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Membranous intermediates in endocytosis are labile, as shown in a temperature-sensitive mutant

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ABSTRACT **Membranous tubules, especially prevalent in mammalian absorptive epithelia and insect oocytes, are one of the pleomorphic endocytic compartments that have a role in receptor-mediated endocytosis. To determine whether these tubules are evanescent, and to investigate their temporal relationships with other endocytic intermediates, we studied these tubules during oocyte vitellogenesis in the temperature-sensitive mutant *Drosophila melanogaster*, shibire^{ts1}. Raising the temperature of shibire oocytes for 1 min from 19°C to 29°C caused a loss of these membranous tubules. The percentage of membrane in tubules decreased from 36% at 19°C to 1.5% after 5 min at 29°C. Concomitantly, the amount of surface membrane increased from 64% at 19°C to 98% after 5 min at 29°C, causing surface membrane invaginations to extend deeper into the cortex. At 29°C the cytoplasmic face of the plasma membrane was studded with coated pits, and the extracellular face was coated with electron-dense material. Return from 29°C to either 19°C or 26°C for 1–2 min produced a rapid reappearance of tubules containing extracellular horseradish peroxidase in the cortex. These data suggest that tubular intermediates are evanescent structures, and that temperature shock (*i*) rapidly blocks their formation from the plasma membrane, (*ii*) causes existing tubules to rapidly recycle to the plasma membrane, and (*iii*) is rapidly reversed, as newly formed tubules derive their membrane and content from the cell surface.**

The uptake of biologically important macromolecules into cells via receptor-mediated endocytosis is ubiquitous. Morphologically, the process involves (*i*) specific binding of ligand to receptors on the plasma membrane or in coated pits, (*ii*) internalization of the receptor–ligand complexes in coated vesicles, and (*iii*) transport of these complexes into a morphologically complex system of vesicles called endosomes, for eventual storage, processing, or exocytosis. Receptor–ligand complexes can be dissociated in the acidic environment within the endosome (1–3); once released, the receptor can either be degraded or recycled back to the cell surface for reloading (4, 5).

In cells of several absorptive epithelia, the endocytic compartments appear as a complex of smooth and clathrin-coated vesicles, membranous tubules, and interconnecting tubulovesicular structures. The tubules observed in rat kidney proximal tubules, rat visceral yolk sac, hamster ductuli efferentes, and suckling rat ileum possess a characteristic ultrastructure consisting of helically disposed parallel rows of particles on the inner surface of the tubule membrane (6, 7). The origin and fate of these tubules is not clear. They may carry receptor–ligand complexes into a cell (8–10) and/or recycle internalized receptors back to the cell surface (11, 12). It is not known what temporal relation these tubules have to other endocytic compartments or whether they are transient or constant endocytic intermediates (13).

To determine (*i*) whether these intermediates are evanescent and (*ii*) their relationship to other endocytic compartments, we chose to extend our previous observations of receptor-mediated endocytosis in the oocyte of the temperature-sensitive mutant *Drosophila melanogaster*, shibire^{ts1} (*shi*) (14). Raising the temperature to 29°C in this sex-linked recessive mutant resulted in a rapid paralysis of the adult (15–17); this was rapidly reversed when the temperature was lowered below 22°C. Kosaka and Ikeda demonstrated that temperature shock (TS) reversibly stopped nerve impulse transmission in presynaptic terminals (18) as well as endocytosis by garland cells (19). In these cells, coated pits did not pinch off into the cytoplasm at 29°C.

Sequestration of vitellogenin by the oocyte is pH sensitive (20), uptake is mediated by coated pits and coated vesicles, and the vitellogenin is endocytosed into membranous tubules (21–24) that are morphologically similar to tubules seen in mammalian absorptive epithelia (6, 7). In *shi* flies we found that TS for 1–5 min causes (*i*) disappearance of all vesicles and membranous tubules from the oocyte cortex and (*ii*) an increase in the surface area of the plasma membrane causing the membrane to fold and project deeper into the oocyte cortex. These modifications to endocytic intermediates in the *shi* oocyte are consistent with a block in the uptake of vitellogenin during TS. Endocytic intermediates present at the time of TS recycle back to the plasma membrane. When returned to permissive temperature, a complete reversal of the block occurs in <2 min, resulting in the restoration of tubular intermediates and the loss of the deep plasma membrane invaginations. Comparably treated wild-type (WT) flies showed no morphological changes in the endocytic elements.

MATERIALS AND METHODS

shi mutant *D. melanogaster* were obtained from the Drosophila Stock Center (Bowling Green State University, Bowling Green, OH). Both the *shi* and WT Oregon R flies were grown at 19°C and used 2–4 days posteclosion.

TS Experiments. *shi* and WT flies were placed into 19°C or 29°C water for 0, 1, 5, 10, 15, and 30 min. The ovaries were removed and immersed in 19°C or 29°C fixative for 2–4 min; subsequent fixation and dehydration was at 4°C, except as noted. *shi* and WT flies at 19°C, and WT flies at 29°C, were anesthetized briefly with ether prior to dissection.

Recovery Experiments. *shi* and WT flies were temperature shocked in a 30°C high humidity chamber and secured, ventral side up, with insect pins. The ovaries were exposed and immersed for 15 min in either 2–5 μl of Robb's phosphate-buffered saline (PBS) (25) or 2–5 μl of 0.7% horseradish peroxidase (HRP) (18). The solutions and flies were cooled to 19°C or 26°C in a high humidity chamber for 0, 0.5, 1, 2, or

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Abbreviations: TS, temperature shock; WT, wild type; HRP, horseradish peroxidase; TL, test line.

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5 min. Fixation of the ovaries was initiated at the recovery temperature (19°C or 26°C) for 30 min and completed at 4°C.

Electron Microscopy. Tissue was fixed for 2 hr in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 1 mM ruthenium red to stain the external face of the plasma membrane (26). After washing, the ovaries were further fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), containing 1 mM ruthenium red, for 1 hr at 4°C and 1 hr at room temperature. Ovaries were washed in five to eight changes of distilled water and stained *en bloc* in 0.5% uranyl acetate for 4 hr or overnight at 4°C. Ovaries exposed to HRP were fixed in 2.5% glutaraldehyde without ruthenium red, and then incubated for 30 min with 0.05% and then an additional 30 min with 0.5% diaminobenzidine tetrahydrochloride at 4°C, prior to postfixation in 2% osmium tetroxide for 2 hr at 4°C. Dehydration with ethyl alcohol, infiltration with propylene oxide resin, and embedding in Epon (27) or Spurr's low viscosity embedding medium (28) were carried out by standard procedures. Oocytes in developmental stages 8–10 (29), characterized by the presence of obvious yolk granules and a partially formed vitelline layer, were thin sectioned, stained with lead citrate and uranyl acetate, and viewed in an Hitachi H-600 or Zeiss 10CA. Tissue exposed to HRP was examined unstained.

Quantitation of Membrane. The relative amount of membrane represented by the oocyte plasma membrane and the tubular endocytic compartments, designated the cortical membrane, was quantitated from 7–10 micrographs for each of two or three oocytes exposed to TS for 0, 1, 5, 10, 15, and 30 min. The micrographs were taken at an initial magnification of 8000–20,000 and enlarged 2–3.5 times. A test line (TL) of known length (in μm), relative to the magnification of each micrograph, was manually drawn along the interface between the oocyte and the overlying vitelline layer. The number of intercepts of a Merz semicircular line grid (31) with the TL, the plasma membranes, and tubular membranes lying within the oocyte cortex, were used to determine the length of the plasma membrane and the amount of tubule membrane per μm of TL. Results were also expressed as percent of cortical membrane that is plasma membrane and percent that is tubular. Clathrin-coated profiles along the plasma membrane were counted and reported per μm of TL. Vesicles, other than nascent yolk granules, were found to represent an insignificant amount of the cortical membrane and are not shown in the data.

RESULTS

Oocyte morphology from WT and *shi* flies reared at 19°C was identical to that in WT flies reared at 25°C. Vesicles, anastomosing tubules, and nascent yolk granules were present predominantly in the first 1 μm of the oocyte cortex, which was consistent with published descriptions of the *Drosophila* oocyte ultrastructure (21–24). Normally, few vesicles of any type were seen immediately beneath the plasma membrane, whereas the tubular intermediates and nascent yolk granules were prevalent deeper in the cortex (Fig. 1). As previously described for *Drosophila* oocytes and other absorptive epithelia (6, 7, 22), the tubular intermediates had a cross-sectional diameter of 50–60 nm and were characterized by a densely packed spiral of particles that occluded all but 15–20 nm of the interior diameter. In longitudinal section, the lumen of the tubules appeared to contain a moderately electron-dense material. The particles observed in cross-section produced linear arrays along the internal face of the tubular membrane (Fig. 1 *Inset*). Often, these anastomosing tubules were confluent with, and interconnected, the smooth membrane of nascent yolk granules. The linear arrays of particles were confined within the tubular lumens; they were never observed on the internal face of confluent yolk granule membrane. Tubules never appeared to contact the plasma membrane (Fig. 2). The plasma membrane was highly folded into microvilli. The bases of the invaginations between microvilli were coated with electron-dense material on the external face of the membrane and coated pits on the cytoplasmic face.

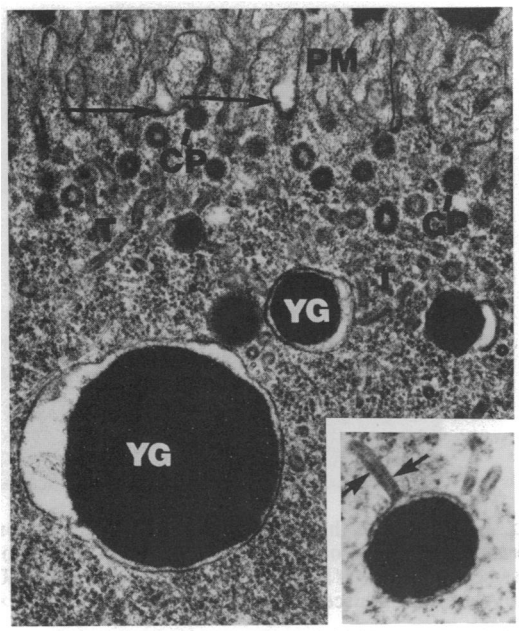

FIG. 1. *shi* oocyte fixed at 19°C with the addition of ruthenium red to stain cytoplasmic structures contiguous with the external face of the plasma membrane (PM). The plasma membrane has microvilli, between which are deep infoldings characterized by ruthenium red-stained patches of electron-dense material (arrows) and coated pits (CP). When present, cytoplasmic vesicles are directly beneath the plasma membrane, while tubules (T) and nascent yolk granules (YG) are located somewhat deeper in the cortex. (×28,000.) (*Inset*) Cortical tubules in longitudinal section. One tubule is fused with a nascent yolk granule. Note the linear arrays of particles protruding from the internal face of the tubular membrane (arrows) and bordering the central lumen, which appears to contain a moderately electron-dense material. (×39,840.)

TS Experiments. When oocytes from *shi* flies were exposed to 29°C for 1, 5, 10, 15, and 30 min, there was an almost complete disappearance of vesicular and tubular intermediates in the oocyte cortex during the first minute. None of the usual endocytic intermediates was present after 5 min (Fig. 3). When the *shi* oocytes were incubated in HRP at 29°C, HRP reaction product was not found in any endocytic intermediates. In contrast, no obvious changes were seen in the WT oocytes at 29°C.

In *shi* flies that were temperature shocked, the loss of oocyte cortical vesicles and tubular membrane was accompanied by an increase in the plasma membrane surface area. This was manifested by a folding of the plasma membrane such that invaginations often extended deeper than 1 μm into the cortex. In addition, the amount of electron-dense material coating the external face of the oocyte plasma membrane accumulated and spread upward from its initial position at the deepest ends of the folds (Fig. 3). Particles similar to those observed within the tubule lumens were not evident on the plasma membrane.

When the amount of membrane in both *shi* and WT oocytes at 19°C and 29°C was quantitated by morphometry, there was a marked difference in the *shi* oocyte as early as 5 min after TS (Table 1). Initially, 36% of the cortical membrane was in the tubular intermediates, but at 29°C this dropped to 1.6% at 5 min, 2.2% at 10 min, 2.8% at 15 min, and 3% at 30 min. There was a concomitant shift of membrane to the oocyte surface from 64% of the cortical membrane at 19°C, to 98%

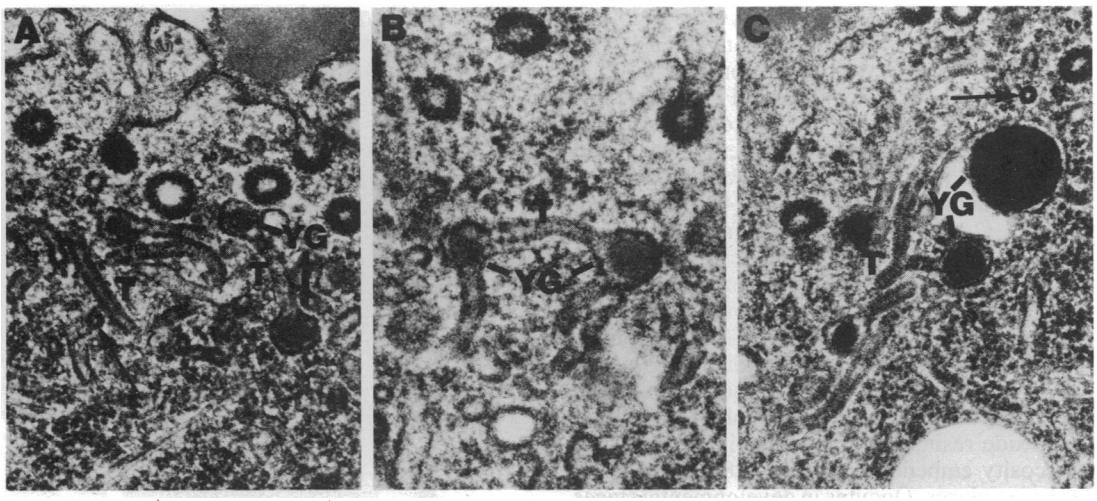

FIG. 2. Tubules in the *shi* oocyte fixed at 19°C. Membranes of anastomosing tubules (T) are fused with, and often interconnecting, the membranes of nascent yolk granules (YG). Direct connections between tubules and the plasma membrane are not observed. Cortical tubules in cross-section (arrows) have an external diameter of 50–60 nm. An array of electron-dense particles rims a 15- to 20-nm central lumen that is relatively electron luscent. (*A*, ×42,670; *B*, ×51,970; *C*, ×42,670.)

at 29°C for 5 min, 97% for 10 min, 97% for 15 min, and 97% for 30 min. In the WT, there was essentially no change in the proportion of membrane that was in the tubular intermediates (21%) and the plasma membrane (79%) throughout the TS regimen. No change was noted in the number of coated pits or coated vesicles in *shi* or WT oocytes, before or after TS (5.37 coated profiles per μm of TL at 19°C as compared with 5.02 coated profiles per μm of TL for *shi* oocytes at 29°C for 15 min). Interestingly, in *shi* oocytes during TS, although coated pits were still found principally along the deepest infoldings of the plasma membrane, there was a change in distribution such that they frequently became clustered in groups of two or three coated pits (Fig. 3).

Recovery from TS. Return from 29°C to 19°C resulted in a rapid reversal of the TS morphology. We attempted to slow recovery from TS so as to better determine the order of reappearance of endosomal intermediates by exposing the oocytes to 26°C. However, we found that at either 19°C or 26°C 1–2 min was sufficient to reinitiate endocytosis and repopulate the cortex with numerous tubular intermediates that contain HRP reaction product (Fig. 4). These tubules were frequently clustered around nascent yolk granules and their membranes were contiguous with, and often interconnected, nascent yolk granules (Fig. 4*B*). At the same time, there was a reduction in the total length of plasma membrane, as evidenced by the return of the plasma membrane to a less convoluted form with shallower invaginations.

CONCLUSIONS

Uptake of vitellogenin into the *D. melanogaster* oocyte occurs by receptor-mediated endocytosis via a system of plasma membrane-coated pits, coated vesicles, and anastomosing membranous tubules. We used the temperature-sensitive mutant *shi* to study the temporal relationships between these components.

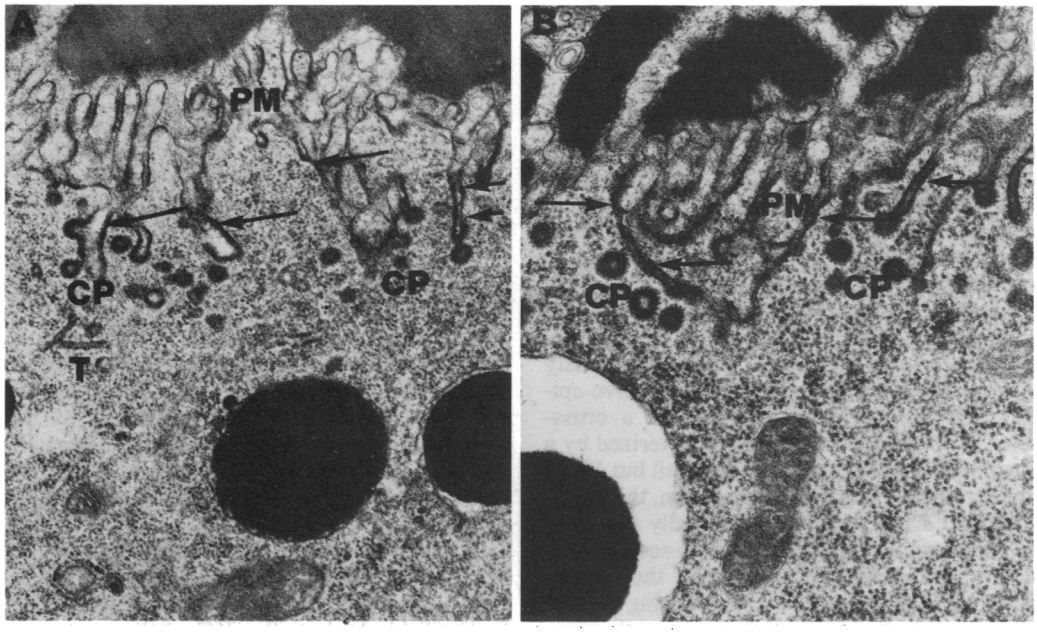

FIG. 3. *shi* oocyte temperature shocked at 29°C for 5 min (*A*) and 15 min (*B*) prior to being fixed at 29°C. Few if any tubules (T), and no vesicles, are seen in the cortex. Note that the plasma membrane (PM) extends deeper into the cortex and that several coated pits are often associated in these deepest portions of the plasma membrane invagination. Ruthenium red-stained electron-dense material (arrows) coats the external face of the plasma membrane on the base and sides of the deep membrane folds (compare to Fig. 1). (*A*, ×21,120; *B*, ×27,840.)

Table 1. Temperature effects on *shi* cortical membrane

t	Time after TS, min	μm per μm of TL		
		Tubular	Plasma	Cortical
19°C	0	2.57 (36%)	4.54 (64%)	7.11
29°C	5	0.10 (1.56%)	6.75 (98%)	6.84
	10	0.24 (2.2%)	9.05 (97%)	9.29
	15	0.21 (2.8%)	7.20 (97%)	7.41
	30	0.15 (5.2%)	5.28 (97%)	5.42

Values are expressed as means. Percent of cortical membrane is shown in parentheses.

TS at 29°C for 1–5 min essentially eliminates vesicular and tubular intermediates from the *shi* oocyte cortex. Concomitant with the loss of endocytic intermediates is a marked deepening of plasma membrane invaginations, with clusters of coated pits localized primarily to the deepest regions of these infoldings. Electron-dense material accumulates on the external face of the plasma membrane. Reversal of TS at 19°C or 26°C results in a dramatic reappearance of all intermediates within 1–2 min, such that membranous tubules contain extracellularly applied HRP reaction product. These data suggest that TS (*i*) blocks pinch-off of surface membrane, thus blocking an initial step in the endocytic process; (*ii*) causes tubular membranes lost from the oocyte cortex to add to the plasma membrane; and (*iii*) is reversible such that the tubules re-form, deriving their membrane and content from the cell surface.

Localization of HRP reaction product, a marker for fluid uptake, within the membranous tubules is consistent with hypotheses that state these pleomorphic tubules are components in the endocytic pathway involved in the transport of vitellogenin into the oocyte cortex (20, 22–24). Since the stage 10 oocytes are known to be active in the uptake of yolk proteins (21–24, 29), it is likely that much of the electron-dense material adsorbed to the external face of the oocyte plasma membrane is vitellogenin. Coupled with the observations of the rapid disappearance/reappearance of the tubular intermediates, the data clearly demonstrate that the tubules are evanescent components of this endocytic pathway. Yet the origin and fate of the membranous tubules within the pathway is not clear. Assuming that TS inhibits the pinch-off of surface membrane into the cytoplasm, formation of subsequent vesicular and tubular intermediates is blocked. This idea is consistent with Kosaka and Ikeda's observations of temperature-dependent vesicle depletion in *shi* presynaptic terminals (18) and diminished numbers of tubules in *shi* garland cells (19). In the latter study, Kosaka and Ikeda also observed an increase in plasma membrane-coated profiles and a decrease in coated vesicles during TS at 30°C. Surprisingly, we did not observe this result in the *shi* oocyte. Perhaps we were unable to observe an increase in clathrin-coated profiles, other than those in coated pits, because they were obscured by the heavy coating of electron-dense material adsorbed to the external face of the plasma membrane and were not counted.

In the *shi* oocyte, tubules could originate either directly from the plasma membrane or secondarily by vesicle fusion. However, tubules are never seen connected to the plasma membrane, and few if any vesicles are present in the vicinity of the plasma membrane when tubules reappear. The only instance in which there appears to be some vesicle fusion is between small vesicles and the nascent yolk granules; however, when viewed in serial sections, almost all of these profiles are found to be cross-sections of tubules. Thus, if tubules arise from vesicles it may be that the vesicles are too evanescent to be preserved during tissue preparation.

These data also suggest that tubules in the *shi* oocyte cortex may have a role in recycling endocytosed membrane, and presumably receptors, back to the plasma membrane. We show (Table 1) that the plasma membrane surface area increases by 5 min at 29°C. This increase could be caused by additions of either newly synthesized membrane or recycling tubular membrane. If membrane from both of these sources was added to the plasma membrane, we would expect its surface area to show a steady increase during extended TS treatment. Instead, the data show that there is an immediate increase in plasma membrane surface area that plateaus for the next 30 min of TS treatment. This implies that the TS-induced increase in the plasma membrane surface area is most probably due to additions from either the tubular membrane compartment or newly synthesized pools, but not both. Since

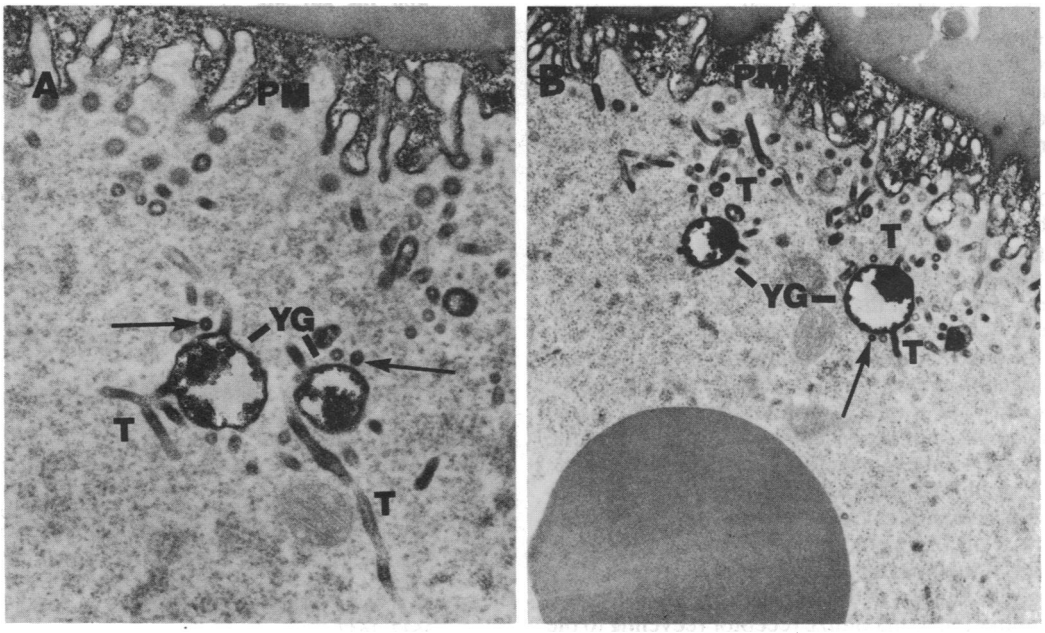

FIG. 4. *shi* oocyte temperature shocked at 30°C in the presence of HRP was then returned to 26°C for 1 min and fixed at 26°C. Anastomosing tubules (T), some in cross-section (arrows), and nascent yolk granules (YG) contain HRP reaction product. The tubules are connected to and clustered around yolk granules. Note that plasma membrane (PM) invaginations are shallower and less convoluted at 26°C compared with those that remain at 30°C (see Fig. 3). (*A*, ×30,860; *B* ×17,140.)

pinch-off of coated pits into coated vesicles is blocked during TS, it is possible that other membrane traffic within the oocyte cortex is also inhibited, thereby stopping or greatly diminishing insertion of newly synthesized membrane into the plasma membrane. This circumstance would leave only tubular membrane available for addition to the surface membrane compartment. In addition, we must consider an alternative fate for the tubular membrane. When membranous tubules are reversibly lost from the oocyte cortex during TS, they might fuse with a later endocytic compartment (e.g., nascent yolk granules) until the TS block to endocytosis is removed. However, in this case we would expect the plasma membrane surface area to decrease, rather than increase, by the amount of tubular membrane that fuses with the nascent yolk granules. We believe the increase in the plasma membrane surface area results only from addition of recycling tubular membrane. Thus, it appears that TS may inhibit endocytosis while permitting the endocytic tubular membranes to fuse into the plasma membrane and complete the endocytic pathway.

If the tubular membrane is adding to the plasma membrane during TS, we might expect to observe particulate arrays on the external face of the plasma membrane that are similar to the particles observed within the tubular lumens. However, this was never seen. It is possible that these particles, once so highly concentrated within the tubular lumens, diffuse when added to the plasma membrane and become obscured by the electron-dense material on the external face of the plasma membrane. Alternatively, if the particles are receptors, they may be a component of the electron-dense material on the external face of the plasma membrane, perhaps as part of the vitellogenin receptor–ligand complex.

When tubules are first observed in the oocyte cortex during reversal, they are connected to and clustered around nascent yolk granules. Thus, it seems reasonable to infer that they may be delivering vitellogenin to nascent yolk granules. However, they may also be involved in recycling of membrane, and presumably receptors, back to the plasma membrane. The morphology of the tubules is consistent with this latter interpretation since the small cross-section of the tubule, being near the minimum curvature of a membrane, would maximize the amount of membrane, and receptors associated with the membrane, while minimizing the void volume available to hold dissociated yolk.

Whether tubules function in vitellogenin transport, membrane recycling, or both, the particles observed on the inner face of the tubules may be arrays of concentrated receptors or ion transport proteins. Consistent with this interpretation is the occurrence of particulate arrays in similar tubules observed in mammalian absorptive epithelia (6, 7) and patches of morphologically similar particles that occur on the plasma membrane of proton-secreting cells (30). If tubules are delivering receptor–ligand complexes to nascent yolk granules, ion pumps could mediate the dissociation of ligands from receptors, after which the vitellogenin would condense to form nascent yolk granules. Alternatively, the ion pump might create an environment that primarily favors condensation of vitellogenin, thus driving the equilibrium toward the formation nascent yolk granules, without necessarily changing the binding constant of the receptors. But if this were the case, one might expect the pumps to reside in the membranes of nascent yolk granules, which are in many ways a specialized form of the endosome. However, we never observed particulate arrays on the membranes of nascent yolk granules. In contrast, if tubules contain primarily receptors, their high concentration coupled with the small void space would be an ideal combination to maximize receptor recycling to the plasma membrane while leaving most of the yolk behind. We favor this latter possibility. Since we have never observed tubules contiguous with the plasma membrane, it is possible that small vesicles that are too evanescent to be fixed may be the last link in recycling.

What is clear from this study is that the membranous intermediates that function in the endocytosis of *Drosophila* vitellogenin are labile rather than permanent structures. The vesicles and tubules form a network through which endocytosed ligands and receptors can migrate. These data are consistent with hypotheses of Kosaka and Ikeda concerning TS block of endocytosis in the *shi* mutant (18, 19) but add to their observations concerning the role of membranous tubules in the endocytic process. We present evidence that the membranous tubules in the *Drosophila* oocyte cortex may be the endocytic compartment through which membrane and receptors are recycled to the plasma membrane. The anastomosing tubular system appears to originate from the plasma membrane, although the relationships between tubules, vesicular components, and nascent yolk granules are sill not clear.

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