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Calmodulin transmitted through gap junctions stimulates endocytic incorporation of yolk precursors in insect oocytes

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Abstract

In ovarian follicles of *Oncopeltus fasciatus*, and of *Xylocopa virginica*, calmodulin (CaM) of epithelial cell origin is required by oocytes for endocytic uptake of yolk precursor molecules. Furthermore, this 17–19 kDa protein is normally transported to the oocytes via gap junctions. Downregulation of gap junctions by treatment with 1 mM octanol or separation of the epithelial cells from their oocytes terminated precursor uptake, and this activity could be rescued by microinjection of 60 μM CaM, but not by injections of incubation medium, nor solutions of other molecular species tested. That endogenous CaM is required was confirmed by incubating otherwise untreated follicles in physiological salt solution (PSS) containing either calmidazolium or W-7, both known antagonists of CaM. By radioimmunoprecipitation, we show that the epithelial cells surrounding an oocyte synthesized 15 times as much calmodulin as did the oocytes they encircled. Neither octanol-treated follicles nor denuded oocytes incubated in medium containing calmodulin were able to resume endocytosis, arguing against an extracellular route. However, fluorescently labeled calmodulin microinjected into oocytes is shown to have crossed through gap junctions, making epithelial cells distinctly fluorescent.

Keywords: Vitellogenesis; *Oncopeltus*; *Xylocopa*; Vitellogenins

Introduction

Insect ovarian follicles are discreet developmental units, each passing through a series of stages during which they become preprogrammed for post-fertilization development. Thus, insect egg production involves a regulated sequence of macromolecular synthesis, transport, and assembly. The recognized sites of synthesis include the fat body, the follicle cells, and in meroistic systems, the nurse cells; the products of these cells are assembled in and around a single cell, an oocyte, lying at the center of the follicle (Telfer, 1975). The egg is precisely structured, containing within it organization that eventually influences post-fertilization development (Malva et al., 1991; Nusslein-Volhard, 1994; Nusslein-Volhard et al., 1987; Sprenger and Nusslein-Volhard, 1992; Wieschaus, 1996). This organized "preprogramming" is achieved within a multicellular complex that must itself be highly and actively regulated. Indeed, the developmental sequence of follicular activities that generate egg structure implies an exacting set of cellular controls.

First insights into these cellular controls have come from studies on the hormonal control of vitellogenesis, the stage of follicular growth during which most of the cytoplasm and all of the yolk are deposited in the oocyte (King and Buning, 1985; Telfer, 1975). In many medically important insects, the stimulus that initiates vitellogenesis is a blood meal, which provides the nutrients needed for one round of egg formation and at the same time triggers, via the central nervous system, the secretion of juvenile hormone (JH) and in some cases ecdysteroids (c.f. reviews by Engelmann, 1979; Giorgi et al., 1999; Hagedorn and Kunkel, 1979; Hagedorn et al., 1979; Raikhel and Dhadialla, 1992). Whatever the exact pattern of hormonal control, the fat body and the terminal ovarian follicles are released from their relatively dormant condition, vitellogenesis begins (Engelmann, 1979; Hagedorn et al., 1979; Hagedorn and Kunkel, 1979; Raikhel, 1987), and the morphological and physiological characteristics of the oocyte change drastically.

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However, this story cannot be true for all insects, for the hormone-regulated system clearly could not function in the multitude of insects in which ovarian follicles of several different stages are simultaneously developing. For many hemipterans, lepidopterans, dipterans, and others, follicles of all stages are in sequential development (Fig. 1). For species in which both previtellogenic and vitellogenic follicles are simultaneously present and active, other control mechanisms must be present.

In *Oncopeltus fasciatus*, *Hyalophora cecropia,* and many other insects, newly formed follicles remain for a period in a slow-growing phase reminiscent of the mosquito and other hemophagous insect follicles that are awaiting activation by a blood meal (Raikhel and Dhadialla, 1992). Termination of this phase is marked not only by the onset of endocytotic activity by the oocyte, but also by extensive and rapid changes in membrane physiology and in the distribution of transcriptional activity between the oocyte and nurse cells (Woodruff and Telfer, 1990). Since in these insects, previtellogenic follicles are exposed to the same hormonal environment as are vitellogenic follicles actively endocytosing Vgs, stimulation to enter into active uptake of Vgs must be developmentally regulated in some other manner.

Anderson and Woodruff (2001) reported the first direct experimental evidence in insects showing that the follicular epithelium and gap junctions are involved in regulating Vg uptake. For follicles of *Oncopeltus*, a diffusible molecular signal able to pass through fully open insect gap junctions was needed for the onset and continuation of endocytic uptake of vitellogenins. (Here and throughout this paper, the term "signal" is used in its broadest sense, indicating a molecular species without which an event cascade does not occur, and in the presence of which the event cascade begins.) Furthermore, follicles blocked from vitellogenin uptake by gap junction antagonists could be rescued by microinjection of the soluble fraction of epithelial cell cytoplasm. Waksmonski and Woodruff (2002) reported that patent gap junctions were a requirement for endocytic uptake of Vgs in insects representing six different orders, confirming that this requirement is wide spread, at least among insects.

In the present study, we report on experiments that implicate the 17–19 kDa protein calmodulin (CaM) as the epithelial cell produced gap junctionally transmitted molecule required by oocytes for endocytic uptake of vitellogenins. We also show direct evidence that despite its molecular weight (and presumably because of its elongate rather than globular structure), this molecule can transit gap junctions.

Materials and methods

Chemicals

Unlabeled calmodulin, the CaM antagonists calmidizolium and W-7, Anti-CaM (sheep against bovine testes), and rabbit anti-sheep IgG/peroxidase were from Calbiochem (La Jolla, CA). Octanol, collagenase (type 1A), and Lucifer yellow CH (E_{max} 535 nm λ) were obtained from Sigma Company (St. Louis, MO). Other fluorochromes used were dextran-bound Texas Red (10,000 Da, E_{max} 615 nm λ) and Alexofluor™ labeled CaM (E_{max} 519 nm λ), both from Molecular Probes (Eugene OR).

Animals

Stock colonies of *O. fasciatus* (Dallas) (milkweed bug) (Carolina Biological Supply Co., Burlington NC) were reared on cracked sunflower seeds in constant light at 30°C to stimulate egg production (Kelly and Telfer, 1977). Newly ecdysed adults were transferred to smaller cages to prevent overcrowding, as lower densities enhance reproduction (Dingle, 1968; Feir, 1974).

Ovarioles to be used were dissected from decapitated females in *Oncopeltus* physiological salt solution (PSS) consisting of 40 mM NaCl, 20 mM KCl, 17 mM $CaCl_2$, 15 mM $MgCl_2$, and 110 mM Tris succinate buffer (pH 6.2) (Kelly and Telfer, 1979). The ovarioles were then transferred to fresh PSS for incubation at room temperature (20–22°C).

Xylocopa virginica: On mornings during May and June, female carpenter bees were captured as they emerged from or returned to their galleries. Bees were anesthetized with CO_2, decapitated, and dissected in a PSS consisting of 20 mM NaCl, 10 mM KCl, 5 mM $CaCl_2$, 7 mM $MgCl_2$, and 50 mM Tris Succinate buffer (pH 6.2) (Waksmonski and Woodruff, 2002). We found that incubated bee follicles required unusually low osmolarity to avoid shrinkage of oocytes.

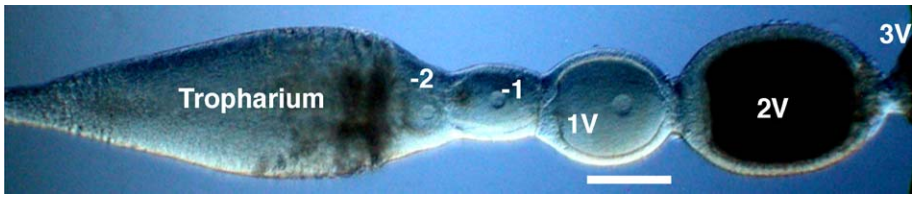

Fig. 1. A single *O. fasciatus* ovariole. At the base of the arrowhead tropharium are formed new oocytes, the youngest several of which are too small to be seen in this micrograph. The youngest visible previtellogenic follicle is labeled −2, while the oldest previtellogenic follicle is labeled −1. Follicle 1V has just begun to endocytose Vgs. Beyond it can be seen an older vitellogenic follicle, 2V, and part of an even older follicle (3V). Scale bar = 100 μm.

Follicles

The sheath surrounding each ovariole was carefully removed using sharp watchmakers forceps. The acellular basement membrane was softened by 3 min (*Oncopeltus*) to 6 min (*Xylocopa*) incubation in collagenase (1 mg/ml) (Singleton and Woodruff, 1994; Sun and Wyman, 1987; Woodruff and Telfer, 1990) after which follicles were washed in PSS and then transferred into the appropriate medium for experimentation.

To obtain viable oocytes denuded of epithelial cells, epithelia were removed from oocytes by using a "skinning" medium consisting of Ca^{2+}-free *Oncopeltus* PSS with EGTA to enforce the low Ca^{2+} (Biczkowski and Dittmann, 1995). In our hands, greatest success was achieved when we also added sucrose to make the solution quite hyperosmotic. Ovarioles were incubated in this medium for approximately 6 min. Using watchmaker forceps, epithelial cells could then be gently peeled from vitellogenic oocytes.

Blood

For any study of receptor-mediated oocyte endocytic activity, it is essential that the blood-born receptor ligands (Vgs) be present in the incubation medium. Female blood was collected by capillary action from the stubs of legs and antennae that had been amputated (Kelly and Davenport, 1976; Kelly and Telfer, 1977). For use, blood was diluted 1:10 with PSS. This incubation medium is hereafter called PSS/bld.

Downregulation of gap junctions

Anderson and Woodruff (2001) found no detectable differences in termination of Vg uptake or rescue thereof when gap junctions were downregulated by treatment with octanol, EMS, or by cytoplasmic acidification. Removal of the epithelium also prevented uptake of Vgs, and uptake by nude follicles could be rescued by microinjection in a manner no different from follicles with intact but downregulated gap junctions. Thus, for uniformity, in the experiments reported here, we used 1 mM octanol. This treatment has frequently been used to disrupt dye coupling between oocytes and their surrounding epithelial cells (Adler and Woodruff, 2000; Bohrmann and Haas-Assenbaum, 1993; Patino and Purkiss, 1993; Woodruff and Tilney, 1998). Octanol was dissolved in dimethyl sulfoxide (DMSO) and diluted to 1 mM with PSS/bld (final concentration of DMSO < 1%).

Microscopy

Observations of fluorescent materials were made in two ways. (1) An Olympus IX-71 inverted microscope (Olympus Optical Company, Tokyo, Japan) equipped with Differential Interference Contrast (DIC), epi-illumination, and the proper dichromatic mirrors, excitation filters, and barrier filters for fluorescence. Images were captured either with a Nikon 995 digital camera, or photographed from a video monitor with a Polaroid screen camera after being enhanced using either an Argus-10 (Hamamatsu Photonics, Japan) or an LKH-9000 (L.K. Hawke, NC). (2) Confocal scanning was done using an Olympus Fluoview FV300 scanner and accompanying software attached to an Olympus IX-70 inverted microscope. The appropriate lasers, dichromatic mirrors, and filters for excitation and viewing of Texas Red fluorescence were used. The unit was also equipped to allow simultaneous viewing on a separate channel of a DIC image.

Microinjections

Putative signal molecules were microinjected into oocytes using a Narishige IM-200 gas pressure microinjection system. Injections were adjusted so that the bolus was equal or less than 1/4 of the volume of the oocyte being injected. A small amount of Lucifer yellow CH was mixed with the injectate to allow visualization of the bolus and to confirm that it did not leak out after injection.

For experiments involving the microinjection of fluorescently labeled CaM, a few follicles from the same animal were first tested to confirm that gap junctions were open to dye coupling. For this purpose, Lucifer yellow CH dye was iontophoretically microinjected into these follicles.

Radioimmunoprecipitation

To quantify the site(s) of CaM synthesis, we labeled proteins with Tran^{35}S-label™ (ICN, Irvine, CA) ([S^{35}]methionine and [S^{35}]cysteine) and then used immunoprecipitation to select newly synthesized CaM. Pairs of first and second vitellogenic follicles were selected, and oocytes were separated from their epithelia as described above. For epithelial cells, follicles were broken open and the ooplasm gently expressed. They were then washed several times to remove any clinging ooplasm or oolemma. Following separation, oocytes or epithelial cells were incubated for 90 min in 100 μl DMEM to which 10 μl radioactive label was added. DMEM (ICN) is a methionine- and cysteine-deficient growth medium that is similar in osmolarity and ionic composition to *Oncopeltus* blood. After labeling, cells were transferred to centrifuge tubes containing 500 μl of a homogenization buffer consisting of 20% w/v sucrose, 2.5% w/v polyvinylpolypyrrolidone, 10 mM $CaCl_2$, 5 mM HEPES, and 5 mM AEBSF protease inhibitor (Calbiochem)(Cole and Woodruff, 1997). Oocytes were gently lysed with appropriate sized pestles. The lysate was centrifuged at 12,000 × *g* for 20 min to remove yolk spheres, nuclei, or other unwanted debris. Pellets were resuspended and examined microscopically. Oocyte samples were

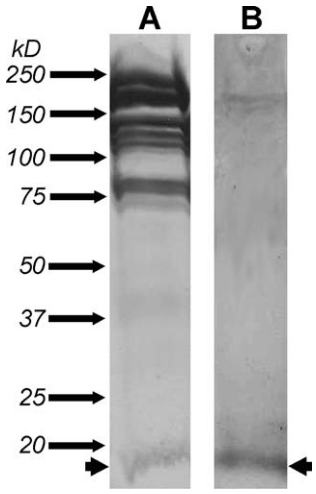

Fig. 2. Immunochemical identification of CaM from vitellogenic follicles of *O. fasciatus* using a polyclonal antibody against CaM. Lane "A" is from a Commassie blue stained 12% SDS PAGE gel. The 17–19 kDa band marked by the large arrowhead comigrated with commercial CaM (not shown). Lane "B" is from a Western blot of a companion gel.

rejected unless their pellets contained intact yolk spheres. Epithelial cells, being smaller, required harsher grinding followed by sonication, after which they were centrifuged as above. To remove heat-labile proteins, all samples were heated in a water bath to 90 °C for 5 min, and then chilled in an ETOH/ice bath and centrifuged three times (Zhang and Kunkel, 1994). To remove any preexisting immunoglobins in the supernatant, G-protein-agarose (Roche, Indianapolis, IN) was added overnight, followed by centrifugation. The supernatant was then treated with anti-CaM (sheep, anti-bovine testes)(Calbiochem) for 3 h. Precipitation of antibody-reacted CaM was insured by overnight treatment with G-protein-agarose. Samples were then twice centrifuged and the supernatant decanted away, the final pellets being resuspended in scintillation fluid and counted in a Beckman model 6500 liquid scintillation counter (Beckman Instruments, Inc. USA).

To test the specificity of our anti-CaM, we performed immunoblot analysis on the heat-stable proteins extracted from first and second vitellogenic follicles. Fig. 2, lane "A", shows the *Oncopeltus* proteins revealed by Coomassie blue staining of a 12% SDS-PAGE gel. The band at about 17–19 kDa comigrated with commercial CaM. Lane "B" is from an immunoblot transferred to nitrocellulose membrane from a companion gel, treated with anti-CaM and labeled with reaction product of rabbit anti-sheep IgG/peroxidase (Calbiochem). Only one distinct band was found, and it matched the 17–19 kDa component in the Coomassie stained gel. As was reported by Zhang and Kunkel (1994) for *Blattella*, there was a faint cross-reaction with a high molecular weight component that was presumably from CaM tightly bound to a large protein. Thus, it appeared that very little protein other than CaM was precipitated by our antibody.

Results

Detection of endocytic uptake of Vgs

Labeling of nascent yolk spheres by nonspecific fluid-phase uptake of fluorescent dye allowed easy confirmation of endocytic activity. This labeling was confined to yolk spheres, as could be seen by confocal scanning microscopy (Fig. 3). That most or all endocytic vesicles formed during the developmental stage known as "vitellogenesis" contain Vgs has been shown by immunolabeling experiments (Dittmann and Biczkowski, 1995; Telfer, 1961); thus, nonspecific fluid-phase labeling was a convenient manner in which to follow uptake of yolk precursor proteins. In all relevant experiments, endosome labeling was for 1 h.

As previously shown (Adler and Woodruff, 2000; Anderson and Woodruff, 2001; Waksmonski and Woodruff, 2002), treatment with 1 mM octanol downregulated the gap junctions connecting *Oncopeltus* oocytes with their surrounding epithelia, and thus terminated uptake of Vgs (Anderson and Woodruff, 2001; Waksmonski and Woodruff, 2002). The current study utilized the same protocol for downregulating gap junctions and for fluorescently labeling any nascent yolk spheres. While new yolk spheres were clearly visible in untreated control follicles, with octanol-treated follicles, neither fixed and sectioned material, in vitro live follicles examined with a standard fluorescence microscope, nor examination by confocal scanning microscopy revealed any sign of nascent yolk sphere formation. This can be seen in Fig. 5A, a confocal scanning fluorescence micrographs of an untreated control follicle, while Fig. 5B shows an octanol-treated follicle.

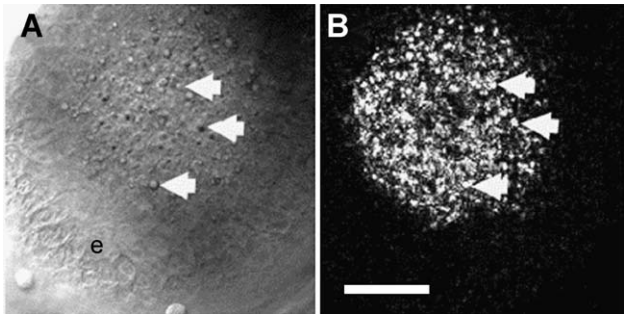

Fig. 3. Paired confocal scanning DIC (A) (Nomarski) and fluorescence (B) images taken simultaneously (separate channels) and showing optical sections of an untreated control ovarian follicle of *Oncopeltus* making nascent yolk spheres by receptor-mediated endocytosis. Taken in by nonspecific fluid phase uptake, fluorescent dye (Texas red conjugated to dextran) in the medium allowed identification of freshly formed nascent yolk spheres. Because of the curvature of the follicle, focus in the center of each micrograph is within the deeper lying oocyte at the depth at which are found the new yolk spheres, while around the periphery, it is follicle epithelial cells (e) that are in focus. Three of the many individual nascent yolk spheres visible in both DIC and fluorescence images are marked by the arrows. Scale bar = 100 μm.

Microinjection of putative messengers

Within 15 min of treatment with 1 mM octanol/PSS, gap junctions begin to downregulate, and by 45 min following octanol treatment, uptake of Vgs no longer occurred. Following octanol disruption of gap junctions and cessation of Vg uptake, we microinjected a series of solutions to determine if they could rescue Vg endocytosis (Table 1). The solutions, each of which contained a very small amount of Lucifer yellow, were PSS alone, PSS/bld, PSS/cAMP, PSS/BSA, and PSS/CaM. In each case, the size of the injection was matched to the size of the oocyte so that the bolus injected was approximately 1/4 the volume of the oocyte or less. In most cases, there was no visible leaking of cytoplasm; follicles that did show damage were removed from the study. Fluorescence of the Lucifer yellow allowed us to confirm that the oocyte had received the bolus, and that post injection leaking had not occurred. In addition to microinjecting oocytes with downregulated gap junctions, we also microinjected cAMP or CaM into denuded follicles.

PSS alone

PSS alone was microinjected into 15 octanol-treated follicles and 7 follicles denuded of their epithelial cells. In concurrence with data presented in Anderson and Woodruff (2001), in none of these was a microinjected follicle seen to regain the ability to endocytose Vgs. Fig. 4A shows a first vitellogenic follicle treated for 1 h with octanol to terminate Vg uptake, and then microinjected with PSS alone. While this follicle had been producing yolk spheres before octanol treatment, it was still small enough to be translucent, and the injection needle and injection bolus can clearly be seen in this brightfield micrograph. Fig. 4B is a fluorescence micrograph (Texas red filters) taken after the injection and a 1-h incubation in PSS/bld/dye. Only autofluorescence levels can be seen, demonstrating a lack of endocytosis.

Table 1
Microinjection of putative signal into downregulated follicles

Microinjected fluid contained	Number injected	Showing fluorescent yolk spheres	Number of fluorescent yolk spheres
PSS only	22[a]	0	22
PSS/bld	22	0	22
PSS/cAMP	20	0	20
PSS/cAMP (epithelia removed)	10	3[b]	7
PSS/BSA	21	1[c]	20
PSS/CaM (Octanol)	39	22	17
PSS/CaM (epithelia removed)	10	10	0

P value of all no-CaM follicles compared to all CaM-injected follicles ≥ 0.001.
[a] Of these, 15 were octanol-treated, 7 had their epithelia removed.
[b] General fluorescence of ooplasm, no compartmentalization.
[c] Only five fluorescent nascent yolk spheres found.

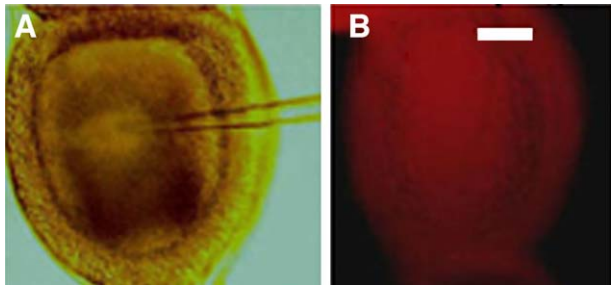

Fig. 4. Microinjections of PSS, PSS + cAMP, or PSS + BSA failed to rescue endocytic activity following downregulation of gap junctions. DIC (left, $t = 0$) and fluorescence (right, $t = 60$ min) micrographs of an octanol-treated first vitellogenic follicle microinjected with PSS. Visible in the DIC image is the microinjection pipette and the injection bolus. The fluorescence image, nearly invisible through the oculars, has been enhanced so that the low autofluorescence can be seen. Scale bar = 30 μm.

PSS/bld

Twenty two follicles were treated with octanol, microinjected with PSS/bld (10/1), and then incubated in PSS/bld/dye. None showed any fluorescent nascent yolk spheres.

PSS/cAMP

Of 30 follicles microinjected with PSS/60 μM cAMP, 27 showed no fluorescence above autofluorescence levels. Three follicles did acquire some fluorescence, but this was not in discreet yolk spheres. Such general uncompartmentalized fluorescence occurs when a breach in the oocyte membrane allows the cytoplasm to be stained by diffusion of dye from the incubation medium.

PSS/BSA

To determine if microinjection of nonspecific larger molecules such as proteins could cause rescue, we used a 60 μM solution of bovine serum albumin (BSA) in PSS. BSA/PSS containing a small amount of Lucifer yellow was microinjected into the oocytes of 21 octanol downregulated follicles, which were then incubated in PSS/bld/dye + octanol. Of these, in only one was there any fluorescent nascent yolk spheres, and in that one, only five fluorescent yolk spheres could be seen.

PSS/CaM

When 39 octanol-treated *Oncopeltus* follicles were incubated in PSS/bld/dye following microinjection with 60 μM CaM, 22 showed distinct fluorescent yolk spheres. Fig. 5A is a confocal scanning micrograph of an untreated control follicle at a focal level equivalent to a cross-section, and showing an area where endocytic vesicles are fusing into small yolk spheres. Fig. 5B is from the same depth in an octanol-treated follicle, while Fig. 5C shows an optical section at this level in an octanol-treated follicle subsequently microinjected with 60 μM CaM.

An additional 10 oocytes were denuded of epithelial cells, microinjected with 60 μM CaM, and incubated in PSS/bld/dye. All 10 became strikingly fluorescent from the

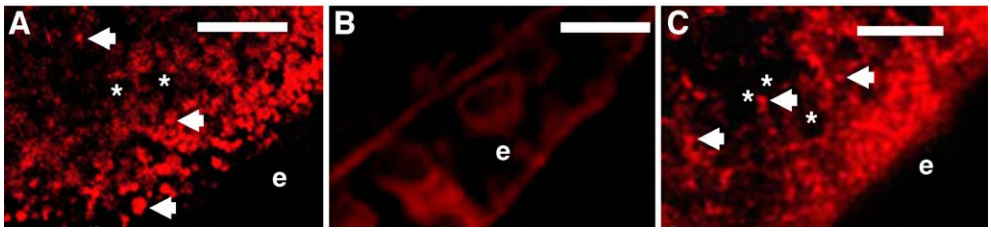

Fig. 5. Confocal scanning fluorescence micrographs of untreated control (A), octanol-treated (B), and octanol-treated–CaM microinjected (C) ovarian follicles of *O. fasciatus* incubated in PSS/bld/dye (Texas red conjugated to dextran). In (A) nascent yolk spheres became fluorescent by nonspecific fluid phase uptake during receptor-mediated endocytosis of yolk precursors. In (B) following treatment with octanol, gap junctions had been downregulated and oocyte endocytosis was terminated. When oocytes were microinjected with 60 μM CaM (C), endocytosis of yolk precursors resumed. Focus is at a level equivalent to a cross section. "e" = epithelial cells, arrowheads indicate nascent yolk spheres. Circular dark regions such as those marked by "*" are older, nonfluorescing yolk spheres. Scale bars = 20 μm.

nonspecific fluid phase uptake of dye into nascent yolk spheres as the oocytes endocytosed Vgs. Fig. 6A shows two denuded *Oncopeltus* follicles after 1 h in PSS/bld/dye. The follicle on the right received a microinjection of 60 μM CaM, while the one on the left did not. Fig. 6B shows the results from a similar experiment performed with *Xylocopa* follicles.

Table 1 shows data from 164 follicles, gap junctions of which were downregulated or absent (epithelia removed). For 95 of these no CaM was provided, and none showed fluorescent yolk spheres. Of 69 follicles into which CaM was microinjected, 52 formed multiple fluorescent nascent yolk spheres. An unpaired *t* test performed on the data yielded a *P* value of > 0.001.

Site of CaM production

Tropharia and vitellogenic follicles were harvested from *Oncopeltus* females, separated and prepared as follows: (1) 15 tropharia, (2) 15 pairs of first and second vitellogenic oocytes carefully denuded of their epithelia, (3) as much of the epithelia striped from these oocytes as we were able to recover, and (4) the intact epithelia from 15 matching pairs of first and second vitellogenic follicles, oocytes of which were sacrificed to insure recovery of complete and largely intact epithelia. Each tissue type was incubated separately in 100 μl DMEM augmented with 10 μCi of [^{35}S]methionine/[^{35}S]cysteine. The tissue samples were labeled for 90 min and then transferred to homogenization medium and prepared for scintillation counting as described in Materials and methods. Results are shown in Tables 2 and 3.

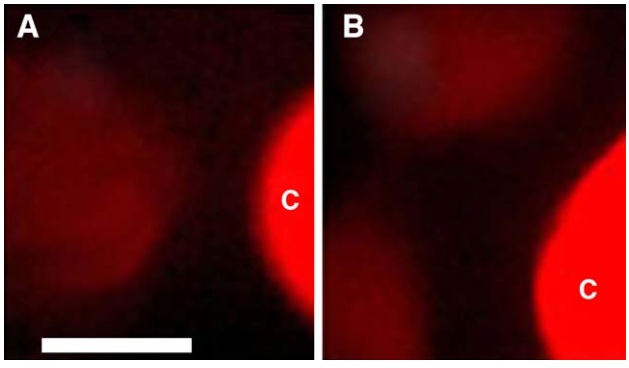

Fig. 6. For oocytes denuded of their epithelium, microinjection of CaM rescued endocytic uptake of yolk precursors. Fluorescence micrographs of *Oncopeltus* (A) and *Xylocopa* (B) oocytes striped of their epithelia. Without epithelia, there were no open gap junctions, and formation of nascent yolk spheres was terminated. In each micrograph, the oocyte marked "C" had been microinjected with 60 μM CaM and had resumed endocytic activity. At the low magnification employed here, individual yolk spheres are not distinguishable because *z* axis resolution (depth discrimination) is poor, and light from both out of focus and in focus "self-luminous" yolk spheres meld together. Scale bar = 50 μm for both micrographs.

Oocytes

The CaM synthesis by 15 pairs of first and second vitellogenic oocytes was determined by scintillation counting following radiolabeling. In these 30 oocytes, uptake and incorporation into CaM resulted in 18,739 cpm.

Tropharia

The 15 tropharia were treated and prepared in the same manner as for oocytes. The final immunoprecipitated CaM was labeled at a level yielding 9655 cpm.

Epithelia

When separating epithelia from oocytes, one or the other cell type suffers. In obtaining undamaged naked oocytes, much of the epithelium surrounding each was unavoidably lost. Yet, even though only a small fraction of the original epithelial cells were available, they still produced sufficient [^{35}S]CaM to produce 30,759 cpm. This was more than 1.5 times the amount from the intact oocytes with which they had once been associated (Table 2).

By sacrificing oocytes, we were able to obtain the intact epithelia from 15 pairs of first and second vitellogenic follicles. These epithelia were incubated in labeling medium and measured against the oocytes from 15 pairs of first and second vitellogenic follicles matched in stage and size to the oocytes from which the epithelia had been harvested. In this experiment, the 30 oocytes contained sufficient label incorporated into synthesized CaM to

Table 2
Synthesis of CaM by different cell types as shown by incorporation of [^{35}S]methionine and [^{35}S]cysteine into immunologically identified labeled CaM

Cell type	15 Tropharia,	30 Naked oocytes (first and second, 15 of each type)	Epithelial fragments from these same oocytes
Counts/minutes	9655	18,739	30,759

produce 10,113 cpm, while CaM synthesized in the epithelial cells produced 152,036 cpm, indicating 15 times greater production of this protein than the oocytes (Table 3).

Incubation in CaM antagonists calmidizolium or W-7

To further test the requirement of endogenous CaM for oocyte uptake of Vgs, follicles were incubated in calmidazolium (Nucifora and Fox, 1998) or W-7 (Gbadebo et al., 2002; Grebenkamper and Nicolau, 1995; Murphy et al., 2002), both of which are CaM antagonists and membrane permeant. Thirty follicles were incubated for 1 h in 10 μM calmidazolium/PSS and then transferred to PSS/Bld/dye/calmidazolium for an additional hour. In the presence of calmidazolium, none of the 30 showed any fluorescence above autofluorescence. In W-7, 15 of 17 incubated follicles showed no sign of labeling. Two of the follicles were fluorescent, but the fluorescence was not in discreet yolk spheres. Careful examination revealed damage, presumably allowing diffusion into the cytoplasm of dye from the incubation medium.

Downregulated follicles incubated in medium containing CaM

To determine if CaM could reach the oocyte by an extracellular route, *Oncopeltus* oocytes were either stripped of their epithelium ($n = 10$) or were incubated in octanol to downregulate their gap junctions ($n = 12$) and then incubated in medium containing 60 μM CaM, PSS/bld/dye. Fig. 7 shows three pealed follicles incubated in medium containing CaM. The bright spots are external debris, the remnants of epithelial cells damaged as those tissues were removed. Additional follicles, either pealed or octanol-treated, were incubated in the same medium, but without added CaM, while seven more follicles were neither pealed nor octanol-treated and served as positive controls (Table 4). Of the pealed oocytes, only three showed some fluorescence, but it was not confined to within yolk spheres. Each also showed minor damage to the oocyte membrane. Only one of the octanol-treated follicles showed fluorescent nascent yolk spheres, and it had very few. Formation of these few yolk spheres may have been enabled by lingering unused CaM. The possibility of this was increased by a similar discovery in one of the six octanol-treated controls, the incubation medium of which had contained no CaM. Here too a few scattered fluorescent yolk spheres were found. Of the untreated control follicles, all but one formed distinct fluorescent yolk spheres.

Table 3
Synthesis of CaM by different cell types as shown by incorporation of [^{35}S]methionine and [^{35}S]cysteine into immunologically identified labeled CaM

Cell type	30 Naked oocytes[a]	Intact epithelia
Counts/minutes	10,113	152,036

[a] From 15 first and 15 second vitellogenic follicles matched in stage and size to those used for epithelia.

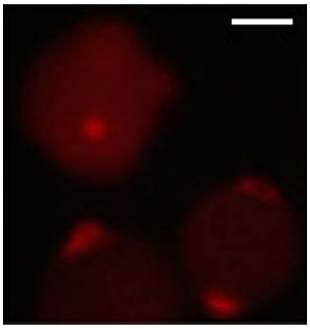

Fig. 7. An extracellular route by which epithelial cell CaM might reach the oocyte was not supported by evidence from incubation of denuded *Oncopeltus* oocytes striped of their epithelia and incubated in PSS + bld + CaM. Standard fluorescence micrograph. Bright spots on each of these oocytes are necrotic epithelial cell debris. Scale bar = 100 μm.

Passage of fluorescently labeled CaM through gap junctions

Calmodulin conjugated to Alexofluor™ was microinjected into the oocytes of 37 follicles, which were then incubated in PSS. Of these, the injected bolus leaked out of 2. Within 1 h following injection, epithelial cells of the remaining 35 became clearly fluorescent (Fig. 8). Eleven other follicles were first treated with octanol before micro-

Table 4
Failure of 60 μM CaM in the incubation medium to stimulate Vg uptake

	n incubated	*n* with fluorescent nascent yolk spheres
Untreated controls	7	6
Octanol downregulated, NO CaM in medium	6	1[a]
Epithelia removed, NO CaM in medium	5	0
Octanol downregulated follicles, 60 μM CaM in medium	12	0[b]
Epithelia removed follicles, 60 μM CaM in medium	10	0[c]

[a] One oocyte had nine visible fluorescent yolk spheres.
[b] One oocyte had six visible fluorescent yolk spheres.
[c] Three oocytes had some fluorescence, but none of it was confined to yolk spheres.

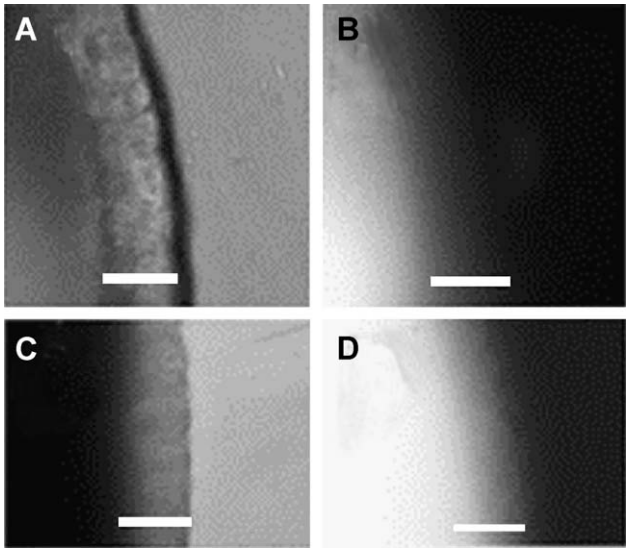

Fig. 8. Fluorescently labeled CaM microinjected into oocytes transited the gap junctions into epithelial cells. In each micrograph, the scale bar = 30 μm, and extends from the outer to inner edge of the epithelium. DIC (A and C) and fluorescent micrographs (B and D) of *Oncopeltus* follicles microinjected with labeled CaM at $t = 0$ (A and B) and after 1 h (C and D). At $t = 0$, there was little or no fluorescence in the epithelium, but by $t = 60$ min, epithelial cells showed marked fluorescence.

injection of fluorescently labeled CaM. None of these showed visible fluorescence in their epithelial cells. Fig. 9A shows a surface view of a follicle in which the oocyte had been injected with fluorescently labeled CaM. Focus is at the level of the follicle epithelium, cells of which are distinctly fluorescent. The follicle shown in Fig. 9B was first treated with octanol to downregulate gap junctions before fluorescently labeled CaM was microinjected into the oocyte. Fluorescence from the ooplasm outlines the nonfluorescing epithelial cells.

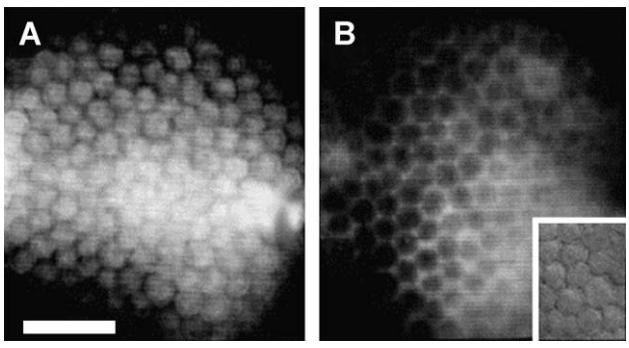

Fig. 9. Octanol, which downregulated gap junctions, changed the ability of microinjected labeled CaM to move from *Oncopeltus* oocytes to epithelial cells. Standard fluorescence micrographs. (A) An untreated control follicle in which, following microinjection of labeled CaM into the oocyte, the epithelial cells became brightly fluorescent. The follicle shown in (B) was first treated with octanol and then microinjected. The only fluorescence visible was from the underlying ooplasm. The inset at lower right is a DIC image taken at the focal level of the epithelial cells, the same level as in the fluorescence micrographs. Because of the curvature of the follicles, focus is deeper at the center of each micrograph. Scale bar = 100 μm.

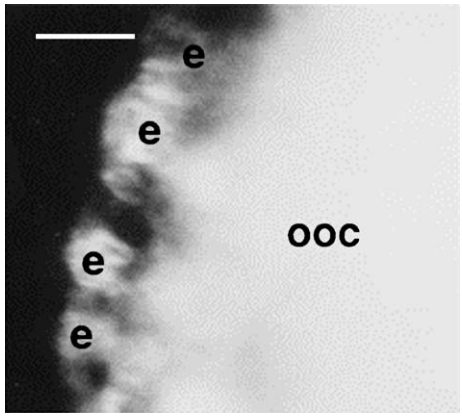

Fig. 10. Fluorescently labeled CaM can transit the gap junctions of *Xylocopa*. Fluorescence micrograph of a young vitellogenic follicle into the oocyte of which was microinjected labeled CaM. The ooplasm is at right. Within 20 min, several follicle cells can be seen to have become brightly fluorescent. e = epithelial cells, ooc = oocyte. Scale bar = 30 μm.

Although Alexofluor™ was covalently bound to CaM, if enzymes in the cytoplasm degraded the CaM, or lysed the bond between the fluorochrome and the protein, epithelial cell fluorescence might have occurred without the transfer of the protein through gap junctions (Simpson et al., 1977). To determine if there was any chance of this, Alexofluor™ CaM was incubated for 30 min in the presence of cytoplasm derived from lysed oocytes. This "treated" CaM, untreated labeled CaM, and unlabeled CaM were run on 1% agarose electrophoresis gels. Gels were run at 100V for 30 min, long enough for the protein to move, but not long enough for any free fluorochrome to have been electrophoresed into a buffer tank. All three proteins traveled the same distance. For the fluorescent CaM, in each lane there was only one fluorescent spot, and each matched the position of the Coomassie stained unlabeled CaM (results not shown).

Xylocopa

Some of the experiments described above using *Oncopeltus* follicles were repeated with *Xylocopa*. Eight untreated control follicles incubated in PSS/bld/dye all accumulated fluorescent yolk spheres, while none of 12 octanol-treated follicles showed any signs of dye uptake, nor did any of the five follicles with gap junctions downregulated by octanol and subsequently incubated in PSS/bld/dye/octanol supplemented with CaM. However, when 12 follicles from which the epithelia had been pealed were microinjected with 60 μM CaM and then incubated in labeling medium, all 12 produced copious amounts of fluorescent nascent yolk spheres (Fig. 6b).

Fluorescently labeled CaM was microinjected into oocytes of 13 *Xylocopa* follicles. In 12 of these, several to all of the epithelial cells became clearly fluorescent. Fig. 10 is a fluorescence micrograph taken 30 min after microinjection of labeled CaM into the oocyte of an early vitellogenic follicle.

Discussion

There are two major issues raised by the data reported here. Firstly, is CaM of epithelial origin an endogenous component of the event cascade leading to Vg uptake in follicles? Secondly, can this 17–19 kDa protein actually pass through the narrow lumena of gap junctions?

A few early observations suggested that gap junctions might play some undetermined role in regulating events during insect vitellogenesis. With in vitro incubation, intact follicles of *Hyalophora* were shown to avidly incorporate Vgs into yolk (Hausman et al., 1971), while oocytes striped of their epithelium would not; even when the epithelia were present in the same culture (Anderson, 1971). Gap junctions between the follicle epithelium and the oocytes were observed in *Locusta* (Wollberg et al., 1976), *Hyalophora* (Woodruff, 1979), *Rhodnius* (Huebner, 1981), and *Oncopeltus* (Woodruff and Anderson, 1984). Shortly thereafter, an 18.5 kDa protein of epithelial origin was implicated as being necessary for *Locusta* oocyte endocytosis (Glass and Emmerich, 1981). Giorgi and Postlethwait (1985) reported for the *Drosophila* female sterile mutation fs(2)A17 that epithelial cell–oocyte gap junctions became abnormal at the time when vitellogenesis should begin, and no Vg uptake occurred. For *Oncopeltus*, in vitro studies showed that the onset of epithelial cell–oocyte dye coupling occurred just before the formation of the first yolk spheres (Woodruff and Anderson, 1984), giving rise to speculation that intercellular communication through gap junctions might have a role in regulating vitellogenic activity. A similar temporal link between gap junctional communication, endocytic uptake of Vgs, and conversion of the active oocyte nucleus into a GV was found in *Hyalophora* (Woodruff and Telfer, 1990).

The effectiveness of octanol in downregulating dye coupling in follicles of *Oncopeltus* has been previously demonstrated and quantified (Adler and Woodruff, 2000), and so has the requirement of open gap junctions for continued uptake of Vgs (Anderson and Woodruff, 2001; Waksmonski and Woodruff, 2002). Furthermore, downregulation of gap junctions by three different methods, as well as by physical removal of the epithelium, each resulted in terminating Vg uptake (Anderson and Woodruff, 2001), strongly suggesting that it was downregulation of the connexons rather than side effects of any one of the treatments that was responsible. In Anderson and Woodruff (2001), we reported that microinjection of the soluble fraction of lysed follicular epithelial cells would rescue follicles in which receptor-mediated endocytosis of Vgs had been terminated by octanol-induced downregulation of gap junctions. In the present study, while other treatments failed to rescue Vg uptake, the success of rescue by microinjected CaM suggests that endogenous CaM from the lysed epithelial cells was the specific component required. For those few CaM-injected follicles that did not resume Vg uptake, the most likely reason was that they did not retain enough of the injected solution. Follicles suffering serious damage could easily be identified, usually be a copious trail of yolk sphere-rich cytoplasm emanating from the point of impalement as the needle was withdrawn. Such follicles were removed from the study at once. The small amount of Lucifer yellow included in the injectate allowed positive confirmation that a bolus of solution had been delivered and that there was no immediate major leakage after microinjection. However, over time, diffusion of the Lucifer throughout the oocyte and up the trophic cord made it ever less useful in determining retention of other components of the injected solution. Thus, there was no way to unequivocally detect slow leakage not obvious within the first few minutes, and this may have accounted for the few CaM-injected follicles in which rescue did not occur. Even so, the overall success rate of microinjected CaM compared with other solutions is obvious from Table 1, and supports the idea that CaM is a specific molecular species that was able to achieve rescue of follicles blocked from endocytosis by downregulation of gap junctions ($P \geq 0.001$).

In PSS/Bld/dye, the presence of the membrane permeant CaM antagonists calmidazolium or W-7 terminated Vg uptake, thus confirming the requirement of endogenous CaM. While two of the 17 follicles exposed to W-7 showed fluorescence, it was not compartmentalized in discreet yolk spheres. When closely examined, both of these follicles showed some damage to the oocyte membrane that may have allowed dye from the medium to enter and stain the ooplasm. Thus, the evidence supports the probability that the rescue accomplished by microinjected CaM was by replacing the required endogenous CaM prevented from entering the oocyte after gap junctions were downregulated.

A link between CaM and receptor-mediated endocytosis of Vgs has been found in *Xenopus* oocytes (Tucciarone and Lanclos, 1982), where inhibition of CaM resulted in decreased endocytic uptake of the blood-born components of yolk bodies. CaM has also been implicated as required for endocytosis in yeast cells (Geli et al., 1998; Riezman et al., 1996; Schaerer-Broadbeck and Riezman, 2000). Endocytosis by cultured insect cells (SF9) as well has been shown to require CaM, and endocytic activity was arrested by treatment with CaM antagonists (Grebenkamper and Nicolau, 1995).

Zhang and Kunkel (1992, 1994) have shown that CaM is involved in uptake of Vgs by oocytes of the cockroach, *Blattella germanica*. Furthermore, they presented evidence that most or all of the CaM in oocytes were produced not in the ooplasm but in the epithelial cells that surround the oocyte. In addition, it was in these epithelial cells that the highest levels of the RNA transcript for CaM were found (Iyengar and Kunkel, 1995). In experiments similar to those performed by Zhang and Kunkel, we found by radioimmunoprecipitation that isolated epithelial cells synthesized more than 15 times as much CaM as did isolated oocytes (Table 3). Thus, results of the present study strongly suggest that like *Blattella*, endocytosis by oocytes of *Oncopeltus*

and *Xylocopa* also requires CaM synthesized elsewhere in the insect.

While these results strongly implicate epithelial cell CaM as a requirement for oocyte endocytosis, they do not directly demonstrate that CaM produced in the epithelial cells reached the oocyte via gap junctions. Previously, it had been noted that oocytes co-incubated with seemingly healthy epithelial cells did not take up Vgs (Anderson, 1971). The possibility existed that the open gap junctions were needed not to pass a signal to the oocytes, but rather for passage of a signal from the oocyte to the epithelial cells stimulating them to produce and secrete CaM into the perioocytic space from whence it might, by receptor-mediated endocytosis specific for CaM, be avidly taken into the ooplasm. To determine the existence of such an extracellular route by which epithelial cell produced CaM might reach the oocyte, we incubated octanol-treated follicles or denuded oocytes in PSS/bld/dye to which was added 60 μM CaM. As can be seen in Fig. 7 and Table 4, CaM provided in the medium had no effect, presumably because it was unable to gain entrance to the ooplasm.

With evidence accumulating that CaM of epithelial cell origin was needed by oocytes for endocytosis of Vgs, but that extracellular CaM had no effect, the question of gap junction transport of this 17–19 kDa protein needed to be examined. In their 1994 study, Zhang and Kunkel pointed out that size calculations of the lumen of an insect gap junction suggested a diameter of 3 nm or slightly larger (Berdan, 1987). They also cited determinations of the physical conformation of CaM: a dumbbell-shaped molecule with each end lobe having a diameter of about 2 nm (Babu et al., 1988), a diameter which makes it theoretically possible for CaM to pass lengthwise through the lumen of a gap junction.

The possibility of CaM moving through gap junctions has here been demonstrated by the movement into epithelial cells of fluorescently labeled CaM microinjected into oocytes of both *Oncopeltus* and *Xylocopa*. Following such microinjections, the presence of labeled CaM in the medium would has suggested the possibility of an extracellular route of transfer. In performing pressure microinjection, some leakage of dye from the needle into the medium is expected. In all cases, to prevent fluorescence from this source obstructing detection of secreted CaM, all microinjected follicles were immediately transferred to fresh medium, at which time no epithelial fluorescence was yet visible. In no case did we subsequently note fluorescence developing in the fresh medium, but epithelial cells became progressively more fluorescent. Furthermore, the fluorescent dye was firmly covalently bound to the CaM, and remained bound even after incubation in the presence of oocyte cytoplasm, making the possibility of epithelial cell fluorescence due to unbound dye quite remote.

Open gap junctions are determined in three ways: by electrophysiological means (electrical coupling), by dye spread (dye coupling), and/or by appearance of metabolic activity in a cell not expressing a required component (metabolic coupling). Determination of the ability of a particular molecular species to transit gap junctions is usually assumed if there is considerable evidence of metabolic coupling. On the basis of metabolic coupling, the evidence of gap junctional transfer of CaM was strong. Not only did CaM rescue oocytes blocked from Vg uptake by downregulating gap junctions, it was clear from treatment with CaM antagonists that endogenous CaM was a normal requirement for endocytosis. Furthermore, CaM synthesis was vastly greater in epithelial cells than in oocytes, nor could the route for CaM have been extracellular. If it were, octanol-treated follicles or denuded oocytes incubated in the presence of extracellular CaM would have been expected to form fluorescent nascent yolk spheres, but did not.

The most rigorous test that a given molecular species can transit gap junctions is demonstration that a labeled molecule introduced in one cell appears in a contiguous cell without being detected in the extracellular medium. On this most demanding criterion, CaM has now been shown, despite its relatively high molecular weight, and presumably because of its elongate configuration, to have been able to pass through the gap junctions between epithelial cells and the oocytes they surround.

Based upon our evidence, we propose that young follicles are prevented from entering into Vg uptake until they are provided with a supply of CaM, that the CaM is provided by the epithelial cells and reaches the oocytes through open gap junctions. Thus, for insects in which follicles of all developmental stages are exposed to the same hormonal environment, follicular epithelial cells are responsible for determining the onset of Vg uptake by upregulating the gap junctions that connect them with the oocyte.

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References

Adler, E.L., Woodruff, R.I., 2000. Varied effects of 1-octanol on gap junctional communication between ovarian epithelial cells and oocytes of *Oncopeltus fasciatus*, *Hyalophora cecropia* and *Drosophila melanogaster*. Arch. Insect Biochem. Physiol. 43, 22–32.

Anderson, L., 1971. Protein synthesis and uptake by isolated *Cecropia* oocytes. J. Cell Sci. 8, 735–750.

Anderson, K.L., Woodruff, R.I., 2001. A gap junctionally transmitted epithelial cell signal regulates endocytic yolk uptake in *Oncopeltus fasciatus*. Dev. Biol. 239, 68–78.

Babu, Y.S., Bugg, C.E., Cook, W.J., 1988. Three-dimensional structure of calmodulin. In: Cohen, P., Klee, C.B. (Eds.), Calmodulin. Elsevier, Amsterdam, pp. 83–89.

Berdan, R.C., 1987. Intercellular communication in arthropods. Biophys-

ical, ultrastructural and biochemical approaches. In: De Mello, W.C. (Ed.), Cell-to-Cell Communication. Plenum, New York, pp. 299–370.
Biczkowski, M., Dittmann, F., 1995. Translation of maternal mRNA during early oogenesis in a telotrophic–meroistic insect (*Dysdercus intermedius*). J. Insect Physiol. 41, 1133–1139.
Bohrmann, J., Haas-Assenbaum, A., 1993. Gap junctions in ovarian follicles of *Drosophila melanogaster*: inhibition and promotion of dye-coupling between oocytes and follicle cells. Cell Tissue Res. 273, 163–173.
Cole, R.W., Woodruff, R.I., 1997. Charge dependent distribution of endogenous proteins within ovarian follicles of *Actias luna*. J. Insect Physiol. 43, 275–287.
Dingle, H., 1968. Life history and population consequences of density, photoperiod and temperature in a migrant insect, the milkweed bug *Oncopeltus*. Am. Nat. 102, 149–163.
Dittmann, F., Biczkowski, M., 1995. Induction of yolk formation in hemipteran previtellogenic oocytes (*Dysdercus intermedius*). Invertebr. Reprod. Dev. 28, 63–70.
Engelmann, F., 1979. Insect vitellogenin: identification, biosynthesis, and role in vitellogenesis. Adv. Insect Physiol. 14, 49–108.
Feir, D., 1974. *Oncopeltus fasciatus*: a research animal. Annu. Rev. Entomol. 19, 18–96.
Gbadebo, T.D., Trimble, R.W., Khoo, M.S., Temple, J., Roden, D.M., Anderson, M.E., 2002. Calmodulin inhibitor W-7 unmasks a novel electrocardiographic parameter that predicts initiation of torsade de pointes. Circulation 105, 770–774.
Geli, M.I., Wesp, A., Riezam, H., 1998. Distinct functions of calmodulin are required for the uptake step of receptor-mediated endocytosis in yeast: the type I myosin Myo5p is one of the calmodulin targets. EMBO J. 17, 635–647.
Giorgi, F., Postlethwait, J.H., 1985. Development of gap junctions in normal and mutant ovaries of *Drosophila melanogaster*. J. Morphol. 185, 115–129.
Giorgi, F., Bradley, J.T., Nordin, J.H., 1999. Differential vitellin polypeptide processing in insect embryos. Micron 30, 579–596.
Glass, H., Emmerich, H., 1981. Immunofluorescent localization of two SDS-soluble proteins in maturing terminal follicles of *Locusta migratoria*. Roux's Arch. Dev. Biol. 190, 27–32.
Grebenkamper, K., Nicolau, C., 1995. Signal transduction in SF9 insect cells: endocytosis of recombinant CD4 after phorbol ester treatment. Biochem. Biophys. Res. Commun. 207, 411–416.
Hagedorn, H.H., Kunkel, J.G., 1979. Vitellogenin and vitellin in insects. Annu. Rev. Entomol. 24, 475–505.
Hagedorn, H.H., Shapiro, J.P., Hanaoka, K., 1979. Ovarian ecdysone secretion is controlled by a brain hormone in an adult mosquito. Nature 282, 92–94.
Hausman, S.J., Anderson, L.M., Telfer, W.H., 1971. The dependence of cecropia yolk formation in vitro on specific blood proteins. J. Cell Biol. 48, 303–313.
Huebner, E., 1981. Oocyte-follicle cell interaction during normal oogenesis and atresia in an insect. J. Ultrastruct. Res. 74, 95–104.
Iyengar, A.R., Kunkel, J.G., 1995. Follicle cell calmodulin in *Blattella germanica*: transcript accumulation during vitellogenesis is regulated by juvenile hormone. Dev. Biol. 170, 314–320.
Kelly, T.J., Davenport, R., 1976. Juvenile hormone induced ovarian uptake of a female specific blood protein in *Oncopeltus fasciatus*. J. Insect Physiol. 22, 1381–1393.
Kelly, T.J., Telfer, W.H., 1977. Antigenic and electrophoretic variants of vitellogenin in *Oncopeltus* blood and their control by juvenile hormone. Dev. Biol. 61, 58–69.
Kelly, T.J., Telfer, W.H., 1979. The functions of the follicular epithelium in vitellogenic *Oncopeltus* follicles. Tissue Cell 11, 663–672.
King, R.C., Buning, J., 1985. The origin and functioning of insect oocytes and nurse cells. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Compr. Insect Physiol. Biochem. Pharmacol., vol. 1. Pergamon, Oxford, pp. 1–134. Chap. 13.
Malva, C., Graziani, F., Cavaliere, V., Manzi, A., Tino, A., 1991. Genetic and molecular analysis of maternal information in region 32 of *Drosophila melanogaster*. Mol. Reprod. Dev. 28, 307–317.
Murphy, T.V., Spurrell, B.E., Hill, M.A., 2002. Mechanisms underlying pervanadate induced contraction of rat cremaster muscle arterioles. Eur. J. Pharmacol. 442, 107–114.
Nucifora, P.G., Fox, A.P., 1998. Barium triggers rapid endocytosis in calf adrenal chromaffin cells. J. Physiol. 508, 483–494.
Nusslein-Volhard, C., 1994. Of flies and fishes. Science 266, 572–574.
Nusslein-Volhard, C., Frohnhofer, H.G., Lehmann, R., 1987. Determination of anteroposterior polarity in *Drosophila*. Science, 1675–1681.
Patino, R., Purkiss, R.T., 1993. Inhibitory effects of *n*-alkanols on the hormonal induction of maturation in follicle-enclosed *Xenopus* oocytes: implications for gap junctional transport of maturation-inducing steroid. Gen. Comp. Endocrinol. 91, 189–198.
Raikhel, A.S., 1987. The cell biology of mosquito vitellogenesis. Mem. Inst. Oswaldo Cruz 82, 93–101 (Suppl.).
Raikhel, A.S., Dhadialla, T.S., 1992. Accumulation of yolk proteins in insect oocytes. Annu. Rev. Entomol. 37, 217–251.
Riezman, H., Munn, A., Geli, M.I., Hicke, L., 1996. Actin-, myosin- and ubiquitin-dependent endocytosis. Experimentia-Basel 52, 1033–1041.
Schaerer-Broadbeck, C., Riezman, H., 2000. Functional interactions between the p35 subunit of the Arp2/3 complex and calmodulin in yeast. Mol. Biol. Cell 11, 1113–1127.
Simpson, I., Rose, B., Loewenstein, W.R., 1977. Size limit for molecules permeating the junctional membrane channels. Science 195, 294–296.
Singleton, K., Woodruff, R.I., 1994. The osmolarity of adult *Drosophila* hemolymph and its effect on oocyte-nurse cell electrical polarity. Dev. Biol. 161, 154–167.
Sprenger, F., Nusslein-Volhard, C., 1992. Torso receptor activity is regulated by a diffusible ligand produced at the extracellular terminal regions of the *Drosophila* egg. Cell 71, 987–1001.
Sun, Y.A., Wyman, R.J., 1987. Lack of an oocyte to nurse cell voltage difference in *Drosophila*. Neuroscience 13, 1139.
Telfer, W.H., 1961. The route of entry and localization of blood proteins in the oocytes of saturniid moths. J. Biophys. Biochem. Cytol. 9, 747–759.
Telfer, W.H., 1975. Development and physiology of the oocyte-nurse cell syncytium. Adv. Insect Physiol. 11, 223–319.
Tucciarone, L.M., Lanclos, K.D., 1982. Effect of calmodulin inhibitor, Stelazine, on the endocytosis of vitellogenin and transglutaminase activity in *Xenopus laevis* oocytes. Mol. Cell. Biochem. 45, 159–161.
Waksmonski, S.L., Woodruff, R.I., 2002. For uptake of yolk precursor, epithelial cell–oocyte gap junctional communication is required by insects representing six different orders. J. Insect Physiol. 48, 667–675.
Wieschaus, E., 1996. Embryonic transcription and the control of developmental pathways. Genetics 142, 5–10.
Wollberg, Z., Cohen, E., Kalina, M., 1976. Electrical properties of developing oocytes of the migratory locust, *Locusta migratoria*. J. Cell. Physiol. 88, 145–158.
Woodruff, R., 1979. Electrotonic junctions in cecropia moth ovaries. Dev. Biol. 69, 281–295.
Woodruff, R.I., Anderson, K.L., 1984. Nutritive cord connection and dye-coupling of the follicular epithelium to the growing oocytes in the telotrophic ovarioles in *Oncopeltus fasciatus*, the milkweed bug. Roux's Arch. Dev. Biol. 193, 158–163.
Woodruff, R.I., Telfer, W.H., 1990. Activation of a new physiological state at the onset of vitellogenesis in *Hyalophora* follicles. Dev. Biol. 138, 410–420.
Woodruff, R.I., Tilney, L.G., 1998. Intercellular bridges between epithelial cells in the *Drosophila* ovarian follicle: a possible aid to localized signaling. Dev. Biol. 200, 82–91.
Zhang, Y., Kunkel, J.G., 1992. High abundance calmodulin form *Blattella germanica* eggs binds to vitellin subunits but disappears during vitellin utilization. Insect Biochem. Mol. Biol. 22, 293–304.
Zhang, Y., Kunkel, J.G., 1994. Most egg calmodulin is a follicle cell contribution to the cytoplasm of the *Blattella germanica* oocyte. Dev. Biol. 161, 513–521.