

Bead Rings at the Endoplasmic Reticulum- Golgi Complex Boundary: Morphological Changes Accompanying Inhibition of Intracellular Transport of Secretory Proteins in Arthropod Fat Body Tissue

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ABSTRACT Golgi complex beads are 10-nm particles arranged in rings on the smooth surface of rough endoplasmic reticulum (ER) making the forming face of the Golgi complex (GC). In arthropod cells they stain specifically with bismuth. Their morphology has been studied after treatment with reagents known to interfere with GC function. Inhibitors of oxidative phosphorylation (antimycin A, cyanide, and anoxia), but not an inhibitor of glycolysis (iodoacetate), both cause the bead rings to collapse and the GC saccules to round up, and inhibit transition vesicle (TV) formation. Cycloheximide blocks protein synthesis on ribosomes but does not stop TV formation or disrupt bead rings, even after prolonged treatment (6 h) to allow emptying of the rough ER cisternae. Thus the collapse of bead rings is not attributable to inhibition of protein synthesis, and the ring structure of beads does not require continued protein synthesis and secretion for its maintenance. Valinomycin has effects on the GC similar to those of antimycin A, but A23187, monensin, and lasalocid do not affect bead ring structure or TV formation. These results are consistent with valinomycin's secondarily uncoupling mitochondria, which collapses bead rings and prevents TV formation. Thus inhibitors of oxidative phosphorylation do not influence the beads through cation movement. Because monensin and lasalocid block secretion at the level of the condensing vacuoles, bead rings are not influenced by blocks in secretion distal to them or by the backup of secretory material. These experiments are consistent with inhibitors of oxidative phosphorylation collapsing bead rings by decreasing intracellular ATP. The concomitant block to TV formation and the collapse of bead rings suggests that integrity of the bead rings is essential for the transport of secretory material from the rough ER to the GC.

Transport of secretory proteins and membrane between rough endoplasmic reticulum (ER) and Golgi complex (GC) saccules appears to be accomplished by a transition vesicle (TV) shuttle in an alternating fission-fusion process (5). This transport is sensitive to inhibitors of oxidative phosphorylation (antimycin A, cyanide, and N_2) (6). The energy-dependent step is at the forming face of the GC between the smooth surface of rough ER and the TVs. Studies on arthropod tissues fixed in glutaraldehyde and reacted with bismuth (9, 10) showed rings of 10-

to 12-nm particles, the GC beads, on the smooth GC-forming face of the rough ER. The beads were found only in this location and frequently associated with forming TVs. This led Locke and Huie (9) to suggest that the beads might be involved in the interaction of TVs with the smooth face of the rough ER. In vitro experiments suggested that bismuth may be bound to the beads through phosphate groups (12). The presence of phosphate groups might indicate a requirement for ATP.

Although GC beads have been seen in vertebrates (11), the

usual experimental systems are unsuitable for this investigation because beads have to be stained with bismuth to allow clear observation. Bismuth shows the beads in unstained thin sections and allows visualization of complete bead rings in unstained thick sections. For this reason, I have used fat body tissue from larvae of an arthropod, *Calpodes ethlius*, during the mid-fifth stage when it is actively secreting hemolymph proteins (8). This tissue is structurally similar and functionally analogous to vertebrate liver.

If the beads are involved in secretion through the energy-dependence of transport to the GC, then inhibitors of oxidative phosphorylation might be expected to alter their structure with the loss of transition vesicle formation. To answer this question, I have investigated the effects of several pharmacological agents upon the ultrastructure of the GC and GC beads. The results show that bead rings collapse after the reduction of intracellular ATP and suggest that the rings are part of the mechanism for TV formation (2).

MATERIALS AND METHODS

Preparation of Test Solutions

Antimycin A (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), valinomycin (Sigma Chemical Co., St. Louis, Mo.), A23187 (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), monensin (a gift from J. Kwong of Eli Lilly Chemical Co., Indianapolis, Ind.) and lasalocid (a gift from Dr. J. F. Meyers of Hoffman-La Roche Inc., Nutley, N. J.) were dissolved in ethanol or dimethyl sulfoxide (DMSO). These solutions were then diluted with distilled water and injected. Controls consisted of the appropriate dilution of ethanol or DMSO. Cycloheximide (Sigma Chemical Co.), sodium cyanide (J. T. Baker Chemical Co., Phillipsburg, N. J.) and iodoacetate (Sigma Chemical Co.) were dissolved in Grace's medium or distilled water.

Injection of Pharmacological Agents

Lepidoptera are convenient experimental animals because they are integumental sacs of hemolymph with fat body floating freely inside. By ligating a larva in the middle and using the anterior ligated abdominal chamber, clearance of test solutions is prevented by excluding the Malpighian tubules posterior to the ligation. These chambers remain viable for days and show no abnormalities in fat body ultrastructure.

Larvae were loosely ligated between the 2nd and 3rd pair of prolegs. The syringe needle was then inserted through one of the 3rd pair of prolegs and pushed forward along the cuticle past the ligature. The ligature was then tightened around the needle and 25 μ l of test solution injected. The needle was withdrawn, the ligature tightened, and the posterior half of the insect removed. These anterior chambers contained \sim 125 μ l of hemolymph and, because 25 μ l of test solution was injected, the final concentration was about one-sixth of the injected concentration.

Electron Microscopy

After the appropriate incubation period, the anterior half of the larvae was inflated with 5.0% glutaraldehyde with 2% sucrose in 0.1 M Na cacodylate buffer at pH 7.4. The abdominal chamber was then sectioned and further fixed in the same fixative at 0–4°C for 1–4 h. The tissue was then washed in 0.1 M Na cacodylate containing 4% sucrose at pH 7.4 and 0–4°C and divided into three portions.

The electron microscopy had three objectives: (a) The general structure of the GC was observed in tissue postfixed in OsO₄ and section-stained with uranyl acetate and lead citrate (part a of all micrograph figures). (b) The relationship between beads and between bead and membrane was viewed in unstained sections of tissue reacted with bismuth and postfixed in OsO₄ (part b of all micrograph figures). In this procedure, fixed tissue was washed in 0.1 M triethanolamine-HCl buffer at pH 7.5, reacted with a bismuth solution at room temperature for 1.5 h at pH 7.5, washed with 0.1 M triethanolamine-HCl buffer, and postfixed with Na cacodylate-buffered 1% OsO₄. The bismuth solution was made according to Locke and Huie (9), except that a 1:2.5 dilution was made with 0.2 M triethanolamine-HCl buffer. (c) The arrangement of beads in rings was observed in thick sections of tissue reacted with bismuth as described above but not postfixed in OsO₄ (part c of all micrograph figures). This allowed sections as thick as 250 nm to be observed at 100 kV.

Over 100 animals were used in this part of the study and the results recorded in over 1,000 micrographs.

ATP Assay

Accurate ATP analysis requires that tissue be quickly removed from the animal and plunged into liquid nitrogen (1, 7). Because dissection of fat body from the animal requires too much time, whole body tissue (minus gut and epidermis) was used for the assay since it can be obtained within 30 s. Fat body constitutes most of this tissue and the results will largely reflect fat body cell ATP levels.

Tissue was quickly removed from the animals, rinsed in Grace's insect medium, and quick-frozen by immersion in liquid nitrogen. Tissues from three or four animals were pooled, pulverized to a powder, and added to cold 6% (wt/vol) perchloric acid. The samples were then homogenized and centrifuged at 86,500 g in a Beckman T60 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) for 30 min. The perchloric acid extracts were neutralized to pH 5–7 with KOH and stored frozen.

A modification of the assay of Lamprecht and Trautshold (7) was used for this study. The incubation medium contained 0.5 ml of perchloric acid extract, 1.925 ml of 0.1 M triethanolamine hydrochloride, 25 μ l of 0.026 M NADP, 25 μ l of 0.5 M MgCl₂, 10 μ l of 0.5 M glucose, 0.5 U of glucose-6-phosphate dehydrogenase (1 U will oxidize 1.0 μ mol of glucose-6-phosphate to 6-phosphogluconate per min at pH 7.4 at 25°C, in the presence of NADP) and 8.4 U of hexokinase (1 U will phosphorylate 1.0 μ mol of glucose per min at pH 8.0 at 25°C). The assay was run at pH 7.6 and 30°C in a Unicam SP1800 spectrophotometer (Pye Unicam Ltd., Cambridge, England). Calculations were made according to Adams (1) from absorbance changes in 340 nm recorded 12–15 min after the start of the assay. Each assay was done in duplicate and the values shown in the graphs are averages of these two assays.

Cell Viability during Exposure to Pharmacological Agents

With the exception of cycloheximide, the effects of all agents were evident within 1 h. At the time of fixation, all treated cells had a normal ultrastructural morphology (except that attributable to the specific effects of the reagent). All treated cells continued to exclude trypan blue beyond the time limits of exposure to all pharmacological agents, unless otherwise indicated in Results.

RESULTS

General Morphology of the GC and GC Beads

Fat body GCs in control tissue consist of three or four saccules filled with dense material (Fig. 1a). Numerous TVs and forming TVs are present in the transition region, and large protein granules, 200–400 nm in diameter, are associated with the innermost saccule.

The beads are found only on the forming face of the GC in the transition region (Fig. 1b) and have a center-to-center spacing of 27 nm and a center-to-membrane spacing of 14 nm (10). Beads are always found around the neck of forming TVs (as in Figs. 1b and 8b). This ring arrangement can most readily be seen in thick sections that may contain most of the TV region of a GC (Fig. 1c).

Inhibition of Oxidative Phosphorylation and the Loss of Bead Ring Structure

Antimycin A is an inhibitor of oxidative phosphorylation (17). After 1 h, 1.7×10^{-4} M antimycin A reduces the tissue ATP level to one-quarter of the control value (Fig. 2). Under these same conditions, the GC saccules round up and the forming TVs disappear (Fig. 3a). These two changes were taken to indicate an effective block to secretion at the level of the forming face of the GC. TVs and tubular structures remain in the transition region after inhibition of transport. These may be derived from the outermost saccule and/or the result of an accumulation of TVs returning to the forming face of the GC but prevented from fusing with it.

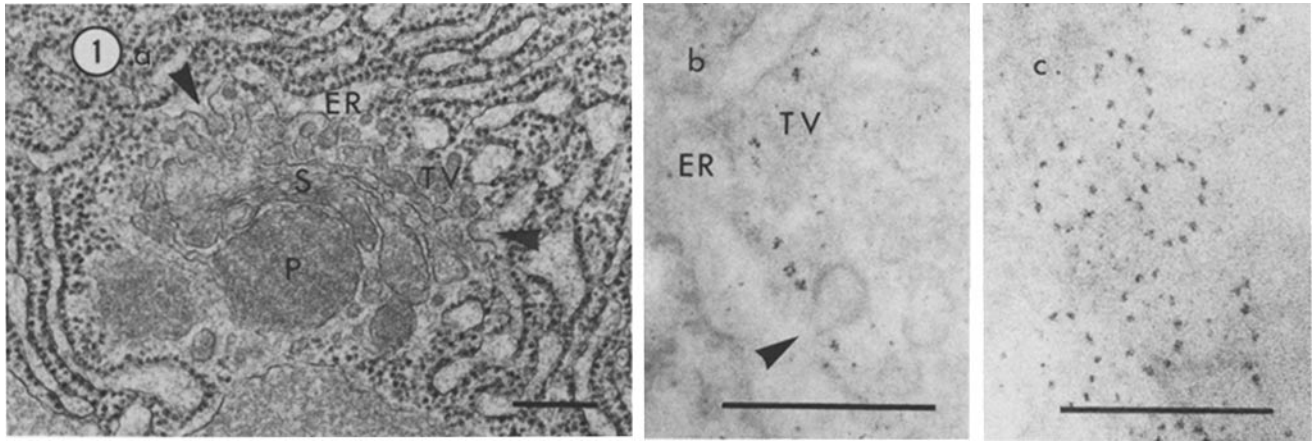


FIGURE 1 The normal morphology of control tissue treated with the carrier for the pharmacological agent (in this instance, 0.8% DMSO in distilled H₂O). (a) Forming transition vesicles (arrowheads), electron-dense and semicircularly arranged saccules (S), and large protein granules (P) are characteristic of fat body GCs of mid-5th-stage larvae. (b) The beads are regularly spaced from one another and the smooth surface of rough ER. Note the presence of a bead on either side of the neck of the forming transition vesicle (arrowhead). (c) The beads are always arranged in rings. For all micrographs, part a is a thin section stained with uranyl acetate and lead citrate and its magnification is $\times 55,000$. Part b is an unstained thin section of tissue reacted with bismuth and its magnification is $\times 140,000$. Part c is an unstained thick section of nonosmicated tissue reacted with bismuth and its magnification is $\times 140,000$. The bar in each part of the figures represent $0.2 \mu\text{m}$. ER, rough endoplasmic reticulum; TV, transition vesicle region.

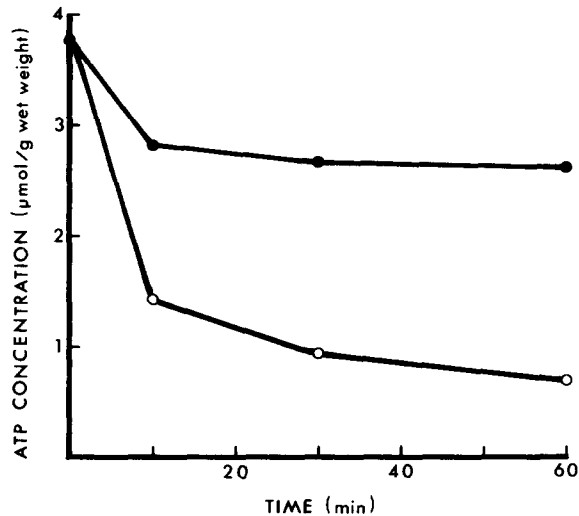


FIGURE 2 Effect of 1.7×10^{-4} M antimycin A (○) on intracellular ATP levels. The control curve (●) is the carrier for antimycin A (0.8% ethanol in distilled H₂O). For all the ATP measurements reported in Figs. 2, 5, 9, 11, and 15 (except for anoxic conditions), the solutions were introduced into ligated anterior abdominal chambers as described in Materials and Methods, and tissue was removed for the assay at the indicated times.

In spite of the gross change in GC morphology, there was no change in spacing between beads or between bead and membrane (Fig. 3b) although the beads had clumped together and lost their ring structure (Fig. 3c). The concentration of the beads into a close-packed sheet may be caused by the loss of membrane, which reduces the area of the forming face of the GC.

To determine the sequence of these ultrastructural changes to the GC, I observed the fat body after a range of periods of exposure to 7.0×10^{-5} M antimycin A. After 5 min, the GC saccules were little changed but forming TVs were absent (Fig. 4a). There was no change in spacing between beads or between bead and membrane (Fig. 4b). Although the rings had col-

lapsed, no large single mass of beads had formed (Fig. 4c). Longer exposures caused greater saccule condensation and bead clumping. 1.4×10^{-5} M antimycin A was much less effective and lower concentrations had no observable effects.

It is possible that antimycin A alters the GC and GC bead rings through nonspecific side effects of the antibiotic. Cyanide and anoxia also inhibit oxidative phosphorylation and block transport between rough ER and GC (6). They reduce the tissue ATP level to one-half of the control value (Fig. 5). After 1 h, 1.7×10^{-2} M sodium cyanide caused changes in GC ultrastructure and GC bead ring arrangements similar to those caused by antimycin A (Fig. 6a-c). Lower concentrations of sodium cyanide had no effect. Fat body cells of animals kept under anoxic conditions for 1-2 h also showed the same changes caused by antimycin A (Fig. 7a-c).

Inhibition of transport is specific to inhibitors of oxidative phosphorylation and not to inhibitors of glycolysis, such as iodoacetate (6). 1.7×10^{-3} M iodoacetate does not lower the tissue ATP level below the control value (Fig. 9) and does not alter the GC or GC bead rings (Fig. 8a-c). Higher concentrations of iodoacetate (1.7×10^{-2} M) cause extensive cytolytic effects as judged by ATP measurements (Fig. 9), ultrastructural morphology and trypan blue dye exclusion tests.

Cycloheximide and Protein Synthesis

Antimycin A inhibits protein synthesis to a variable extent depending upon the dose applied (6). To investigate the possibility that antimycin A might affect bead rings by inhibiting protein synthesis, I exposed tissue to 2.4×10^{-3} M cycloheximide for 0.5-6 h. This concentration of cycloheximide is sufficient to stop silk synthesis in *Calpodex* larvae (M. Locke, personal communication) but as much as 6.0×10^{-3} M cycloheximide does not reduce the ATP level below the control value (Fig. 9). By 1-2 h, the GC saccules had increased in number to four or five and became electron lucent, as if empty (Fig. 10a). No protein granules or condensing vacuoles were present but TVs and forming TVs remained. There was no change in spacing between beads or between bead and membrane (Fig. 10b) and the beads remained in rings (Fig. 10c).

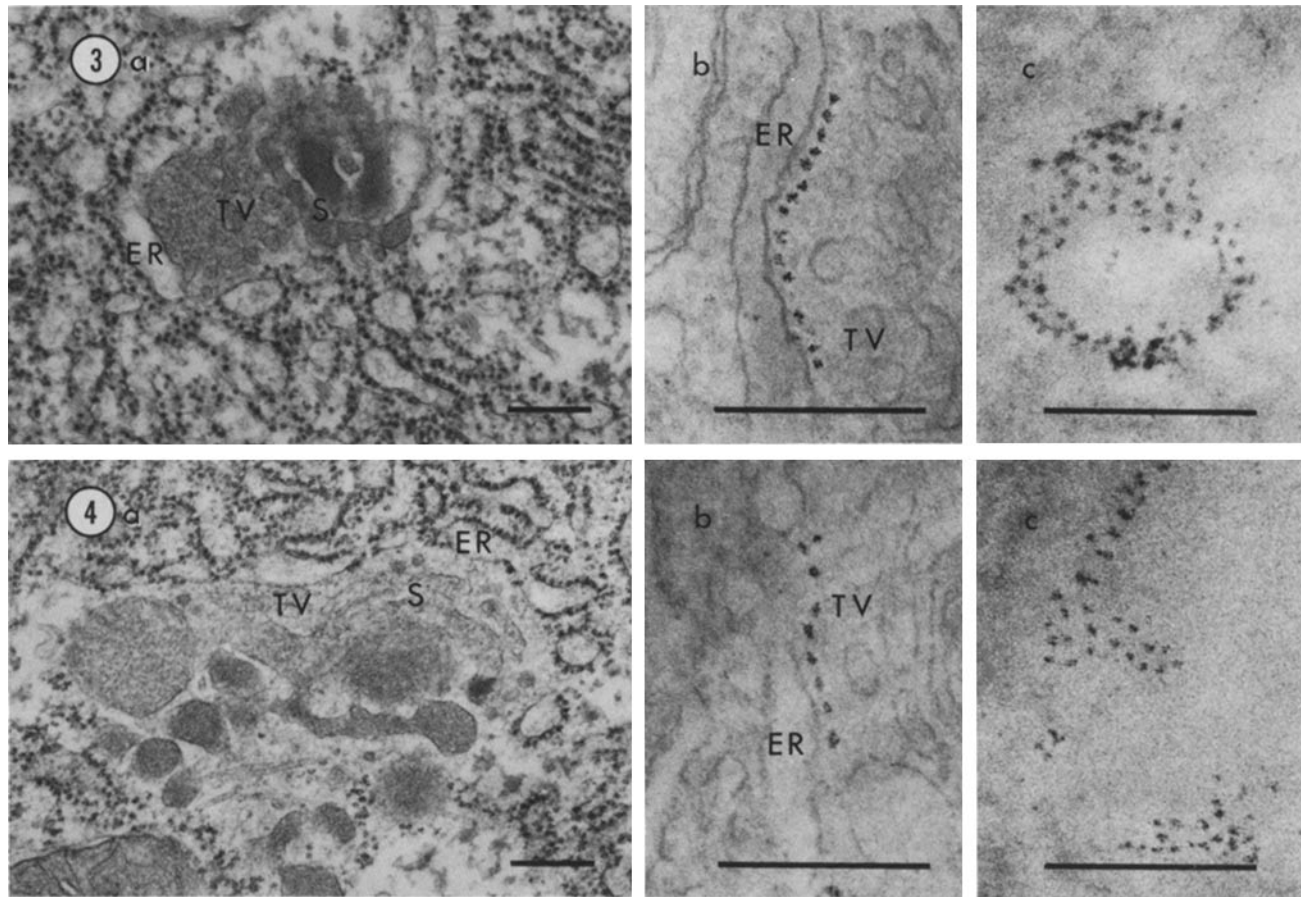


FIGURE 3 (a) 1.7×10^{-4} M antimycin A for 1 h prevents formation of transition vesicles and causes GC saccules (S) to round up. (b) The spacing between beads and between bead and membrane is not changed. (c) The bead ring arrangement is lost and a mass of beads is formed instead.

FIGURE 4 (a) 7.0×10^{-5} M antimycin A for 5 min prevents formation of transition vesicles, but GC saccules (S) have changed very little. (b) The spacing between beads and between bead and membrane is not changed. (c) The bead ring arrangement is lost but no large mass of beads is formed.

Valinomycin, A23187, and Ion Balance

Antimycin A and other mitochondrial inhibitors deplete intracellular calcium stores (4, 19), and ion balance across membranes requires energy (15, 20). If antimycin A's effects on the GC and bead rings are caused by alterations in free intracellular ion levels, then valinomycin and A23187 should cause similar changes in these structures.

Valinomycin selectively transports K^+ across membranes (15) but may also uncouple mitochondria (18). After 1 h, 7.0×10^{-5} M valinomycin reduced the tissue ATP level to one-third of the control value (Fig. 11) and caused the same morphological effects as antimycin A (Figs. 1 a-c). The effects of valinomycin are consistent with K^+ redistribution across the mitochondrial envelope secondarily reducing the intracellular ATP concentration that collapses bead rings. Some rings remain in the mass of beads (Fig. 12c), which may reflect the fact that the treatments used in this study to block transport at the forming face of the GC do not completely eliminate ATP from the cell.

A23187 specifically transports Ca^{2+} and Mg^{2+} (15). Exposure to 1.3×10^{-4} M A23187 did not lower the tissue ATP level below the control value (Fig. 11) but after 30 min the saccules

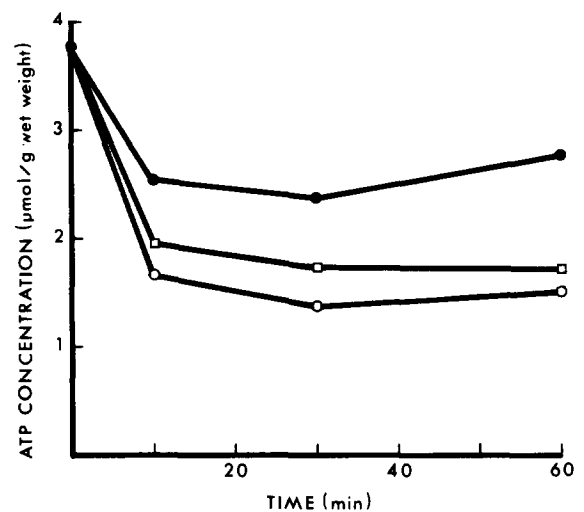


FIGURE 5 Effect of 1.7×10^{-2} M sodium cyanide (○) and anoxia (□) on intracellular ATP levels. The control curve (●) is for the distilled water solvent of cyanide. Anoxic conditions were made by maintaining the animals in an air-tight holding tank under a constant stream of nitrogen. The control for anoxia is the time 0 value.

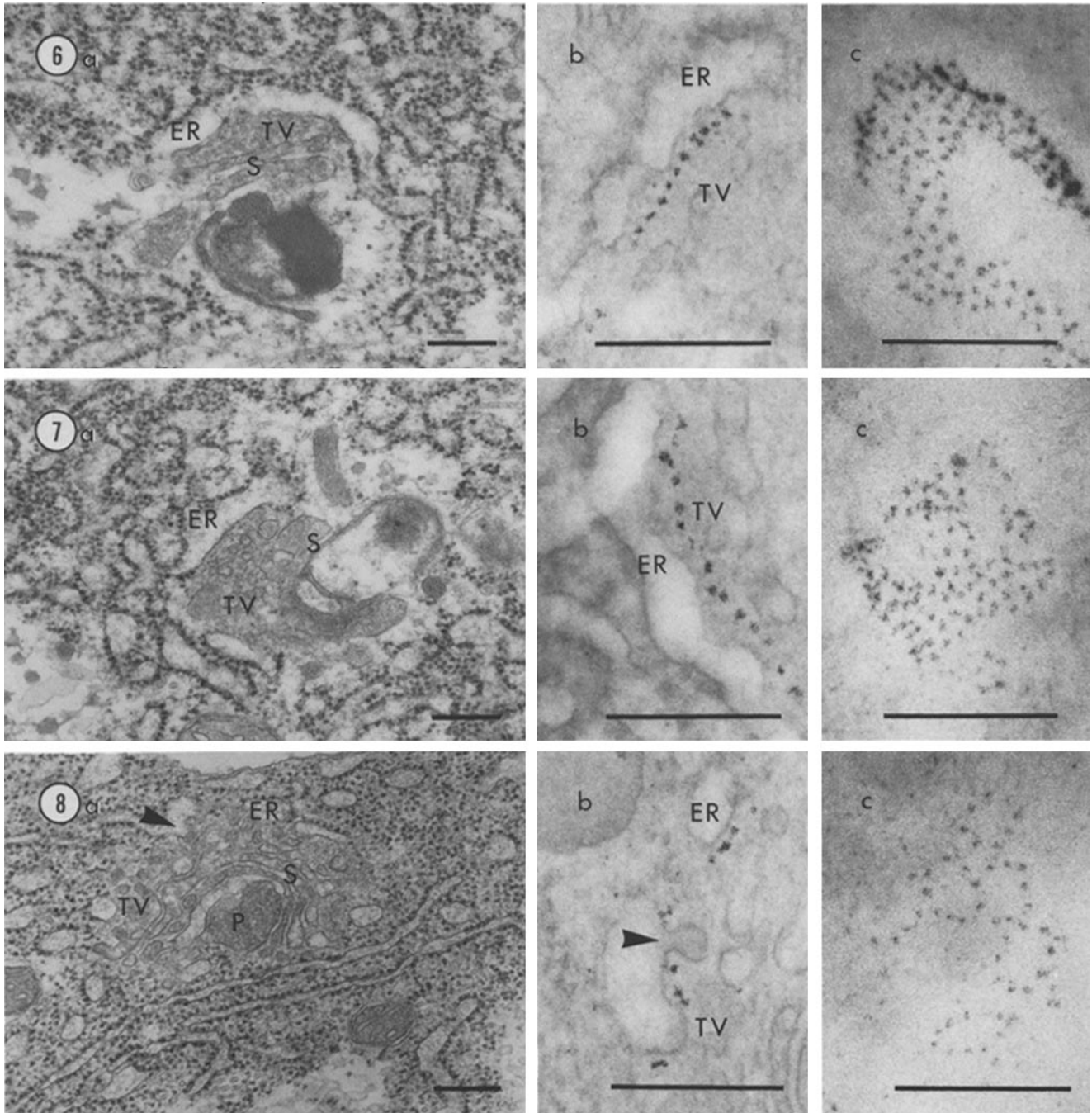


FIGURE 6 (a) 1.7×10^{-2} M sodium cyanide for 1 h prevents transition vesicle formation and causes GC saccules (S) to round up. (b) The spacing between beads and between bead and membrane is unchanged. (c) Bead rings have collapsed.

FIGURE 7 (a) Anoxia for 1 h prevents transition vesicle formation and causes GC saccules (S) to round up. (b) The spacing between beads and between bead and membrane is not changed. (c) Bead rings have collapsed.

FIGURE 8 (a) 1.7×10^{-3} M iodoacetate for 1 h does not prevent transition vesicle formation (arrowhead), and GC saccules (S) and protein granules (P) resemble control images. (b) The spacing between beads and between bead and membrane remains the same as control values. Note the presence of a bead on either side of the neck of the forming transition vesicle (arrowhead). (c) Bead rings are not affected.

of GCs did decrease in size and content to a variable extent, although forming TVs were still present (Fig. 13 *a*). The center-to-center spacing, center-to-membrane spacing, and ring structure of the beads were not changed (Fig. 13 *b* and *c*). Lower concentrations of A23187 had no effect. Longer incubations allowed increasing degrees of recovery to control conditions, although protein granules remained poorly developed (Fig. 14 *a-c*). This recovery was expected because intercellular coupling also recovers in epidermal cells of *Tenebrio molitor* in the continued presence of A23187 (3).

Monensin and Lasalocid and Blocks to Other Parts of the GC

Secretion can be blocked at the level of protein granules by monensin (22) and lasalocid (21). Monensin is a Na⁺ ionophore and lasalocid has a broad specificity encompassing virtually every known cation (15). Neither of these ionophores lowers the tissue ATP level below the control value (Fig. 15). Exposure for 0.5–2 h to 1.3×10^{-4} M monensin caused the GC saccules

to swell but forming TVs and beads were unaffected (Fig. 16 *a-c*). 1-h exposure to 7.0×10^{-5} M lasalocid gave the same results (Fig. 17 *a-c*).

DISCUSSION

There is much evidence to show that the lipid and protein compositions of rough ER and GC membranes are different (13, 14). There must therefore be a mechanism for the qualitative and quantitative control of membrane flow in the transition region. The beads are located in the appropriate position encircling the base of forming TVs to act as such a gating mechanism.

Transport of secretory proteins was blocked at three successive levels: (*a*) at the ribosome (cycloheximide treatment), (*b*) at the forming face of the GC (antimycin A treatment), (*c*) at the protein granule (monensin and lasalocid treatment). Because bead rings collapsed only when transport was blocked at their level, the beads are independent of protein synthesis and of the late GC processing of secretory protein. In addition, they

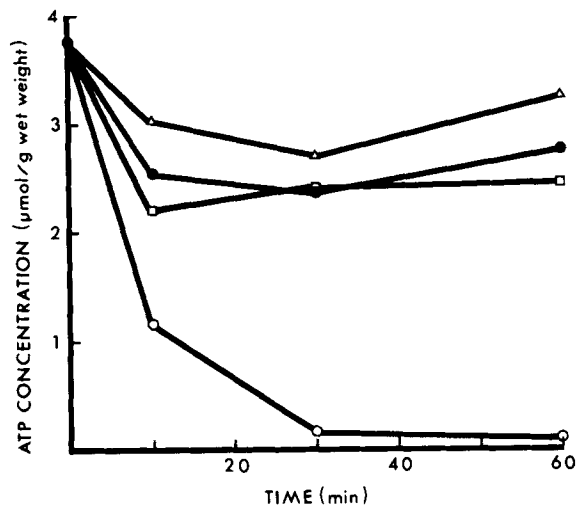


FIGURE 9 Effect of 6.0×10^{-3} M cycloheximide (Δ), 1.7×10^{-3} M iodoacetate (\square), and 1.7×10^{-2} M iodoacetate (\circ) on intracellular ATP levels. The control curve (\bullet) for all of these agents is for the distilled water solvent.

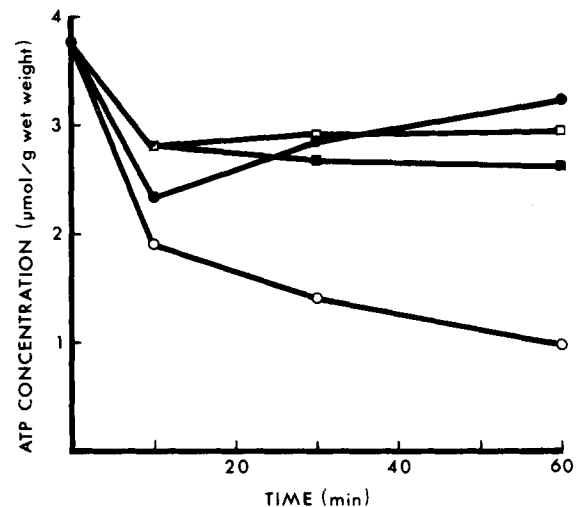


FIGURE 11 Effect of 7.0×10^{-5} M valinomycin (\circ) and 1.3×10^{-4} M A23187 (\square) on intracellular ATP levels. The control curve for valinomycin represents 0.8% DMSO in distilled water (\bullet) and for A23187 represents 0.8% ethanol in distilled water (\blacksquare).

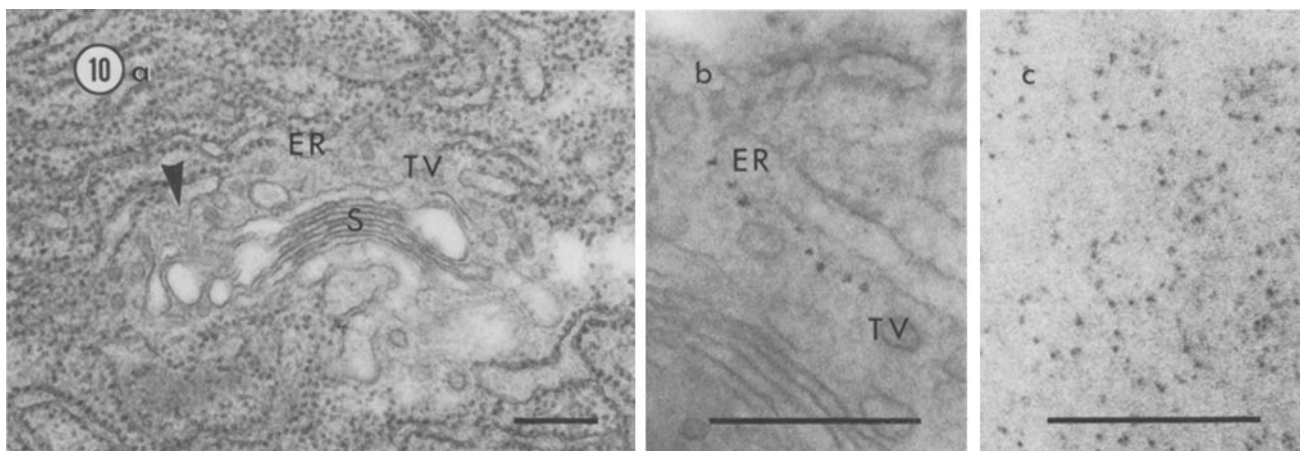


FIGURE 10 (*a*) 2.4×10^{-3} M cycloheximide for 6 h does not prevent transition vesicle formation (arrowhead) but GC saccules (S) are increased in number (in this case, five) and electron lucent. (*b*) The spacing between beads and between bead and membrane remains the same as in the controls. (*c*) The bead ring arrangement is intact.

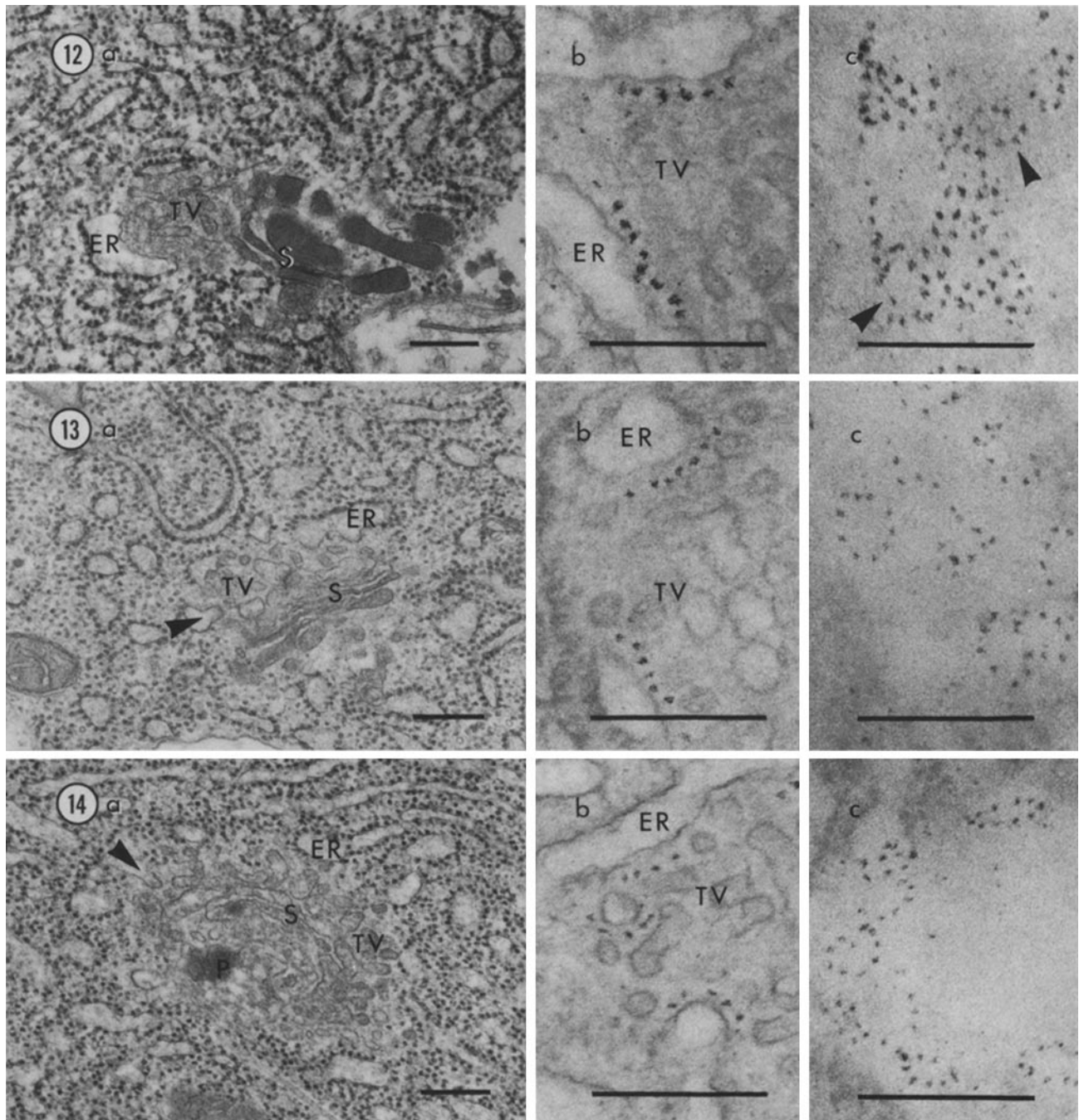


FIGURE 12 (a) 7.0×10^{-5} M valinomycin for 1 h prevents transition vesicle formation and causes GC saccules (S) to round up. (b) The spacing between beads and between bead and membrane is unchanged. (c) Bead rings can still be seen (arrowheads), although a large mass of beads has formed.

FIGURE 13 (a) 1.3×10^{-4} M A23187 for 30 min does not prevent transition vesicle formation (arrowhead) but the GC saccules (S) are deformed to some extent. (b) The spacing between beads and between bead and membrane is unchanged. (c) Bead ring arrangement is intact.

FIGURE 14 (a) 1.3×10^{-4} M A23187 for 4 h does not prevent transition vesicle formation (arrowhead), and GC saccules (S) resemble control images. Protein granules (P) are poorly formed. (b) The spacing between beads and between bead and membrane is unchanged. (c) Bead ring arrangement is intact.

are not correlated with the movement of cisternal secretory proteins, because the absence of these proteins (cycloheximide treatment) or their backup through the GC saccules (monensin

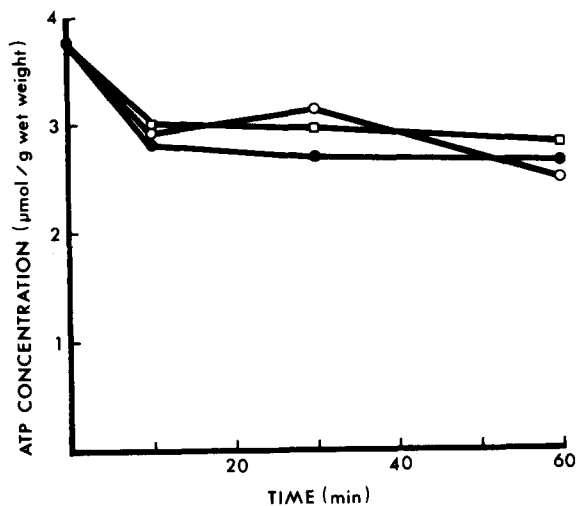


FIGURE 15 Effect of 1.3×10^{-4} M monensin (○) and 7.0×10^{-5} M lasalocid (□) on intracellular ATP levels. The control curve (●) for both ionophores represents 0.8% ethanol in distilled water.

and lasalocid treatment) did not alter bead ring formation.

Nine treatments were used in this study but only those that lowered intracellular ATP levels altered bead ultrastructure. Furthermore, only inhibitors of oxidative phosphorylation (antimycin A, cyanide, and anoxia) and not an inhibitor of glycolysis (iodoacetate) were effective. This is in keeping with transport from rough ER to GC being supported by long-chain fatty acids as substrate for the Krebs cycle rather than pyruvate of the Embden-Meyerhoff pathway (6).

Ionophores seldom change the equilibrium concentration of just one ion across a membrane (16), a conclusion that may apply to any chemical treatment of living cells. The inhibitors of oxidative phosphorylation and valinomycin affect the intracellular ATP concentration, protein synthesis, and ion balance across membranes (4, 6, 18, 19). Inhibition of protein synthesis can be ruled out because cycloheximide treatment did not affect the bead rings. Although valinomycin does alter the K^+ distribution across membranes, it also lowers intracellular ATP levels. Monensin, lasalocid, and A23187 also alter equilibrium concentrations of a wide spectrum of cations across membranes (15, 16) but do not affect the bead rings. Thus the results of this study are consistent with the integrity of the bead rings being ATP dependent. The block to TV formation concomitant with the collapse of bead rings suggests that formation of TVs

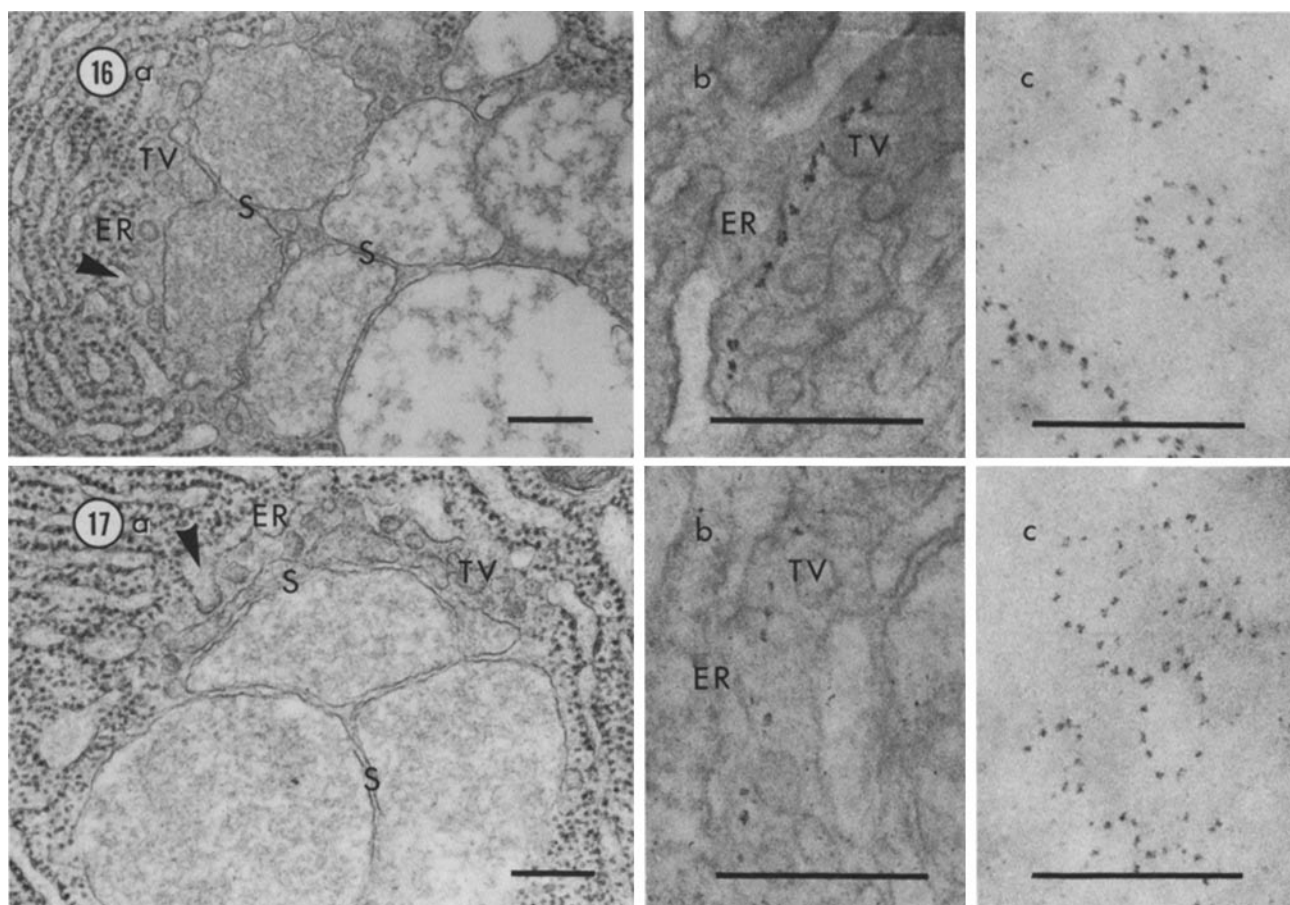


FIGURE 16 (a) 1.3×10^{-4} M monensin for 30 min does not prevent transition vesicle formation (arrowhead) but the GC saccules (S) have swelled enormously. (b) The spacing between beads and between bead and membrane is unchanged. (c) The beads remain in ring formation.

FIGURE 17 (a) 7.0×10^{-5} M lasalocid for 1 h does not prevent transition vesicle formation (arrowhead) and the GC saccules (S) have swelled to an extent similar to that for monensin treatment. (b) The spacing between beads and between bead and membrane is unchanged. (c) The beads remain in ring formation.

may be dependent upon the integrity of the rings. The beads may therefore be the structural correlate to the energy-dependent lock-gate of transport in the GC-ER transition region.

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