Journal of Cellular Biochemistry 25:99–107 (1984)

Calmodulin, a Calmodulin Acceptor Protein, and Calcimedins: Unique Antibody Localizations in Hamster Sperm

Pamela B. Moore and John R. Dedman

Bureau of Biological Research, Rutgers University, Piscataway, New Jersey 08854 (P.B.M.) and Department of Physiology and Cell Biology, University of Texas Medical School, Houston, Texas 77025 (J.R.D.)

A calmodulin acceptor protein has been identified in isolated hamster caudal sperm by immunofluoresence and Western transfer techniques. The protein shows a localization in sperm heads identical to calmodulin. Fluorescence of both calmodulin and the acceptor protein are lost by treatment with $MgCl_2$, conditions which release the acrosome. These results are consistent with the proposed function of calmodulin in a sperm function.

Key words: calmodulin, calmodulin acceptor protein, calcimedins, sperm acrosome, antibody localization

Mammalian spermatozoa are a unique tissue for biochemical studies since functional and morphological compartmentalization are discrete and complementary. Thus the spermatozoan can be considered to be divided into two major functional portions. The head contains the acrosome, a caplike structure which encases the genomic material. It serves two functions: as protection for the nucleus with its tightly packaged DNA and as a "modified lysosome." The diverse hydrolytic enzymes are thought to be essential for sperm penetration of the egg zona pellucida [1]. Cytochemical and immunologic methods have detected peroxidase [2], hyaluronidase [3], acrosin [4,5], and cyclic nucleotide phosphodiestase [6] in sperm heads.

The other major portion, the flagellum has a microtubular core surrounded by dense fiber and mitochondrial sheaths. Its major function is for motility.

Recently, calmodulin (CaM) has been localized in rabbit and guinea pig [7] and bull sperm [8]. Both reports localize CaM in the acrosome, in the postacrosomal and equatorial regions, and at the neck and tip of the flagellum. Feinberg et al [8] also reported CaM staining along the tail in the midpiece and principle-piece regions, whereas Jones et al [7] did not find staining in these regions. Localization of CaM in sperm together with data showing Ca^{++} dependence of the acrosome reaction has fostered the hypothesis that CaM functions in the acrosome reaction.

Received November 7, 1983; revised and accepted February 6, 1984.

Cyclic nucleotide phosphodiesterase has also been localized in heads, tightly bound to chromatin [6], and associated with the midpiece [9]. Unfortunately, the enzyme was not tested for CaM depletion or stimulation. Thus the CaM dependence or independence of the enzyme is not established and to date no report has firmly shown the presence of a calmodulin acceptor protein (CAP) in sperm. The results presented here show that a CAP isolated by affinity chromatography on a calmodulin-Sepharose matrix localizes in sperm heads in a region which also stains for CaM. This pattern is contrasted to that obtained with antibodies directed against two calcimedins [10].

METHODS

Isolation of Calmodulin Acceptor Protein

Chicken gizzard extracts were prepared by homogenizing the tissue in 0.075 M NaCl, 0.4 M TRIS-HCl pH 7.3, 0.5% NaN_3, and 2 mM edetic acid (EDTA;300 gm/750 ml). The homogenate was centrifuged at 9,000 rpm (Beckman J21, JA10 rotor) followed by 14,000 rpm (JA20 rotor) for 30 min each. The supernatant was divided into 100–125-ml portions and chromatographed in the same buffer on an ACA34 column (LKB resin). The column was 7.5 cm × 180 cm and flow rate was 30 ml per hr; 8-ml fractions were collected and the A280 was determined. The fractions were divided into 14 pools according to the protein peaks. Each pool was made 1-mm final concentration in $CaCl_2$ and chromatographed on a calmodulin-Sepharose affinity matrix.

The affinity matrix was prepared by suspending 1.5 gm CNBr-activated Sepharose 4B (Pharmacia) in 10 ml purified calmodulin (3 mg/ml) in 0.1 M $NaHCO_3$, 0.1 M NaCl, pH 8.5 The mixture was rocked gently at 4°C for 16 hr, washed, and any sites remaining blocked by 2 ml 2-aminoethanol in 10 ml 0.1 M $NaHCO_3$, 0.1 M NaCl, pH 8.3. Greater than 95% of the protein was coupled. The resin was packed into a 1.0 cm × 10 cm column. The $CaCl_2$ -adjusted pool (20–80 mg protein) was chromatographed at a flow rate of 20 ml per hour. The column was washed with 0.5 M NaCl, 0.04 M TRIS-HCl, pH 7.3, 0.5% NaN_3, 1mM $CaCl_2$ followed by 0.075 M NaCl, 0.04 M TRIS-HCl, pH 7.3, 0.5% NaN_3, 1 mM $CaCl_2$. A protein peak of 0.5–3 mg protein was eluted with 0.075M NaCl, 0.05 M TRIS-HCl, pH 7.3, 0.5% NaN_3, 1 mM EDTA.

Isolation of Polyclonal Antibody

Protein peaks from the calmodulin-Sepharose affinity column were analyzed by sodium dodecylsulfate (SDS) polyacrylamide gels and several isolations were combined. New Zealand white rabbits were injected with protein in Freund's complete adjuvant weekly for 4 wk (1 mg/ml, 0.1 mg/ml, 0.1 mg/ml, 0.1 mg/ml, each of 1.5 ml). Booster injections were of 1 mg protein. Blood was collected from the marginal ear vein. Polyclonal antibodies were obtained by chromatographing the crude serum on a CAP-Sepharose column.

The CAP proteins were coupled using the same procedure; 10–15 mg protein were coupled per gm of Sepharose-4B and packed into 0.7 cm × 10 cm columns. The column was washed with 0.1 M boric acid, 0.025 M sodium borate, 0.075 M NaCl, pH 8.0, followed by 0.2 M glycine, pH 6.0. Protein was eluted with 0.2 M

glycine, pH 2.8, and collected into 100-μl 1.0 M TRIS, pH 7.5, and the peak pooled and dialyzed against the borate saline buffer.

Isolation of Sperm

Male Syrian Golden hamsters aged 4–5 mo were anesthetised with ether and decapitated. The caudal epididymis was dissected free and washed in Dulbecco phosphate-buffered saline (DPBS). The tissue was transferred to 4 ml fresh DPBS and minced and teased with flat blade forceps to extrude mature sperm. Sperm were pipetted off and collected at 500g for 10 min. They were washed once. Heads were broken from the flagella by addition of DTT (2 mM final concentration) to the sperm in PBS. They were incubated for up to 90 min at 25°C followed by several rapid passages through a 26-gauge needle. Heads were recovered by sedimentation through 25% Percoll. Tails floated in a layer above the Percoll. The sperm heads were further treated as described in the text.

Immunofluorescence

Samples of whole untreated sperm and DTT-treated and $MgCl_2$ treated heads and flagellae were pipetted onto clean slides and fixed with 3% paraformaldelhyde in DPBS. After 30 min, the slides were washed three times and treated with 0.005% digitonin in DPBS for 3 min. The slides were again washed three times (20 min each; 30–50-μl antibody was added and the slides incubated at 37°C for 60 min followed by DPBS washes. The appropriate second antibody was added (30 μl) and the slides incubated at 37°C for an additional 30 min. Either fluorescein isothiocyanate (FITC) goat antirabbit or FITC-rabbit antisheep antibodies were used (Boehringer Mannheim). After additional washes with DPBS, the slides were mounted and sealed. Microscopic observation employed a Nikon F fluorescence microscope from 40× glycerin immersion ocular at the labortory of Dr. Fred Roisen, UMDNJ. Images were recorded on either Kodak Tri-X 400 ASA or Ektachrome 400 ASA film.

Calmodulin and calmodulin antibodies prepared in sheep and affinity column purified were prepared according to Dedman et al [11]. All antibodies were adjusted to $A_{280} = 0.1$ for use.

Analytical Procedures

Protein concentrations were determined using the Coomassie dye assay [12]. Electrophoresis followed the SDS disc gel system of Laemmli [13]. Antibody detection used the Western transfer method of Towbin et al [14]. Proteins were transferred for 16–20 hr at 12 V onto 0.2-μm nitrocellulose paper (Schlecter and Schuell). Blocking of nonspecific sites was with 5% (BSA) in DPBS. Specific antibody (35 μl) was diluted with 50 ml DPBS and incubated with the paper strip for 5 hr at 4°C. The paper was then washed for 2 hr with four changes DPBS. Peroxidase-coupled rabbit antisheep or peroxidase-coupled goat antirabbit was added (0.1 ml in 100 ml DPBS) and incubated 1 hr at 25°C. The strips were washed 1 hr in four changes of DPBS. Color was developed with o-dianisidine and hydrogen peroxide.

RESULTS

Chicken gizzard tissue was used as a source of calmodulin acceptor proteins (CAPs). Low salt extracts were divided into several protein size classes by chroma-

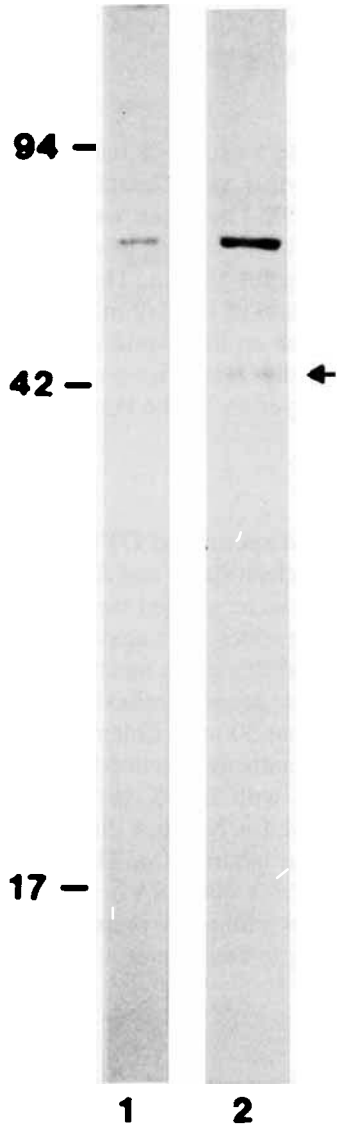

Fig. 1. Isolation of the 51–53 -Kd calmodulin acceptor protein (CAP) protein. Chicken gizzard tissue fractionated on an AcA34 column was pooled into several molecular weight ranges and rechromatographed on a calmodulin-Sepharose column with $CaCl_2$. Proteins were eluted by chelation with EGTA. Lane 1) A protein of apparent Mr 70–75 Kd Lane 2) A fraction containing proteins of ranges 80–40 Kd showed two protein bands, the 70–75-Kd protein and a less-abundant protein of 51–53-Kd apparent size.

tography on an AcA34 resin column in the presence of EDTA. Each pool was chromatographed on a CaM-Sepharose affinity matrix in the presence of calcium. Calcium-specific binding proteins were eluted with EGTA as in Methods. One major protein of apparent molecular weight 51–53 Kd was eluted along with a protein of 70–75 Kd (Fig. 1, lane 2). A higher Mr pool from the AcA34 column contained this higher Mr protein exclusive of the 51–53-Kd protein (Fig. 1, lane 1).

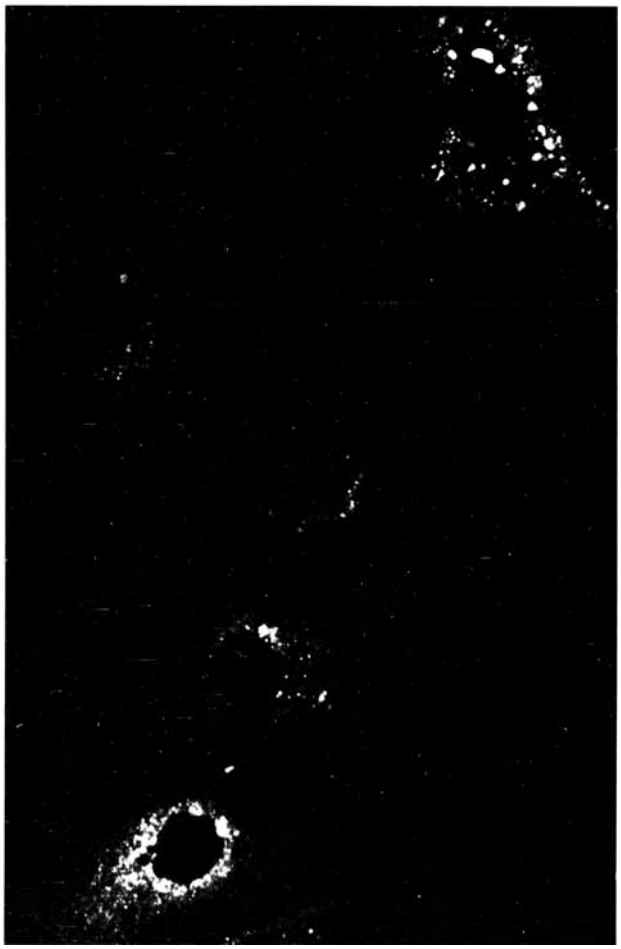

Fig. 2. CAP staining of cultured PtK_2 cells. Cells on coverslips were fixed, permeabilized and stained by indirect immunofluorescence with 30 μl 51–53-Kd CAP antibody.

Antibody to the 51–53 Kd protein was prepared by passage of crude immune sera over an affinity matrix made by coupling the higher Mr protein to CNBr-activated Sepharose. Antibody passing through the column was then passed through a column prepared using the two proteins shown in Figure 1, lane 2. In this way the antibody to the lower Mr protein was obtained.

The antibody to the 51–53 Kd protein was localized in PtK_2 cells fixed to coverslips and stained according to that outlined under Methods for sperm. Figure 2 shows intense staining in what appears to be cytoplasmic, perinuclear vesicles (phase-dense). The staining intensity was variable from cell to cell and could be seen to extend well into the cell periphery in many cells. Western transfer analysis of PtK_2 cell homogenates, rabbit muscle myofibrils, and rat liver plasma membranes and microsomes did not show crossreactivity to any protein band in these fractions using the purified CAP antibody.

Since sperm have well-defined structural compartmentalization of specific organelles, sperm were isolated and prepared for microscopy. The staining of the acceptor protein antibody was compared with that for calmodulin and two of the recently reported calcimedin proteins [10].

Figure 3 shows a composite of the staining pattern for calmodulin and its

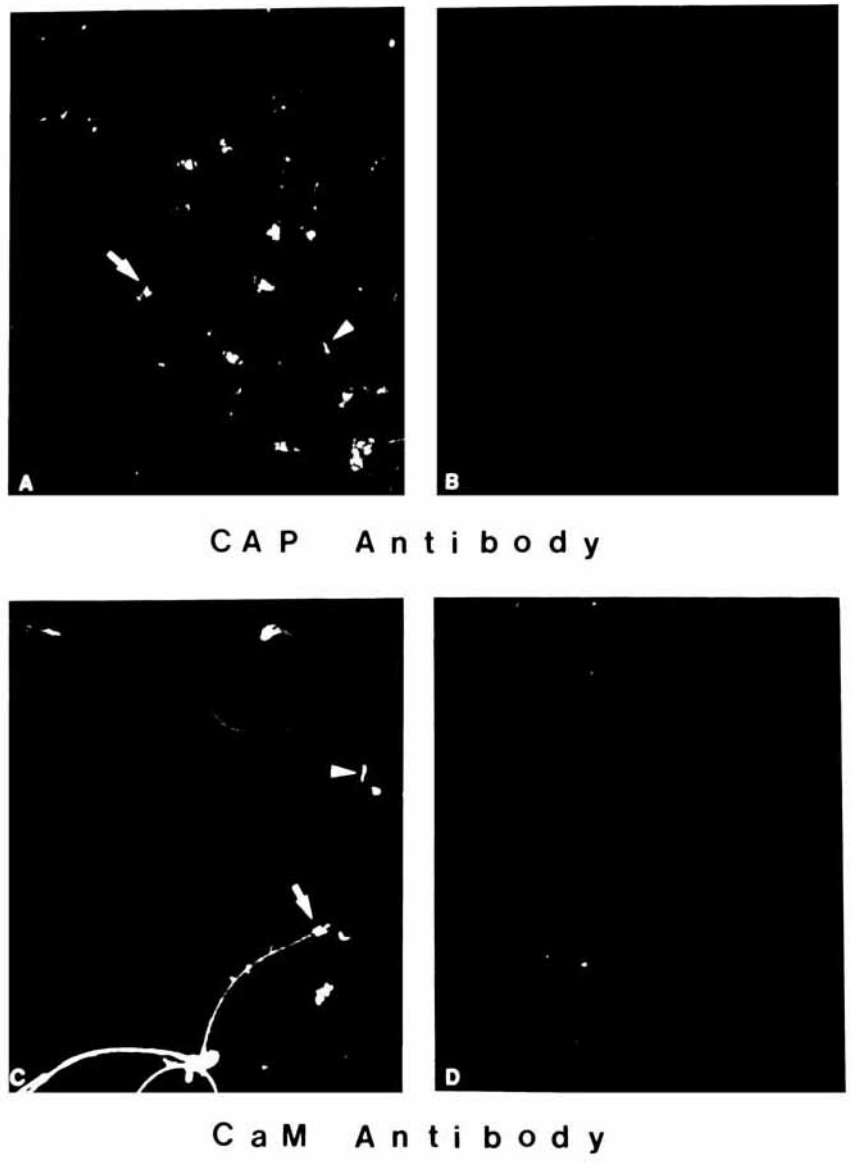

Fig. 3. Comparison of staining patterns for the CAP and calmodulin (CAM) in hamster sperm (50-μl CAP antibody, 30-μl CaM antibody). A) CAP staining in isolated sperm heads after treatment with DTT. B) Sperm heads treated with 2 mM $MgCl_2$ in DPBS for 5 min prior to staining with CAP antibody. C) Whole sperm stained with anti-CaM. D) Sperm treated with $MgCl_2$ prior to anti-CaM staining. Arrows point to equatorial region of sperm heads. Arrowheads point to anterior acrosomal regions.

acceptor protein. Sperm heads were severed from the flagellae as in Methods. The heads fraction was recovered and washed free of Percoll and divided into two portions. One portion was incubated with 5 mM $MgCl_2$ for 15 min followed by a brief vortexing to dislodge the acrosome. The second portion was untreated. In panels A and B, control sperm heads and sperm heads treated with $MgCl_2$ were stained with antibody to the CAP. A distinct band is present in the equatorial region (arrow) and the anterior most region of the cap (arrowhead). The staining was removed by $MgCl_2$ treatment. Anticalmodulin staining is shown in panels C and D. Both the antrior portion of the cap (arrowhead) and the equatorial region (arrow) also stained with anti-CaM. The acrosome staining was abolished by the $MgCl_2$ treatment, although the flagellum staining appeared to partially remain after $MgCl_2$.

Figure 4 contrasts the above results with two calcimedin antibodies: anti-67-K protein and anti-35-K protein. Both antibodies appeared to stain the flagellum but neither stained the head regions of the sperm. Arrows point to separated sperm heads in the preparation. They are unstained.

The two fractions of sperm, heads and tails, were isolated and solubilized for Western transfer. Figure 5 shows these results. Gels 1 and 2 are Coomassie-brilliant-blue-R250-stained lanes and 3 and 4 are corresponding lanes which were transferred to nitrocellulose for immunoblotting instead of visualization by Coomassie. As can be seen, a protein band of apparent Mr of 51–53 Kd was apparent in the heads portion. This band stained with the CAP antibody. Lane 2 did not have a major protein of this molecular size nor did the antibody cross-react with proteins in the tail fraction.

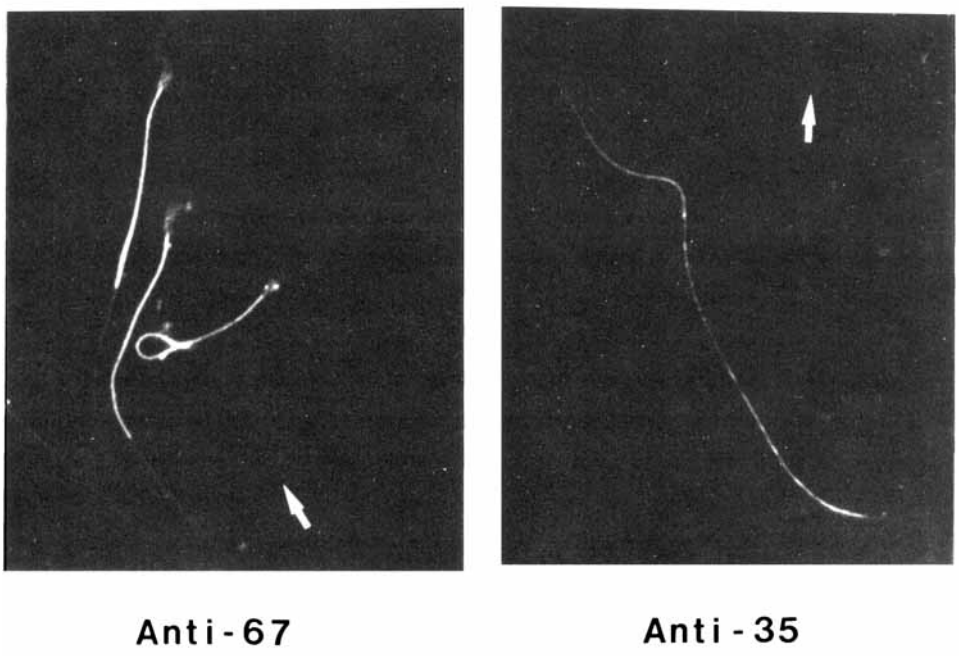

Fig. 4. Sperm staining with anticalcimedins. Left panel) Antibody to the 67-Kd calcimedin (30 μl). Right panel) Antibody to the 35-Kd calcimedin (30μl). Arrows point to sperm heads lying in the same field as the intact sperm.

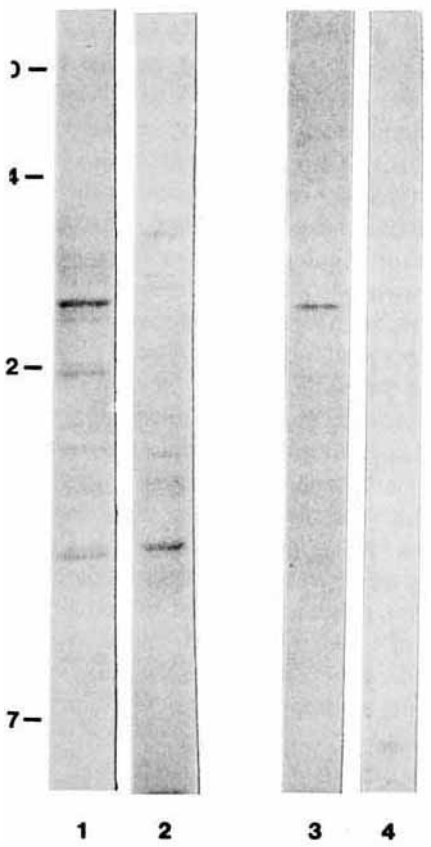

Fig. 5. Western transfer staining of sperm fractions. Sperm heads and flagella fractions were solubilized in Laemmli sample buffer and electrophoresed on 11.5% acrylamide gels (4.5% stacker) under conditions for Laemmli disc gels. Gel tracks 1 and 2 were fixed and stained with Coomassie brilliant blue R250. Gel tracks 3 and 4 were not fixed but transferred to 0.2-μm nitrocellulose paper and reacted for antibody staining as under methods. Gel 1) heads fraction of sperm, 27.0 μg protein. Gel 2) tails fraction of sperm, 21.5 μg protein. Gel 3) head proteins stained by anti-CAP, 27.0 μg protein. Gel 4) tail proteins stained by anti-CAP, 21.5 μg protein.

DISCUSSION

Calmodulin is widely recognized as a major calcium regulatory protein in eukaryotic cells, especially nonmuscle cells. These results are in agreement with the localizations for CaM previously reported [7,8], although they differ slightly from Jones et al [7] in that our anti-CAM also stained the midpiece portion. Of significance, however, is the colocalization of a CAP in the anterior acrosomal cap region and the equatorial region of the sperm head. These results significantly add to a presumed role of CaM in a sperm process. It is interesting that the equatorial region is also that region initially covered with microvilli at fertilization [9]. The staining pattern for both CaM and the CAP were lost by Mg^{++} treatment but not by DTT. Since $MgCl_2$ but not DTT has been reported to remove the acrosomal cap [15,16] it is likely that both CaM and the CAP are associated with this structure. Two calcimedin antibody

fractions, one specific for the 67 Kd gizzard protein and one for the 35 Kd protein [manuscript in preparation] were also used to stain the sperm. Neither antibody showed staining in the acrosomal region but rather stained the mid-piece and principle-piece areas. This staining pattern is consistent with their proposed separate function distinct from CaM. The staining also further supports the CAP-CaM relationship for CaM function.

Attempts to identify the CAP protein with a specific intracellular organelle fraction have, thus far, failed. It is a possibility that the protein is lost during the isolation procedures here employed. The staining pattern in both PtK_2 cells and sperm acrosomes suggests a possible lysosomal localization. Attempts to identify the protein in a lysosomal rich liver fraction have also been unsuccessful as yet. The strong staining patterns seen for the CAP, however, suggest that the protein is involved with a specific CaM-regulated process.

ACKNOWLEDGMENTS

The authors acknowledge Drs. William Dubinsky and Naomi Kraus-Friedmann of the Department of Physiology and Cell Biology, U.T. Houston, for generous gifts of plasma membranes and microsomes. We also thank Dr. Rita Colella, Rutgers, for lysosome preparations. PtK_2 cells grown on coverslips were the gift of Dr. Charles Connor, U.T. Houston. We acknowledge the excellent assistance of Ms. Jane Sherwood in preparation of the manuscript.

This work was supported by the Busch Memorial Fund and PHS RR07058-18 (P.B.M.) and NIH GM29323 and Research Career and Development Award (J.R.D.).

REFERENCES

1. Yanagimachi R: In "Fertilization and Embryonic Development *in Vitro*," Mastroiani L, Biggers JD (eds): NY Plenum: 1981, pp 81-82.
2. Graham RC, Lundholm U, Karnovsky MJ: J Histochem Cytochem 13:150-152, 1965.
3. Morton DB: J Reprod Fertil 45:375-378, 1975.
4. Garner DL, Easton MP: J Exp Zool 200:157-162, 1977.
5. Flechon JE, Huneau D, Brown CR, Harrison AP: Ann Biol Anim Biochim Biophys 17:749-758, 1977.
6. Bhatnager SK, Chaudhry RS, Anand SR: J Reprod Fertil 56:133-139, 1979.
7. Jones HP, Lenz RW, Palevitz BA, Cormier MJ: Proc Nat Acad Sci USA 77:2772-2776, 1980.
8. Feinberg J, Weinman J, Weinman S, Walsh MP, Harricane MC, Gabrion J, Demaille JG: Biochim Biophys Acta 673:303-311, 1981.
9. Bhatnager SK, Anand SR: Biochim Biophys Acta 716:133-139, 1982.
10. Moore PB, Dedman JR: J Biol Chem 257:9663-9667.
11. Dedman JR, Welsh MJ, Means AR: J Biol Chem 253:7515-7521, 1978.
12. Bradford MM: Anal Biochem 721:248-254, 1976.
13. Laemmli UK: Nature 227:680-685, 1970.
14. Towbin H, Staehelin T, Gordon J: Proc Natl Acad Sci 76:4350-4354, 1979.
15. Srivastava PN: J Reprod Fertil 33:323-326, 1973.
16. Millett CF, Spear PG, Gall WF, Edelman, GM: J Cell Biol 58:662-675, 1973.