M_r 210,000 and 80,000 membrane proteins remaining in the supernatant, whereas the M_r 190,000 and most other membrane proteins sediment. These data demonstrate that this AC is a relatively insoluble protein that only breaks down to a soluble subunit form when boiled in SDS and mercaptoethanol.

Even though electrophoretically purified M_r 190,000 pro-

tein was used as the immunogen, the crude rabbit antiserum cross-reacted with proteins at M_r 230,000 and 50,000 (Fig. 1). The fact that immunoglobulins to these two proteins could be removed by absorption to living cells shows that they are membrane components. This also shows that the antibodies to these two proteins are all directed to epitopes on the extracellular side of the cell membrane. We have no knowledge of the M_r 50,000 protein. However, we have previously shown that the M_r 230,000 protein (termed 210 kD in previous papers; reviewed in reference 29) is the most potent antigen on the extracellular surface of the sea urchin sperm plasma membrane (25, 29). By using sperm plasma membranes as the immunogen for monoclonal antibody production, 85 out of 96 positive hybridomas reacted with the protein (25). Of interest to this study is the finding that monoclonal antibodies to the M_r 230,000 ("210 kD") protein activate both calcium channel opening (26) and adenylate cyclase in live sea urchin sperm (29).

The absorbed "anti-190 serum" must react with multiple epitopes of the sea urchin sperm AC. In sea urchin sperm lysates, and the CaM-El, the absorbed serum is a potent inhibitor of AC activity. The same level of inhibition of AC activity is found in lysates of horse sperm, showing that at least part of the immunoglobulins are blocking the AC active site. Immunoaffinity chromatography of the sea urchin sperm CaM-El on an anti-190 kD protein A–Sepharose column depletes the AC from the flow through fraction (Fig. 6). If done in the presence of BSA, the washed antibody beads are a rich preparation of AC activity, showing that at least a portion of the immunoglobulin molecules are not directed against the AC active site. However, elution of the pure AC from the antibody column (Fig. 6) results in the total inactivation of AC activity. By densitometry the M_r 190,000 protein is ~10% of the total CaM-El protein. The enrichment for AC in the CaM-El is ~42-fold above that of the 40,000 *g* supernatant. The pure AC band eluted from the antibody column is thus enriched roughly 420-fold over the 40,000 *g* supernatant.

Regulation of the sea urchin sperm AC by CaM cannot be shown directly by a loss of activity in the presence of EGTA, and the subsequent reactivation of activity after addition of Ca^{2+} and CaM (3). To date, the only two sperm AC that have been shown to be regulated by Ca^{2+} and CaM in broken cell preparations are from the abalone (5, 6) and the horse (4). The calmidazolium and chlorpromazine inhibition curves (Figs. 7 and 8) for the AC activity in the CaM-El, and the restoration of AC activity by addition of bovine brain CaM are very similar to the data obtained for the CaM-sensitive AC of horse sperm (4). Our data are suggestive, but not direct proof, that the sea urchin sperm AC may be CaM regulated. One fact known about the sea urchin sperm AC is that its activation in live cells that encounter egg jelly depends on the presence of extracellular calcium (3). The activation of AC and the induction of the sperm acrosome reaction are temporally correlated and both depend on the entrance of Ca^{2+} through Ca^{2+} channels (3, 13). Calmodulin inhibitors such as trifluoperazine inhibit both the acrosome reaction and the AC activity (see reference 3 and Table I). However, there is as yet no direct evidence that AC activation and increased cAMP concentrations are required for the induction of the acrosome reaction.

An M_r of 190,000 for sea urchin sperm AC is in agreement with the size of AC from other cells. The AC of rat olfactory cilia, identified by use of a monoclonal antibody, has an M_r of 180,000 (17). Yeast AC catalytic subunits are M_r 180,000–205,000 (31).

The apparent concentration of sperm AC in the proximal region of the sea urchin sperm flagellum (Fig. 9) is a novel finding. This location is the part of the flagellum closest to the site of ATP production in the single large mitochondrion midpiece, which is at the posterior part of the sperm head. Localization of AC to the flagellum is in agreement with recent data showing that membranes of the flagellum compared to membranes from the sperm head are enriched 28-fold in AC activity (24). Flagellar membranes, relative to head membranes are also enriched 14-fold in cAMP phosphodiesterase and 6.5-fold in sites for the calcium channel antagonist D-600 (24). Values for enrichment of guanylate cyclase in flagella versus heads of sea urchin sperm vary from two- (24) to eight- (19) fold. Membrane preparations derived from sea urchin sperm flagella are enriched 8–15-fold in AC activity (13). In addition to components of the axonemal complex, other sea urchin sperm components located exclusively in the flagellum are the M_r 145,000 creatine kinase (23) and a transmembrane glycoprotein of unknown function defined by monoclonal antibody J17/30 (15, 25).

The initiation and maintenance of sperm motility in all types of animal sperm appears to be regulated by cAMP-dependent protein kinase (1, 3, 8, 11, 21, 22). The concentration in the flagellum of enzymes regulating cAMP levels (AC, cAMP phosphodiesterase) is probably involved in the motility process. One model addressing the role of A-kinase in flagellar motility (21) suggests that the phosphorylation of axonemal proteins is involved in microtubule sliding, and that a calcium-activated protein phosphatase (calcineurin) reverses the process. In this model a heterogeneous distribution of regulatory elements along the flagellum would be important (21). The data presented (Fig. 9) on the existence of a concentration gradient of AC along the sea urchin sperm flagellum supports such a model.

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