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Most Egg Calmodulin Is a Follicle Cell Contribution to the Cytoplasm of the *Blattella germanica* Oocyte

YUJUN ZHANG AND JOSEPH G. KUNKEL

Biology Department, University of Massachusetts, Amherst, Massachusetts 01003

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A high concentration of calmodulin (CaM) appears in mid- to late vitellogenic cockroach follicles which composes 1.5% of total protein. CaM levels during oogenesis were estimated by densitometric analysis of immunoblots using anti-*Blattella germanica* egg CaM antibody as a probe. CaM accumulates in the follicle throughout the yolk deposition phase maintaining its highest level of accumulation during the later 6-fold increase in oocyte volume. Evidence suggests that this later accumulated CaM is synthesized by the follicle cells and deposited in the oocyte. *In vitro* experiments with [^{35}S]-met showed that the highly abundant CaM accumulating in vitellogenic follicles may not all be synthesized by the oocyte. Isolated follicle cells incorporate 13-fold more [^{35}S]-met into CaM than the oocytes themselves but do not accumulate the product. The follicle cells are capable of producing all the CaM observed in newly ovulated eggs. No CaM is detectable in transit in the hemolymph of the female. These facts argue that CaM produced by follicle cells is the most likely source of CaM in the vitellogenic oocyte. Indirect immunofluorescent staining with anti-egg CaM demonstrated that in early- and midvitellogenic follicles CaM is localized in the cytoplasm of follicle cells and the cytoplasmic compartment surrounding yolk granules of oocytes but is excluded from yolk granules. Immunofluorescence was most intense in the cortex of the oocyte and outside the membranes of yolk granules. Transport of CaM into the cytoplasmic compartment of the oocyte is possible without invoking traditional adsorptive endocytosis.

INTRODUCTION

In the oocytes of most insects, protein-yolk deposition is a process of receptor-mediated endocytosis, which requires both the maternal donation of serum vitellogenin (Vg) and the acquired competence by the oocyte to promote adsorptive endocytic uptake (Raichel and Dhadialla, 1992). Whatever the mechanism controlling the initiation of yolk deposition, vitellogenesis requires the oocyte to be fully equipped to take in vitellogenin and to process a large amount of storage molecules in an efficient manner. Calcium is involved in both binding of Vg to its receptor and internalization of Vg-receptor complexes in *Xenopus* (Opresko and Wiley, 1987) and in several insects, locust (Rohrkasten and Ferenz, 1985) and cockroach (König *et al.*, 1988).

However, calcium often does not act in its free ionic form but rather requires the presence of a binding protein, calmodulin (CaM), to mediate its effect (Means and Dedman, 1980). Indeed most germ cells are rich in CaM (Klee and Vanaman, 1982; Zhang and Kunkel, 1992a).

Abundant egg CaM may mediate events that are unique to or be exaggerated during oogenesis or early embryogenesis. The high level of CaM may be a prerequisite for oocyte commitment toward vitellogenesis. CaM could play several traditional roles in receptor-mediated endocytosis such as (i) assisting the assembly of coated vesicle structure, (ii) modulating interactions between coated vesicles and cytoskeletal elements (Linden *et al.*, 1981), (iii) regulating the Ca^{2+}-ATPase reported in coated vesicles (Blitz *et al.*, 1977), (iv) mediating the clustering of receptor-ligand complexes into coated vesicles (Salisbury *et al.*, 1980), (v) regulating active movement of the vesicles to areas of coalescence.

In order to answer these questions we have isolated and characterized CaM from newly ovulated eggs of *Blattella germanica* and prepared antibodies directed against this protein (Zhang and Kunkel, 1992a). We ask whether egg CaM is at high levels in the oocyte before ovulation and where and when it is synthesized and accumulated during oogenesis. Determining the site of synthesis and cytological localization of CaM may provide clues to its special role in oocytes and germ cells in general.

MATERIALS AND METHODS

Animals and Tissues

Developmentally synchronous cohorts of the cockroach *B. germanica* were used for experimentation (Kunkel, 1966). The stage of the terminal follicle is mea-

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sured in length which is highly correlated with days after feeding the adult at 30°C (Kunkel, 1973). In the text "follicle" will be used to indicate the intact oocyte including the surrounding follicle cell layer, and "oocyte" will refer to the follicle minus its follicle cell layer. "Follicle cells" are the separated follicular epithelium. The follicle cells were mechanically separated from the oocyte (Anderson and Kunkel, 1990).

Materials

L-[^{35}S]methionine (1066 Ci/mmole) was from New England Nuclear. Rhodamine-conjugated goat-anti rabbit secondary antibodies were from Sigma Chemical Co. A polyclonal monospecific anti-*B. germanica* egg CaM was prepared (Zhang and Kunkel, 1992a).

Gel Electrophoresis and Immunoblotting

Electrophoresis in the presence of 0.1% SDS was performed as described by Laemmli (1970) using a minigel system. CaM from follicles was estimated from immunoblots using purified egg CaM as a standard as described earlier (Zhang and Kunkel, 1992a). Immunoblot identified protein was quantified by densitometric scanning of the colored product. The data were collected and analyzed using a Megavision image analysis system. It should be noted that the polyclonal anti-egg CaM antibodies used in immunoblot analysis also have a weak cross-reaction with two high-molecular-weight SDS gel bands in the heat-stable fraction of crude oocyte extracts. It is suspected that these bands are actually CaM tightly bound to other proteins since these bands are also stained by commercial anti-bovine brain CaM (not shown). Total oocyte protein contents of different stages were measured according to Bradford (1976) using BSA as a protein standard.

Preparation of Heat-Stable Proteins

CaM is in general heat-stable and this property is used in an initial step of purification and analysis to eliminate the majority of other proteins (Zhang and Kunkel, 1992a). Tissues were obtained from Day 3-vitellogenic females by dissection in orthopteroid insect tissue culture medium S20 (Landureau, 1976). Extracts of tissues were done in 8 volumes of buffer H (40 m*M* Tris, 5 m*M* EGTA, 1 m*M* 2-mercaptoethanol, pH 7.5). The supernatants from centrifugation were brought to 90°C for 5 min and then rapidly cooled in an ethanol–ice bath (−20°C). Denatured proteins were removed by centrifugation and heat-stable proteins were precipitated by addition of four volumes of ethanol (−20°C). The ethanol precipitate was collected, dried under a stream of nitrogen, and resuspended in SDS sample buffer for electrophoresis.

Hemolymph was collected from Day 4-vitellogenic females and diluted with an equal volume of PBS (phosphate-buffered saline, pH 7.5) containing 0.5 m*M* phenylmethylsulfonyl fluoride. The diluted hemolymph was centrifuged to remove hemocytes and processed as described above to prepare heat-stable proteins.

In Vitro Labeling of Proteins

Medium S20 (− met) was formulated identical to medium S20 but minus methionine to facilitate labeling experiments. For each experiment tissues from Day 3- or Day 4-vitellogenic females were isolated and examined separately. They were incubated for 1 hr in 100 μl of culture medium S20 (− met) supplemented with L-[^{35}S]-methionine (0.5 mCi/ml, 1066 Ci/mmol). After incubation tissues were washed three times with culture medium S20.

Isolating Labeled CaM

After *in vitro* [^{35}S]methionine labeling, tissues were homogenized in 100 μl of buffer H, and heat-stable proteins were prepared. In a gel-separation approach, CaM was immunoprecipitated from supernatants derived from 40 oocytes using rabbit anti-egg CaM. The resulting pellets were washed and resuspended in SDS sample buffer (Laemmli, 1970). After 15% SDS–PAGE the gels were stained with Coommassie blue and fluorographed for 24 hr.

In an affinity column approach, the heat-stable proteins from follicles cells and oocytes derived from 70 follicles were bound to a W-7 agarose column and eluted with EGTA as described previously (Zhang and Kunkel, 1992a). The CaM fractions were pooled and disintigrations per minute were estimated by liquid scintilation spectrometry. The four residues of methionine per mole of CaM (Zhang and Kunkel, 1992a) and specific activity of the methionine in the culture medium were used to estimate the amount of CaM synthesis. Assuming no knowledge of the endogenous pool of met allows an estimate of the minimum CaM that each tissue can synthesize; the actual rate of synthesis can only be higher.

Immunohistochemistry of Vitellogenic Follicles

Vitellogenic follicles (Day 3, about 0.5 mm long and Day 4, about 1 mm long) were fixed in 4% paraformaldehyde + 0.1% glutaraldehyde for 2 hr and then permeabilized with 0.15% Triton X-100 for 5 min. The follicles were then infiltrated with 0.8 *M* sucrose for 2 hr, 1.6

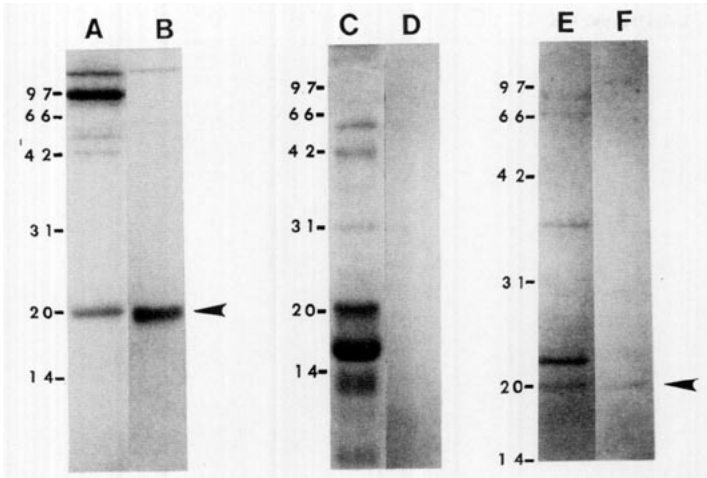

FIG. 1. Immunochemical identification of CaM from vitellogenic follicles, hemolymph and fat body using a polyclonal antibody against egg CaM. (Lanes A, C, and E) Coomassie blue stained SDS-PAGE of heat-treated Day 4 vitellogenic follicle extract, vitellogenic female hemolymph and fat body, respectively. (Lanes B, D, and F) Immunoblot analysis on lanes A, C, and E (1:5000 dilution of anti-egg CaM). The SDS-PAGE acrylamide concentration is 15% for lanes A and C and 13% for lane E.

M sucrose for 3 hr, and 2.4 *M* sucrose overnight. These follicles were cryosectioned and semithin sections (about 6 μm) were examined by indirect immunofluorescence as described below.

Sections were air-dried for 2 hr, postfixed in 4% paraformaldehyde + 0.1% glutaraldehyde for 10 min, put into 0.1% NaBH4 (in PBS) for 30 min, washed twice (5 min each) in PBST (phosphate-buffered saline containing 0.1% Triton X-100), incubated for 1 hr on ice in aldehyde-quenching buffer (PBST prepared with 150 m*M* glycine), and washed three times in PBST (3 × 5 min). Before labeling with primary antibody, sections were preincubated for 2 hr in blocking solution (6% BSA + 0.2% Tween 20 + 5% goat serum in PBS) to reduce nonspecific binding sites. Sections were then stained with rabbit anti-egg CaM (primary antibody staining, 1:25 dilution) overnight at 4°C (about 16 hr), rinsed thoroughly in PBST (5 × 5 min) and incubated for 2 hr with rhodamine-conjugated goat anti-rabbit antibodies (secondary antibody staining, 1:100 dilution). Stained sections were washed three times and mounted in antibleaching mounting buffer (5% *n*-propyl gallate in 50% glycerol). Immunofluorescence was viewed with a Nikon Optiphoto microscope equipped with differential interference contrast and epifluorescence, and recorded on Kodak T-MAX 400 film. Pictures were taken of control sections which were incubated with either primary or secondary antibody alone. Control pictures showed no fluorescence at exposure times equaling or exceeding that for the experimental material (not shown).

RESULTS

Egg CaM Originates in Follicles

To explore the likelihood that the abundant CaM of eggs preexisted in vitellogenic follicles, immunoblot analysis of CaM was conducted on Day 4 follicles (~1 mm long), just prior to the peak of vitellogenesis. Anti-egg CaM antibody identifies one heat stable major protein band in vitellogenic follicles (Fig. 1, lanes A and B (arrowhead)) with a molecular weight appropriate to CaM. A high-molecular-weight minor band cross-reacts weakly with anti-egg CaM antibody. This band may represent CaM bound to a CaM-binding protein present in the crude follicle extracts since anti-bovine brain also identifies such a band and CaM is known to be strongly bound to a number of high-molecular-weight proteins.

CaM Content during Oogenesis

Using anti-*B. germanica* egg CaM antibody as a probe, CaM levels in follicles during oogenesis were estimated by densitometric analysis of immunoblots. CaM levels change as a proportion of total follicle proteins during the vitellogenic cycle (Fig. 2). CaM content was about 0.6% relative to the total protein in Day 1 and Day 2 early vitellogenic follicles (length under 0.4 mm), and rapidly increased up to 1.6% of total protein during Days 3 and 4. From Day 4 through Day 6 vitellogenic follicles (length between 0.5 and 2.0 mm), CaM was maintained at about 1.5% of total protein. This indicates that CaM had been continuously synthesized and

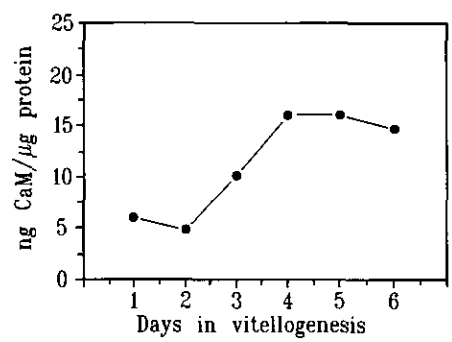

FIG. 2. Quantitative analysis of CaM during the vitellogenic phase of oogenesis. For each stage of development, the total protein of two ovaries was extracted with 200 μl buffer. After 90°C heat-treatment the heat-stable proteins from 200 μl crude extract were resuspended in 40 μl electrophoresis sample buffer and 5 μl of heat-stable proteins was loaded per well. Purified *B. germanica* CaM was used as standard. The densitometric quantization of the immunoblots demonstrate that substantial CaM is present in early vitellogenic follicles (Day 1 and Day 2). The dramatic increase of CaM correlates with the onset of rapid yolk deposition (after Day 2), and is maintained during latter vitellogenesis (from Day 4 to Day 6).

accumulated during the adsorptive endocytotic incorporation of Vg into *B. germanica* oocytes.

Search for CaM in Vitellogenic Fat Body and Hemolymph

The high content of CaM accumulated during vitellogenesis raised the question of its tissue of origin. We explored the possibility that CaM originates from fat body and hemolymph using anti-egg CaM antibody in immunoblotting technique. In order to concentrate CaM, thus increasing the sensitivity of the assay, extracts of these tissues were heat-treated, and only heat-stable proteins (Fig. 1, lanes C and E) were probed with anti-egg CaM. CaM was found in small amounts in vitellogenic fat body (Fig. 1, lane F), but was absent from hemolymph (Fig. 1, lane D). Thus while fat body may synthesize CaM for its own function, apparently it does not secrete, nor does any other tissue secrete, CaM into the hemolymph.

Ovarian Sites of CaM Synthesis and Accumulation

To delineate further the site of CaM synthesis in vitellogenic ovaries the follicular epithelium and oocytes were separated before being incubated in culture medium containing [^{35}S]methionine. Fluorescence microscopy demonstrated that mechanical separation of the two tissues did minimal damage. Follicle cells, which distinctly fluoresce more highly due to abundant actin (Zhang and Kunkel, 1992b) were not seen on the defolliculated oocyte surface.

CaM synthesis was determined by separate *in vitro* labeling of follicle cells and defolliculated oocytes followed by immunoprecipitation with anti-egg CaM antibody. L-[^{35}S]methionine-labeled proteins were visualized by SDS–PAGE and fluorography (Figs. 3A–3D). Labeled CaM (20-kDa band) was seen only from immunoprecipitates of follicle cell preparations (lane A) not from defolliculated oocyte preparations (lane C). Immunoblot analysis demonstrated, however, that abundant CaM was present in both follicular epithelium and in defolliculated oocytes by staining with Coomassie blue (Figs. 3E and 3F) as well as Western blot (Figs. 3G and 3H). These results suggested that the follicle cells were an active site of CaM synthesis and that the CaM found in oocytes was not under active synthesis there and must result by accumulation from outside the oocyte. Several high molecular weight radioactive proteins were also present (Fig. 3A) in the immunoprecipitate of follicle cell CaM. These may be tightly associated CaM-binding proteins which coprecipitate with CaM and antibody to CaM. These findings suggest follicle cells to be a possible source of oocyte CaM.

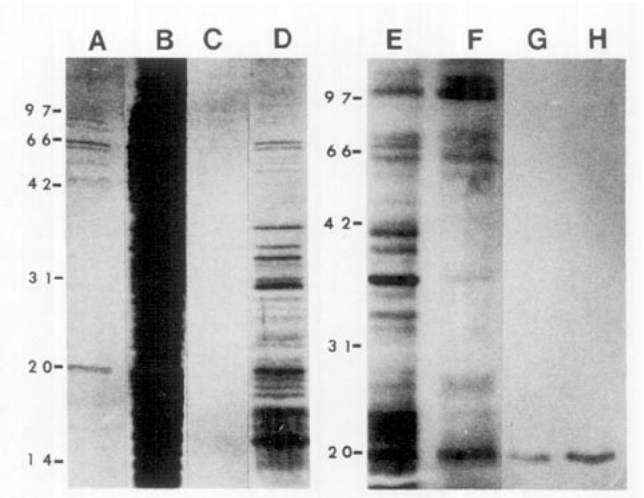

FIG. 3. Calmodulin synthesis in follicle cells and defolliculated oocytes. Follicle cell layer and defolliculated oocytes derived from ~60 Day 3 follicles were separately incubated in culture medium containing 50 μCi [^{35}S]-met (1066 Ci/mmol) for 1 hr at 30°C. After labeling the tissues were homogenized in 100 μl of homogenizing buffer and centrifuged at 10,000 rpm for 20 min. The supernatants were heated to 95°C for 5 min and the heat-stable fractions were immunoprecipitated with 40 μl anti-egg CaM antibody overnight. The immunoprecipitated pellets were resuspended in SDS sample buffer. (Lanes A–D) ^{35}S-met-labeled proteins were visualized by fluorography after 15% acrylamide SDS–PAGE. (Lanes A and C) ^{35}S-met-labeled proteins immunoprecipitated with anti-egg CaM antibody. (Lanes B and D) Total ^{35}S-met-labeled proteins. (Lanes A and B, follicle cells; lanes C and D, defolliculated oocytes.) (Lanes E–H) CaM in heat-stable extracts of follicle cells and defolliculated oocytes analyzed by immunoblot. (Lanes E and F) Coomassie blue staining patterns of heat-stable proteins from follicle cells and defolliculated oocytes, respectively, separated by 12% acrylamide SDS–PAGE. (Lanes G and H) Immunoblot of lanes E and F using a 1:5000 dilution of anti-egg CaM.

Synthesis of CaM in Oocyte and Follicle Cells during High CaM Accumulation Phase?

A minimum rate of CaM synthesis in Day 3.5 oocytes and follicle cells (~84th hour of vitellogenic cycle) was estimated by purifying CaM synthesized by the separated follicle cells and oocytes from 70 follicles in organ culture in culture medium with labeled methionine. While we have not yet determined the pool sizes for CaM in either the oocyte or the follicle cell layer, we proceeded to calculate a CaM synthesis rate assuming that rapid equilibrium of the internal pools were achieved in the tissue culture medium. This assumption leads to a lower limit estimate for CaM synthetic rate, leaving open an upper limit. Assuming uniform labeling of the four residues of methionine in CaM allowed calculating a minimum amount of CaM synthesized by each tissue in 1 hr (Table 1). Thus the follicle cells surrounding one follicle, which are a minor part of the mass of the follicle, can produce at least 1.28 ng of CaM in 1 hr. A similar estimate for the oocyte is 13-fold lower; however, this esti-

TABLE 1
ESTIMATION OF THE LOWER LIMIT OF CALMODULIN SYNTHESIS IN ORGAN CULTURE

Treatment	Synthesis by follicle cells	Synthesis by oocyte
dpm ^{35}S-met incorporation	41,827	3379
nCi of ^{35}S-met incorporated	18.84	1.52
pm CaM synthesised in one hour	4.71	0.38
ng CaM/follicle (70 follicles)	1.28	0.10

mate may be adversely affected by endogenous unlabeled met pool sizes.

Localization of CaM in Vitellogenic Follicles

Is the location of CaM in ovaries consistent with CaM being synthesized in follicular epithelium and accumulating in developing oocytes? The localization of CaM at different stages may help to understand the path this protein takes during vitellogenesis. In previtellogenic follicles (Fig. 4a) and in Day 3 follicles (about 0.5 mm long, with newly formed yolk spheres, Y, at the periphery of the oocyte, Fig. 4b) CaM appears in follicle cells, at the periphery of the oocyte, and in the central core at similar staining intensities, suggesting CaM was present in the oocyte prior to yolk deposition. Immunoblot analysis (Fig. 2) also supports this conclusion and further suggests that the level of CaM is lower at these earlier times.

Immunostaining of vitellogenic follicles with anti-egg CaM antibody revealed a diffuse distribution of CaM through the cytoplasm of follicle cells (Figs. 4c and 4d) and ooplasm (Figs. 4e and 4f). However, CaM is strikingly concentrated in the oocyte cortex (Fig. 4d arrow) and also localized as a rim around yolk granules (Fig. 4f, arrow). The insides of yolk granules, in contrast to the ooplasm, are CaM free. At two times, Days 3 and 4 and after chorionation, isolated follicle cells could be examined. Isolated follicle cell layers at Day 4, the beginning of rapid vitellogenesis, demonstrated a diffuse distribution of CaM throughout the cytoplasm (Fig. 4c). In follicle cells isolated postchorionation there were randomly distributed brightly stained punctate distributions of CaM on top of the uniform background cytoplasmic distribution (Fig. 4d). The double nuclei (N) present in each follicle cell, a peculiarity of this terminally differentiated tissue (Zhang and Kunkel, 1992b), appear unlabeled by the CaM antibody, an internal negative control for CaM.

Newly formed yolk spheres, both large and small, are free of CaM immunostaining. Immunofluorescence is found in the ooplasm and closely applied to the outside of yolk granules. This suggests that *in vivo*, despite the reported ability of Vt to bind CaM (Zhang and Kunkel, 1992a), CaM is not associated with vitellin inside the yolk granules of the developing oocytes, at least until Day 5 of vitellogenesis.

DISCUSSION

Much of the cell machinery and stored material of the early embryo is clearly of maternal origin (the sperm brings very little to the egg) but where it is synthesized can vary widely. During early embryogenesis the first detection of nuclear transcription in most insects is at the blastoderm stage and the newly synthesized proteins, "embryonic proteins," originate most likely from maternal mRNA (Sander *et al.*, 1985). The major yolk storage proteins, found in the yolk granules, are mainly synthesized outside the oocyte and transported in by adsorptive endocytosis. But what of the myriad of cytoplasmic proteins which we generally do not think of as yolk proteins? The majority of the CaM found in newly ovulated eggs of *B. germanica* (Zhang and Kunkel, 1992a) is synthesized prior to ovulation and likely outside the oocyte since the endogenous rate of oocyte CaM synthesis was found to be low. This low rate of CaM synthesis, even if corrected for a larger pool size of methionine, may be insufficient to produce the CaM present by ovulation; however, the rate of synthesis by the follicle cells at 84 hr into the vitellogenic cycle could provide the observed CaM.

If that rate were maintained proportional to the size of the growing oocyte over hours 72 to 144 of the reproductive cycle the follicle cells would have synthesized at least 336 ng of CaM while the oocyte would have synthesized at least 26 ng of CaM. This minimum amount which the follicle cells are calculated to be able to produce is close to the amount of CaM found per egg at ovulation (Zhang and Kunkel, 1992a). If pool sizes of unlabeled met are very high, particularly in the bulky oocyte, then the small amount demonstrated to be produced by the oocyte might be hiding a greater capacity for the oocyte to produce its own calmodulin.

CaM exists in both previtellogenic and early vitellogenic follicles but a marked increase in its relative titer occurs during the peak of vitellogenesis. It is continually accumulated at high levels during yolk deposition. While CaM is of general importance in regulating a variety of cellular functions (Means and Dedman, 1980; Cheung, 1980), the massive accumulation and titer changes in oocytes and storage in newly ovulated eggs must reflect important roles that CaM plays in exagerated events of vitellogenesis or early embryogenesis.

Potential Roles for the CaM of Oocytes

In insects the massive deposition of yolk proteins occurs primarily via receptor-mediated endocytosis (Kun-

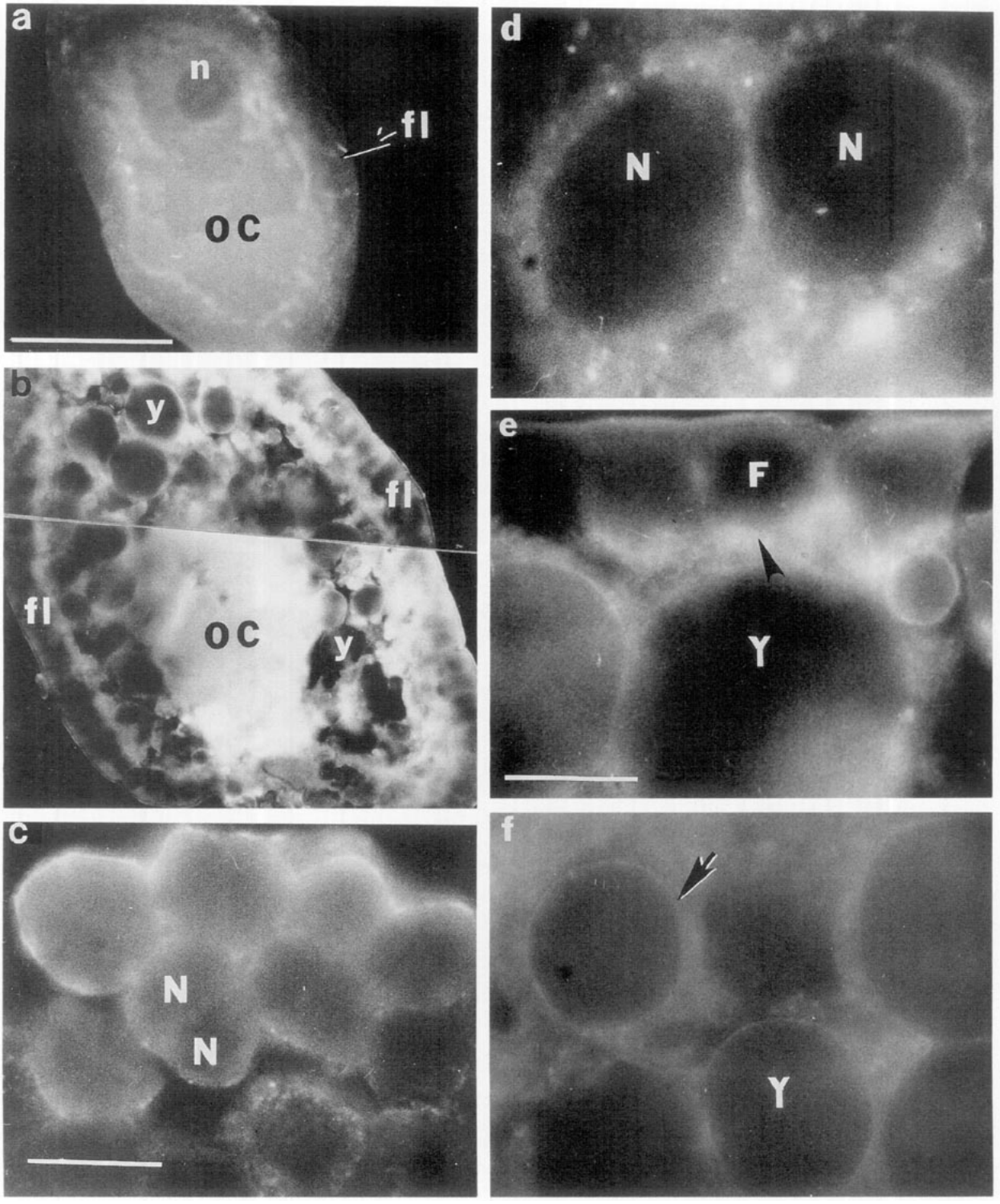

FIG. 4. Localization of CaM in cryosections of oogenic follicles (a, b, e, f) or isolated follicle cells (c, d) from *B. germanica*. (a) Localization of CaM in a previtellogenic (subterminal) follicle shows a diffused distribution of CaM present in cytoplasm with unlabeled nucleus. Because follicle cells are interlocked with oocyte plasma membrane at this stage it is hard to distinguish them under this low magnification. (b) Localization of CaM in an early (Day 2) vitellogenic oocyte show the brightly stained ooplasm, the cortex of oocyte, and follicle cell layer (fl) as well as unstained yolk granules and follicle cell nuclei. At this stage the yolk granules only appear in the ooplasm along the follicular epithelium. Note the bright rim of immunostain around individual yolk granule. fl, follicle cell layer; n, oocyte nucleus; OC, oocyte; y, yolk granules. (c, d) Fluorescence micrographs demonstrate the even distribution of CaM through the cytoplasm of vitellogenic follicle cells (c) as well as puctate localization in postvitellogenic follicle cells (d). Nuclei of late Day 6 follicle cells are free of CaM (d). Bright staining of nuclei in c

kel and Nordin, 1985; Raikhel and Dhadialla, 1992). While the fat body of insects is the major source of extrinsic yolk proteins, minor and major follicle cell contributions to yolk have also been documented in several insect species particularly in the Diptera and Lepidoptera (Raikhel and Dhadialla, 1992). It has been demonstrated elsewhere that CaM plays an important role in receptor-mediated endocytosis (Salisbury *et al.*, 1981). But CaM is a minor component of the coated vesicles (Salisbury *et al.*, 1980) constituting about 1% of total protein in purified coated vesicles (Linden *et al.*, 1981). This level of involvement, or better, would be necessary throughout the oocyte for the 1.5% composition to be explained. The localization of CaM in the ooplasmic compartment is consistent with a potential role in the machinery of endocytosis. During active deposition of yolk numerous coated pits pinch off from the oolema and coated vesicles migrate through the ooplasm. Although the cell membrane and coat materials may be recycled to oolema (Goldstein *et al.*, 1979), the formation of new coated pits, new coated vesicles, and the increase of oocyte size demand large cytoplasmic pools of membrane precursors, receptor proteins, clathrin, and other coat materials. The movement and recycling of these resources and storage material in vesicles or associated with the cortical cytoskeleton may be dependent on microfilament or microtubule-based motility systems which both use CaM in their structure and function (Salisbury *et al.*, 1981).

Even after considering such other functions associated with the machinery of endocytosis, the amount of CaM in the oocyte still seems excessive. The total protein in the oocyte, of which CaM composes 1.5%, includes the vitellin which makes up about 85% of the total egg protein. Since CaM is excluded from the yolk granules its percentage of the cytosolic protein most likely is an order of magnitude higher (i.e., 10%) in the cytosol. This amount of CaM is sufficient to bind in a 1:1 stoichiometry with the oocytes M_r 1,500,000 vitellin trimers. We have previously demonstrated using a gel overlay method that CaM binds to the M_r 95,000 subunit of *B. germanica* vitellin (Zhang and Kunkel, 1992a). There are six of these subunits per vitellin trimer. However we have shown that CaM is excluded from the vitellin compartment prior to ovulation. CaM may gain access to the vitellin compartment later during Day 5 of embryogenesis when vitellin utilization begins and this may be an important regulatory step in yolk degradation at that time (Purcell *et al.*, 1988a,b). A role in a massive process such as yolk degradation might explain the large amounts of CaM in oocytes.

Follicle Cells as the Source of CaM

We suggest that the follicle cells supply much of the CaM of the oocyte during mid to late vitellogenesis. The oocytes of panoistic ovaries are surrounded by a single layer of follicle cells. During oogenesis the participation of the germ nucleus in protein synthesis is reported by many workers to be minimal. This has been corroborated for CaM by our protein incorporation experiments in *B. germanica* oocytes (Fig. 3B and D) and by ultrastructural studies of oogenic follicles (Kimber, 1980).

Several follicle cell products which may interact with Vg and/or stimulate its endocytotic uptake have been postulated (Anderson and Telfer, 1969, 1970; Deloof *et al.*, 1972). Akin to our observations, Glass and Emmerich (1981) isolated a 18.5-kDa polypeptide from the vitellogenic follicular epithelium of locust. They suggested that this polypeptide plays an important role in the endocytotic uptake of locust Vg into the growing oocyte.

Our *in vitro* synthesis experiments suggest that oocyte CaM is more likely synthesized by follicle cells than by the oocyte itself. While CaM is evenly distributed in the ooplasm of previtellogenic follicles, it appears concentrated at the cortex of the oocyte during yolk deposition. We interpret this to indicate that the lower amount of CaM present in previtellogenic oocytes might be an endogenous oocyte product but during yolk deposition the larger amounts of CaM would be provided by follicle cells. The enhanced immunofluorescence of CaM in the oocyte cortex and adjacent cytosol of follicle cells is circumstantial evidence that CaM synthesized in follicle cells is actively transferred to the oocyte, since it does not accumulate in the follicle cells. The continually expanding volume of the oocyte cytoplasm is a large sink for the follicle cell CaM. In addition, the intense immunofluorescence at the periphery of oocytes is consistent with a cell cortex site of CaM accumulation and action.

Route of CaM Entry into Oocytes

Permeable gap junctions are a possible direct entry for CaM into the ooplasm from the follicle cells. In the mammalian ovary the oocyte–granulosa cell gap junctions have been suggested as an avenue of maintaining meiotic arrest in the oocyte by transmitting an inhibitor from the follicle cells (Anderson and Albertini, 1976). In

is due to the thickness of the follicle cells at this stage (the onset of vitellogenesis). N, follicle cell nuclei. (e, f) Day 4 (~1 mm)-vitellogenic follicle with anti-CaM localization of intense cortical staining of oocytes (e, arrowhead). The inside of yolk granules (Y) appear unlabeled, while the cytoplasm adjacent to yolk granules are brightly stained particularly in deeper ooplasmic areas (f, arrowhead). F, follicle cells; Y, yolk granules. Scale bar for a and b is 100 μm, for c and d is 5 μm, for e and f is 25 μm.

amphibians, follicle cells must be present on the surface of the oocyte for initiation of the *in vitro* uptake of Vg; it is possible that an "initiation factor" is transmitted from the follicle cells to the oocyte directly by means of gap junctions (Browne and Wiley, 1978). In insects it has been postulated that onset of vitellogenesis could be controlled by a regulatory molecule transferred *via* gap junctional contacts (Mazzini and Giorigi, 1985). Gap junctional contacts between follicular epithelium and oocyte of the stick insect, *Bacillus*, occur at mid and late previtellogenic stages and are retained after vitellogenesis has started (Mazzini and Giorgi, 1985). It is suggested that CaM in conjunction with calcium participates in the gating properties of gap junctions and perhaps operates on both surfaces of junctions interacting closely enough to change circular dichroic properties of gap proteins in response to Ca^{2+} (Peracchia, 1987). This role of CaM in regulating junctions between cells could be reflected in the polarized steady currents observed surrounding vitellogenic follicles (Bowdan and Kunkel, 1990; Kunkel, 1991). But would such junctions allow the flow of a small protein such as CaM itself?

Insect gap junctional channels are less restrictive than mammalian junctional channels. Some probe molecules (linear peptides up to 1.8 kDa), however, do not pass (Newton *et al.*, 1979). Calculations based on model compounds (Simpson *et al.*, 1977; Berdan, 1987) suggest that spherical molecules up to 3 nm in diameter could pass through insect gap junctions. According to SDS–polyacrylamide gel electrophoresis CaM has a molecular weight between 18.5 and 20 kDa and in physical conformation looks somewhat like a dumbbell. The length of the molecule is about 6.5 nm, with each end lobe having approximate dimensions of $2.5 \times 2.0 \times 2.0$ nm (Babu *et al.*, 1988). Based on the size of these lobes, it would be possible for CaM to pass lengthwise through the gap junctional channels.

The high level of CaM found in *B. germanica* oocytes and its intracellular localization in the cortex of the oocyte and outside the bounding membrane of yolk granules suggest that CaM might be involved in the massive endocytotic uptake mechanism of yolk proteins into the developing oocyte and the ooplasmic transport of yolk vesicles toward the oocyte center. CaM appears to be synthesized in large excess in the follicle cells and becomes highly concentrated in the ooplasm particularly during vitellogenesis. CaM may be being stored for a later function during embryogenesis, as are other yolk molecules, supplying the embryo with CaM for the rapid cell division characteristic of early embryogenesis (Zhang and Kunkel, 1992a) or as a modulator of yolk protein utilization (Purcell *et al.*, 1988a,b). The extra CaM might be used to mediate an exaggerated immediate event such as the high rate of vitellogenesis that is unique to oocytes including the onset of vitellogenesis, the uptake of yolk proteins, and the massive movement of yolk vesicles from the surface to their sites of aggregation into yolk platelets.

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