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Evidence of Microfilament-Associated Mitochondrial Movement

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The mitochondria in the lower Malpighian tubule of the insect Rhodnius prolixus can be stimulated by feeding in vivo and by 5-hydroxytryptamine in vitro, to move from a position below the cell cortex to one inside the apical microvilli. During and following their movement into the microvilli, the mitochondria are intimately associated with the microfilaments of the cell cortex and microvillar core bundle. Bridges approximately 14 nm in length and 4 nm in diameter are observed connecting the microvillar microfilaments to the outer mitochondrial membrane and microvillar plasma membrane. Depolymerization of all visible microtubules with colchicine does not inhibit 5-HT-stimulated mitochondrial movement. On the other hand, treatment with cytochalasin B does block mitochondrial movement, suggesting that microfilaments play a role in the mitochondrial motility. We have labeled the microvillar microfilaments, which are 6 nm in diameter, with heavy meromyosin, which supports the contention that they contain actin. A model of the mechanism of mitochondrial movement is presented in which mitochondria slide into position in the microvilli along actin-containing microfilaments in a manner analogous to the sliding actin-myosin model of skeletal muscle.

Key words: microvilli, Malpighian tubule, cytoskeleton, actin, cell motility

The Malpighian tubules of insects are the site of primary urine formation [1]. In the bloodsucking insect Rhodnius prolixus the Malpighian tubules are divided into two functional units: the upper tubule, which is the site of blood filtration by means of an osmotic gradient, and the lower tubule, where KCl resorption and urine acidification occur [2]. The periodic intake of a liquid meal in this insect imposes a cyclic nature on diuresis in the Malpighian tubules.

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We have shown that substantial ultrastructural modifications occur in the lower tubule as ion transport is initiated, ie, in response to feeding in vivo or to 5-HT stimulation in vitro [3, 4]. These modifications include microvillar growth, changes in mitochondrial conformation, and the movement of mitochondria from a position inside the cell below the cell cortex to one inside the microvilli. Because both the physiological and morphological changes can be induced rapidly (in 10 min) in vitro by 5-HT, this system has proved very useful.

The microvilli of cells of the lower Malpighian tubule, which form a striated border, differ from more conventionally studied microvilli such as vertebrate intestine in that, under certain conditions, they may contain mitochondria or endoplasmic reticulum (ER) [5]. They share with all microvilli the characteristic that they are apical cell projections enclosed by a unit membrane surrounding a core of microfilaments that extend down out of the base of the organelle into a terminal web.

Based on ultrastructural examination of 5-HT-stimulated tubules, we have suggested that mitochondrial movement in the lower tubule is dependent upon interactions with the microvillar microfilaments [5]. This evidence includes the close and regular association of microvillar core microfilaments with extended mitochondria and the movement of mitochondria into microvilli in which no microtubules are visible.

In this paper we provide further experimental support for this hypothesis. We demonstrate for the first time that the core microfilaments of insect cell microvilli, like vertebrate cell microvilli, contain actin. In addition, we show that drugs such as cytochalasin B, which interferes with microfilament-associated movement, affect mitochondrial extension, whereas drugs such as colchicine do not.

MATERIALS AND METHODS

Animals were reared and dissected as described elsewhere [6]. Dissected tubules were maintained in vitro in normal Rhodnius Ringer [6] in small drops suspended under mineral oil. Tubules were fixed for 1 h in 4% glutaraldehyde in 0.5 M cacodylate buffer (pH 6.9), followed by further fixation in 1% O_5 O_4 in 0.5 M cacodylate containing 0.1 M sucrose, 10 min en block staining in 1% uranyl acetate in 70% ethanol, ethanol gradient dehydration, and embedding in Epon 812. Sections were cut on a Reichert ultramicrotome, stained with uranyl acetate in 50% ethanol followed by Reynolds lead stain, and observed in a Siemens 101 electron microscope.

The effect of cytochalasin B on mitochondrial movement was examined by treating cells for 15 min with 10 μm cytochalasin B (Sigma) in dimethylsulfoxide (DMSO) solvent dissolved in Rhodnius Ringer (final DMSO concentration, 1%). Control preparations were placed for an equal time in Ringer's containing 1% DMSO. Treatment with colchicine was carried out at room temperature for 30 min, using 10 mM colchicine. Tubules treated with colchicine were either poststimulated with 5-HT or the two agents were added together. Parallel samples from the same animal were run as controls. Fixation and preparation for EM were as described above.

Heavy meromyosin (HMM) was prepared from rabbit skeletal muscle following the procedure of Kielly and Harrington [7] with only minor modifications. All procedures up to fixation were carried out at 0°C. Tubules were made permeable by a series of glycerine solutions (25%, 10 min; 33%, 10 min; 50%, 1 h; 25%, 10 min) made up in solution A (100 mM KCl, 5 mM $MgCl_2$, pH adjusted to 7.0 with 7 mM sodium phosphate buffer). After

glycerine treatment, the tubules were treated with 10 mM sodium pyrophosphate for 10 min, washed 3 times in 25% glycerol in solution A, and placed overnight in solution A with 12.5% glycerol and 2.8 mg/ml HMM. After 3 additional rinses with solution A, fixation and preparation of the tubules for EM was as described above, with the exception that 1% tannic acid was added to the glutaraldehyde and osmium fixation was carried out at pH 6.0 for 20 min.

RESULTS

Colchicine Experiments

The morphology of the lower Malpighian tubule has been described in detail elsewhere [3–5]. Treatment of the lower Malpighian tubules with 10 mM colchicine does not result in gross nonspecific changes (Fig. 1), so that, for example, the basal infoldings of the cell membrane, basal mitochondria, nuclei, and ER appear normal. However, microtubules in the cells are substantially reduced in frequency within 10 min of exposure to colchicine. After 30 min, 75–100% of the microtubules formerly present have been depolymerized, with many cells, upon EM observation, containing no visible microtubules. Probably the most striking effect of colchicine is the gradual disappearance of the microtubule-containing axopods that lie among the apical microvilli [6]. Microtubules also depolymerize in other microtubule-rich regions, such as the apical subcortical area, the periphery of the nucelus, and between the basal infolds.

Treatment with colchicine does not block 5-HT-stimulated mitochondrial movement. This is true whether the tubules are 1) treated with colchicine for 30 min and then stimulated or 2) treated with colchicine and 5-HT simultaneously for 30 min. In the latter case, mitochondrial movement occurs within 10 min after stimulation and is unaffected by the continued presence of colchicine. The tubule shown in Figure 1 is of particular interest, because this section and adjacent sections were found to contain no microtubules when exhaustively searched under the electron microscope. Yet, when stimulated with 5-HT subsequent to the colchicine treatment, mitochondrial movement into the microvilli was normal (Fig. 1). Similar results with many other tubules demonstrate that mitochondrial movement is not dependent on intact arrays of cytoplasmic microtubules and can proceed even when no microtubules are observed near the apical microvilli.

Evidence for Microfilament Involvement

Treatment of the tubules with cytochalasin B has no effect either on gross cell morphology or on the appearance of microfilaments in the apical microvilli. Nevertheless, upon subsequent stimulation with 5-HT, mitochondrial movement is entirely blocked (Fig. 2). In control tubules treated only with DMSO, 5-HT-stimulated mitochondrial movement is normal (Fig. 3).

In nonstimulated tubules, microvillar core microfilaments are evenly spaced throughout the microvillus (Fig. 4a). Following stimulation with 5-HT and mitochondrial movement into the microvilli, there is a redistribution of the microvillar microfilaments such that a sheath of microfilaments is observed surrounding the mitochondria. In microvillar cross-sections this is visualized as a ring of points around the mitochondrion (Fig. 4b). In DMSO-treated tubules, where mitochondrial movement is normal, bridges are observed between the microvillar microfilaments and the outer mitochondrial membrane as well as between microfilaments and the microvillar plasma membrane (Fig. 4c). The length of

the bridges average around 14 nm (13.7 ± 1; n = 16). In images where the bridges are free of surrounding stained material (white arrows, Fig. 4c) the thickness of the bridges can be seen to be thinner (∿ 4 nm) than the core microfilaments (∿ 6 nm).

In nonstimulated tubules the mitochondria are associated with microtubules in the subcortical region of the cell. When tubules are treated with cytochalasin B before stimulation, the mitochondria remain in that region (Fig. 5), suggesting that the mechanism whereby the mitochondria are drawn away from the microtubules and into the microvilli has been inhibited.

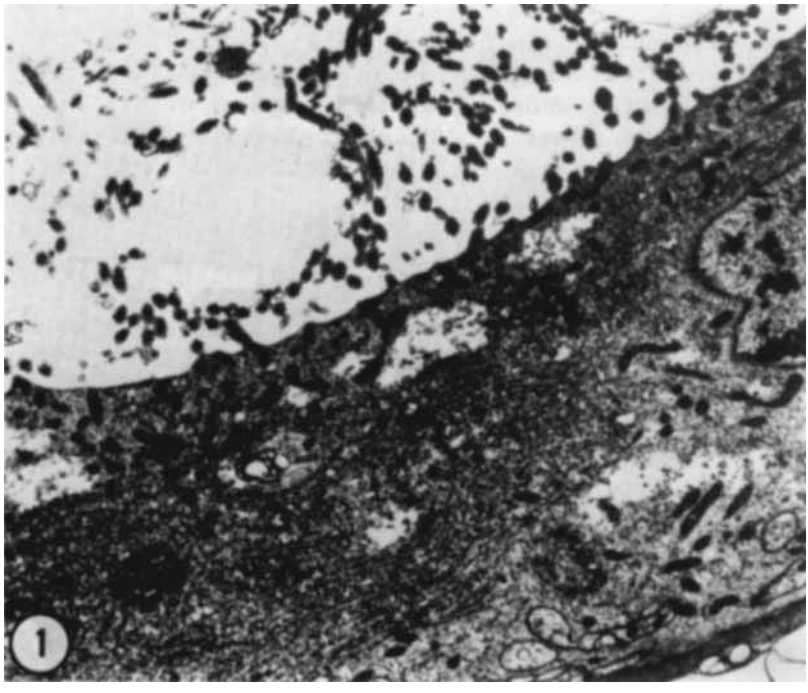
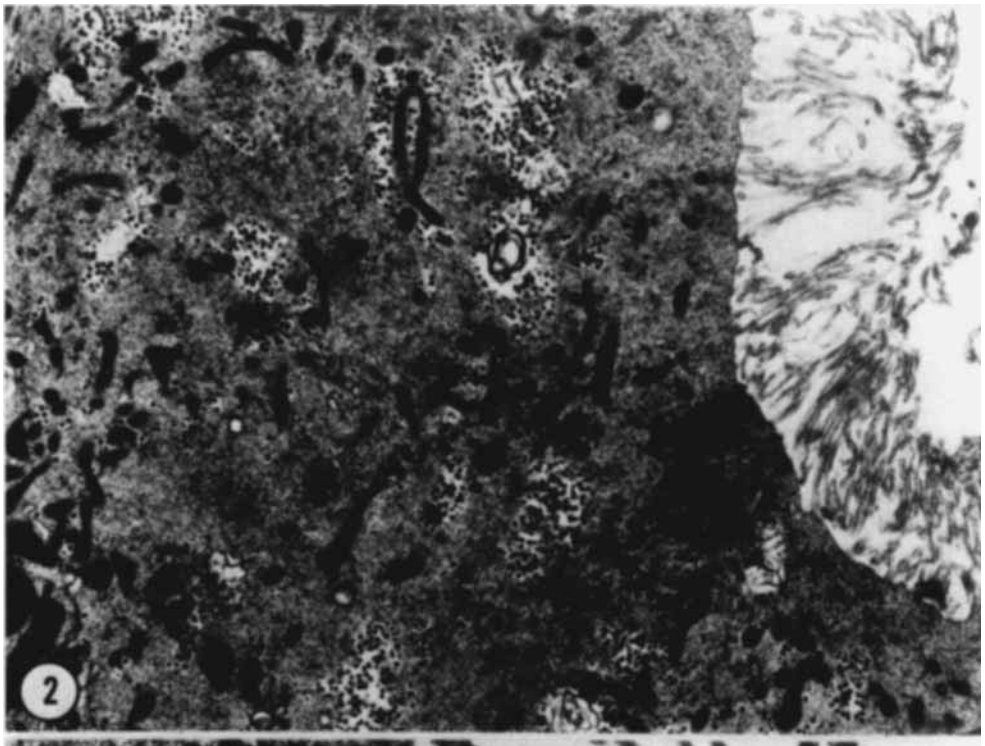


Fig. 1. A tubule treated for 30 min with 10 mM colchicine followed by 5-HT stimulation. Notice that mitochondrial movement into the microvilli is not blocked. Several sections of the tubule shown were examined at high magnification, and no microtubules were observed. Although some microtubules may have been overlooked, one can conservatively state that over 95% of the microtubules previously in the cell were depolymerized by the colchicine treatment; yet mitochondrial movement is unaffected (magnification, × 8,200).

Fig. 2. A tubule treated with 10 μm cytochalasin B in 1% DMSO for 15 min, followed by stimulation with 5-HT. Mitochondria have not moved into the microvilli but remain in the subcortical region as in nonstimulated cells (magnification, × 11,400).

Fig. 3. A tubule treated with 1% DMSO followed by 5-HT stimulation. This is a solvent control for the cytochalasin experiments. Mitochondrial movement into microvilli is normal (magnification, × 12,300).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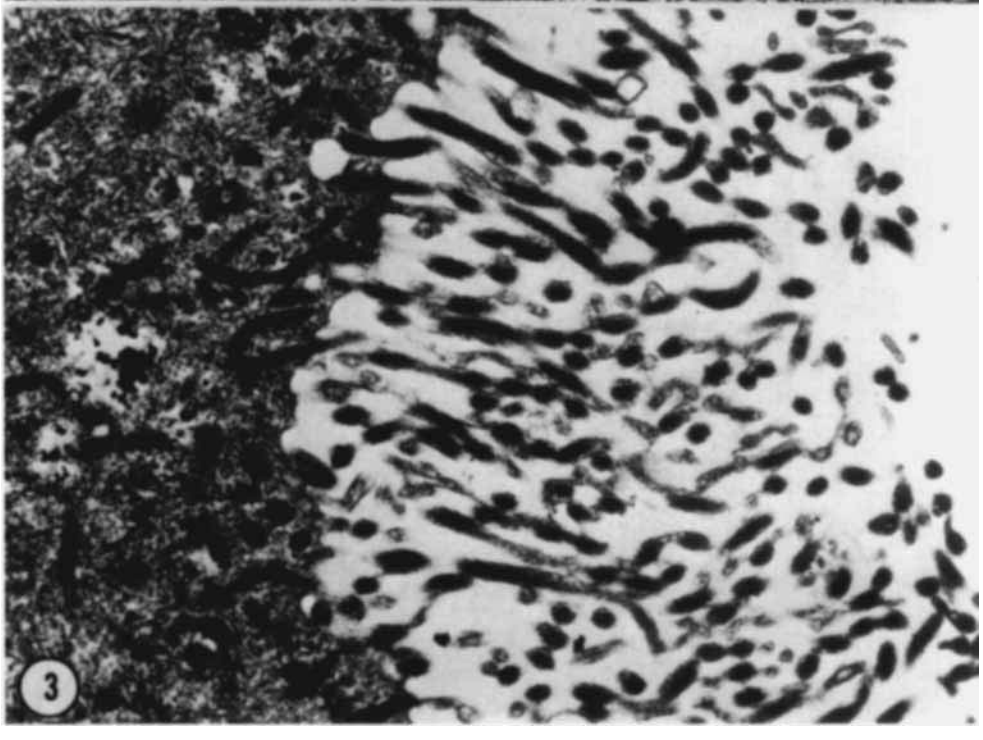
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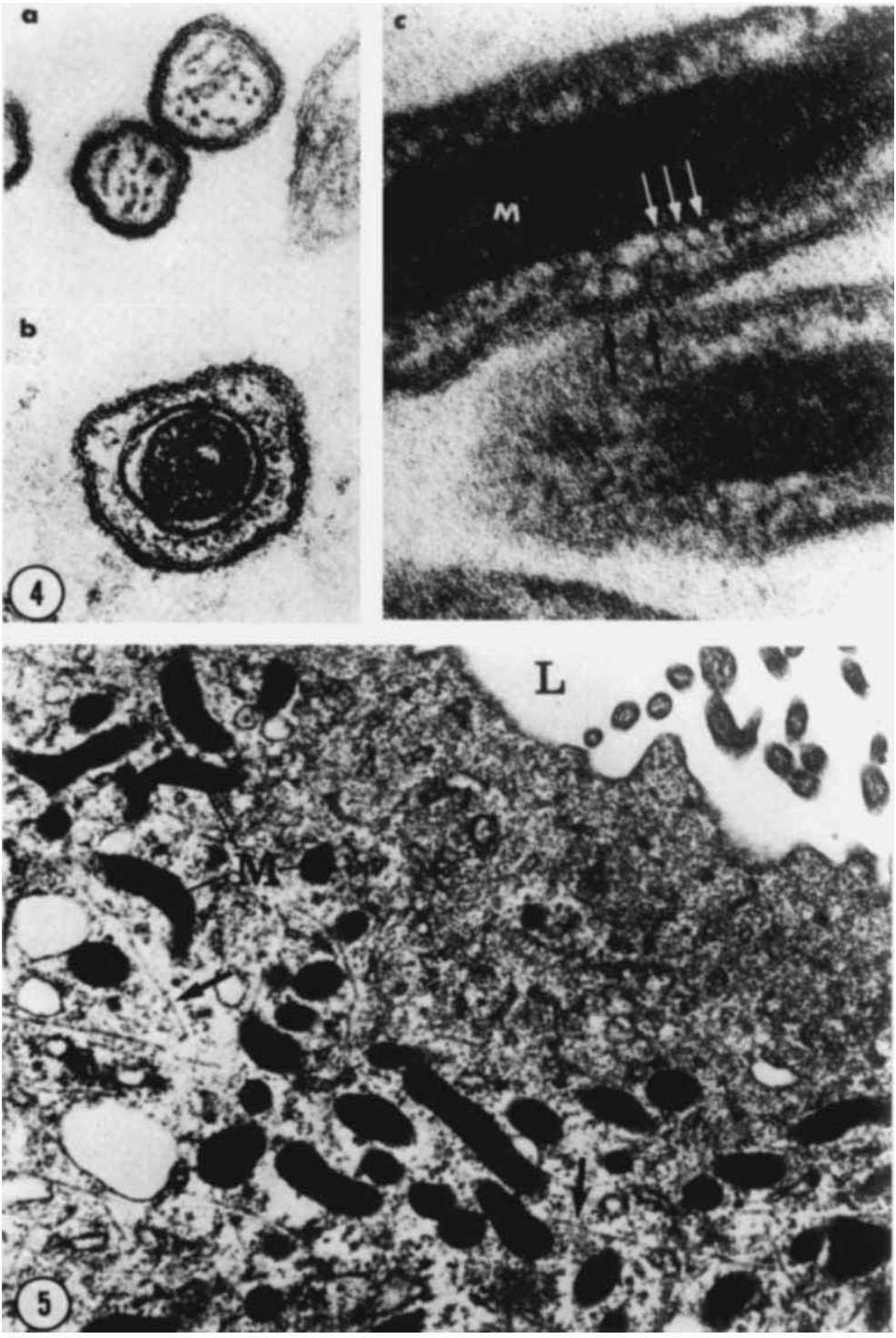
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HMM Labeling

We have succeeded in decorating microfilaments in the lower tubule with rabbit skeletal muscle HMM to form arrowheads (Figs. 6 and 7). This finding confirms our hypothesis that the microfilaments in the central cell region and in the microvillar core bundle contain actin. Although the direction of some of the arrowheads in the microvillar core bundles is clearly toward the cell, we are unable to state definitely that all the microfilaments have identical polarity in these preparations.

DISCUSSION

Movements of cell organelles have repeatedly been shown to be associated either with microtubules [8–13] or with microfilaments [14–17]. Based on morphological associations, it has been suggested that mitochondrial movement in the Malpighian tubules of insects is associated with microfilaments [4, 18]. Our work reported here with Rhodnius Malpighian tubules is the first to demonstrate experimentally the role of microfilaments in mitochondrial movement.

5-HT-stimulated mitochondrial movement in the lower tubule clearly is not microtubule dependent, since mitochondria can enter microvilli where no microtubules are visible and depolymerization of approximately 95% of the cellular microtubules with colchicine does not inhibit subsequent mitochondrial movement. This clearly differentiates the mechanism of mitochondrial movement into microvilli in the lower tubule from that postulated for mitochondrial movement in nerve axons, where microtubules are implicated on morphological grounds [19, 20] and on the basis of the blockage of movement by colchicine and vinblastine [21]. It is not clear, however, if a component in positioning and holding the mitochondria below the microvilli in the subcortical region involves microtubules and might be comparable to movement along the axon.

Instead, 5-HT-stimulated mitochondrial movement into the microvilli in the lower tubule evidently involves an interaction with actin-containing microfilaments: 1) We have shown that when mitochondria enter the microvilli in response to 5-HT, there is a reorganization of the microvillar core bundles into a sheath of microfilaments which surrounds the mitochondrion along its entire length within the microvillus. This close association of microfilaments with the mitochondria is observed wherever extended mitochondria are seen, both in the microvilli and in the axopods [5]. Bridges 14 nm in

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Fig. 4. a. Cross-section of a microvillus from a nonstimulated tubule. The microvillar core microfilaments are evenly spaced (magnification, × 131,250). b. Cross-section of a microvillus from a tubule stimulated with 5-HT. The core microfilaments are organized in a sheath around the mitochondrion. In cross-section the microfilaments of the sheath are seen as a ring of points (magnification, × 137,500). c. Bridges are observed connecting the microfilament 1) to the mitochondrion (white arrows) and 2) to the microvillar plasma membrane (black arrows) in tubules stimulated in 1% DMSO (mitochondrion, M; magnification, × 204,000).

Fig. 5. In tubules stimulated following cytochalasin B treatment the mitochondria (M) remain below the cell cortex (C) in close association with microtubules (arrows) (tubule lumen, L; magnification, × 28,250).

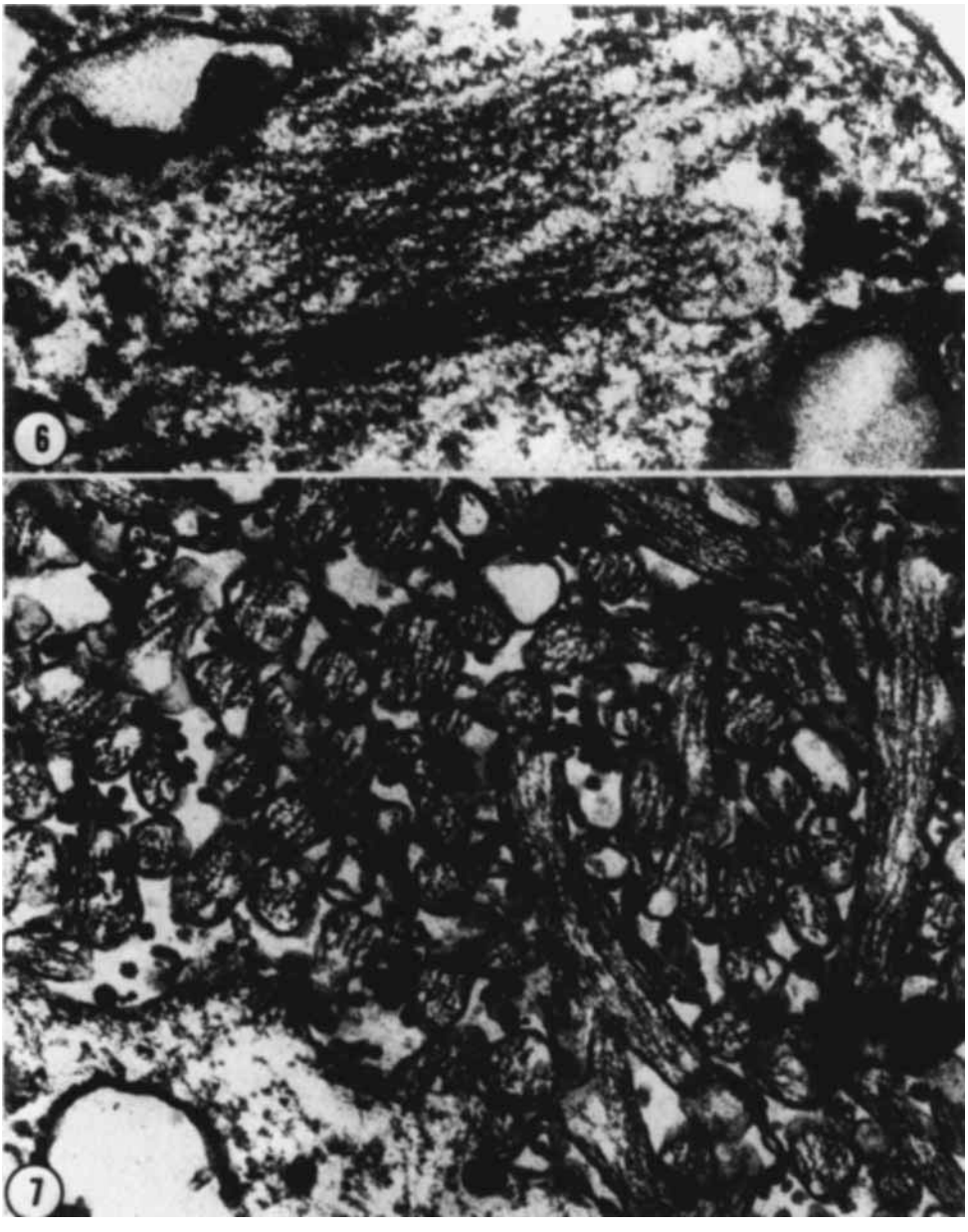

Fig. 6. HMM isolated from rabbit skeletal muscle labels microfilaments throughout the lower tubule cells with an "arrowhead" pattern (magnification, × 87,500).

Fig. 7. The core microfilaments of the microvilli in the lower tubule label with HMM, suggesting that they contain actin (magnification, × 47,500).

length connect the microfilaments to the mitochondrial outer membrane and to the microvillar plasma membrane. The appearance of these bridges is not an artifact induced by DMSO treatment, since they can also be observed, albeit with less contrast, following fixation in the presence of tannic acid where no DMSO is used. The increased clarity of the bridges following DMSO treatment may be due to traces of DMSO remaining during fixation with glutaraldehyde, thereby facilitating the rapid entry of fixative. 2) Treatment of lower tubules with cytochalasin B completely blocks mitochondrial movement upon subsequent stimulation with 5-HT. Other workers have shown that cytochalasin B, although it is not completely specific, blocks microfilament-associated cell and cell organelle motility in vivo [22] and inhibits gelation in vitro of purified actin [23, 24]. 3) We are able to decorate microfilaments throughout the lower Malpighian tubule cell with HMM. This demonstration of actin in the microvillar core microfilaments is the first using insect microvilli. In the microvilli of chicken intestine [25] and sea urchin egg [26, 27] the core microfilaments all label identically with HMM such that the "arrowheads" point toward the base of the microvillus. This orientation has important implications for assembly and function. We are unable to confirm this point in our material at present. We are, however, carefully investigating the orientation of the core microfilaments in the microvilli of the lower tubule, since mitochondria not only move into the microvilli upon stimulation but also move out when stimulation ceases (unpublished observation). We have no information about the presence or location of myosin in these cells.

We summarize our results in a model for 5-HT-stimulated mitochondrial movement in the lower tubule (Fig. 8). In the nonstimulated cell, the mitochondria are located below the cell cortex in close association with microtubules. Occasional bridges are observed between microtubules and mitochondria (Fig. 8a), and we suggest that microtubules may be important in positioning the mitochondria in the subcortical region. At this time the microvillar core microfilaments are evenly spaced within the microvillus, perhaps structurally connected to each other and to the plasma membrane, as is also the case in intestinal microvilli in vertebrates [25, 28].

Upon stimulation (Fig. 8b) the mitochondria partially detach from the microtubules to become associated with the actin-containing microfilaments extending down from the microvillar core bundles. We suggest that this lateral association of the mitochondria with the microvillar core bundles allows the mitochondria to enter the microvilli (Fig. 8c) via an actin-myosin sliding mechanism. During this movement there is a fundamental rearrangement of the microvillar microfilaments from a microvillar core pattern to a regularly spaced single row surrounding the mitochondria. Bridges form links from the outer mitochondrial membrane to the plasma membrane via the microfilaments.

Intestinal microvilli do not contain myosin [29], and it has been proposed that the connections between microfilaments in the core bundle are composed of another protein [28]. Because the location of myosin in the lower tubule has not been determined, it is not clear whether the bridges observed perform a myosin-like function in moving the mitochondria or whether they are composed of other proteins like those in vertebrate microvilli. Further refinements or modifications of our model should therefore result from our present investigations regarding the presence and localization of myosin in the microvilli of the lower tubule and the mechanism by which stimulation associated with 5-HT binding at the basal cell surface is communicated across the cell to influence cytoskeletal events in the apical region.

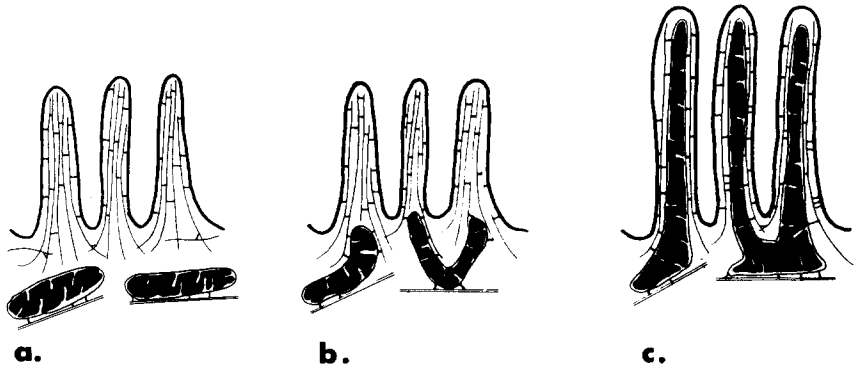

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Fig. 8. This diagram presents our conception of the events associated with 5-HT-stimulated mitochondrial movement in the lower tubule. In nonstimulated tubules (a) the mitochondria are located in the subcortical region attached to anchoring microtubules. The core microfilaments, which are evenly spaced in the microvilli, may be positioned by bridges to each other and to the microvillar membrane. Following stimulation (b), the mitochondria become laterally associated with microfilaments which extend down from the microvillus. As mitochondrial movement into the microvillus proceeds (c), the core microfilaments reorganize into a sheath surrounding the mitochondrion. Bridges are observed attaching the microfilaments to the mitochondrial outer membrane and microvillar plasma membrane. Mitochondrial extension proceeds by an actin-based sliding mechanism (see text). Basal regions of the mitochondria remain anchored to the subcortical microtubules.

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