J. Membrane Biol. 185, 43–56 (2002)
DOI: 10.1007/s00232-001-0105-2

The Journal of
Membrane
Biology

Membrane Dynamics in the Malpighian Tubules of the House Cricket, *Acheta Domesticus*

S.R. Hazelton[1,*], **V.R. Townsend, Jr**[2], **B.E. Felgenhauer**[1], **J.H. Spring**[1]
[1]Department of Biology, University of Louisiana at Lafayette, P.O. Box 42451, Lafayette, Louisiana 70504-2451, USA
[2]Division of Natural Sciences and Mathematics, Virginia Wesleyan College, 1584 Wesleyan Drive, Norfolk Virginia Beach, Virginia 23502, USA

Received: 21 February 2001/Revised: 31 July 2001

Abstract. In *Acheta domesticus*, the Malpighian tubules (Mt) are composed of three morphologically distinct regions (proximal, mid and distal), each consisting of a single cell type. The bulk of the Mt is composed of the midtubule, which shows the greatest response to corticotropin releasing factor-related diuretic peptides (CRF-DP). We know from previous laboratory studies that the second messenger cAMP and its analog dibutyryl cAMP (db-cAMP) cause an approximate doubling in the secretion rate and that this is accompanied by notable ultrastructural changes in the midtubule, especially membrane reorganization in the basal area and extensive vesiculation of the cytoplasm. In this study, we examined the morphological changes in membranes both at the cell surface and internally. By enzymatically removing the basal lamina, we examined the increase in spacing between infolded membranes initiated by db-cAMP stimulation. To examine the intracellular membranes, we used a technique developed for use in invertebrate tissues. This allowed the removal of the cytoplasm for high resolution scanning electron microscopy (HR-SEM) while maintaining the integrity of the lipid constituents of the cell. By using HR-SEM and confocal laser scanning microscopy (CLSM), we gained a unique three-dimensional perspective of the complexity of the internal membrane system of the *A. domesticus* Mt in both the unstimulated and db-cAMP-stimulated states.

Key words: Malpighian tubules — Vesicle — Endomembrane — db-cAMP — Endoplasmic reticulum — Vacuoles

*Present address: Division of Sciences, Louisiana State University at Eunice, 2048 Johnson Hwy. P.O. Box 1129, Eunice, LA 70535

Correspondence to: S.R. Hazelton Robichaux; email: rerobich@lsue.edu

Introduction

Investigators have recognized for over 50 years that Malpighian tubules (Mt) are the major excretory and osmoregulatory organs of insects (reviewed by Wigglesworth, 1972). Structurally, the Mt represent a tubular monolayer epithelium with both the basal and apical membranes elaborated to increase surface area for fluid transport (Bradley, 1985; Hazelton, Parker & Spring, 1988; Spring & Felgenhauer, 1996). Thus far, researchers have concentrated on the ionic forces governing transport, mainly the movement of Na^+, K^+ and Cl^-, with water following passively (Beyenbach, 1995; Bradley, 1985; Pannabecker, 1995). However, the ultrastructural modifications that occur to facilitate the rapid movement of large volumes of fluid through the cells and into the lumen are not well documented.

The Mt of the house cricket, *Acheta domesticus* (Orthoptera: Gryllidae), are an excellent model system to investigate the physiological and subcellular response to rapid fluid transport concurrently. The house cricket is a small (15–20 mm), yellowish brown insect found worldwide. It is a detritivore, feeding on material ranging from paper and cloth to food scraps. The excretory system of the house cricket is well suited to experimental manipulation. In the "classic" insect model, the Mt are connected directly to the gut at the midgut-hindgut junction. However, in *A. domesticus* the approximately 100 Mt join to a bladder-like ampulla that is connected to the gut via a muscular ureter (Hazelton et al., 1988). This arrangement is advantageous for *in vitro* manipulation, allowing experimentation on the entire mass or the removal of individual tubules.

The Mt are regionally segmented (proximal, mid and distal; Hazelton et al., 1988) with the midtubule displaying the greatest response to endocrine stimulation (Spring & Kim, 1993). The midtubule is 50–70

μm in diameter and is composed of a single layer of cuboidal epithelial cells joined near the apical surface by septate junctions (Bradley, 1985; Hazelton et al., 1988). Ultrastructurally, the midtubule displays characteristics of a typical transport epithelium with extensive infolding of the basal surface and a dense brush border surrounding the tubule lumen (Bradley, 1985; Hazelton et al., 1988). Much is known concerning the response of *A. domesticus* Mt to various secretagogues (e.g., corpora cardiaca homogenates, db-cAMP, achetakinins, CRF-DPs) in terms of secretion rate and ionic content of the secreted fluid (Spring & Hazelton, 1987; Clark & Spring, 1992; Kim & Spring, 1992; Spring & Kim, 1993; Coast, Kay & Wheeler, 1993; Spring & Kim, 1995). The basic ultrastructure of the Mt of *A. domesticus* has also been well documented in both stimulated and unstimulated states (Hazelton et al., 1988; Spring & Kim, 1993; Spring & Felgenhauer, 1996; Hazelton, Felgenhauer & Spring, 2001a). However, the correlation of the subcellular changes with the physiological response and the explanation of the mechanisms underlying them are lacking.

In insects, the Mt function in both excretion, to rid the organism of waste, and in osmoregulation, to prevent osmotic imbalances from occurring. Insects have an open circulatory system and the Mt lie free in the hemocoel, bathed in hemolymph. Primary urine is formed through the process of secretion and is nearly isosmotic to the hemolymph with Na^+, K^+ and Cl^- as the major osmolytes (Pannabecker, 1995). Secretion is directly linked to the active transport of cations (Na^+ and K^+) with the movement of water following local osmotic gradients. The Mt remain relatively quiescent until stimulated to undergo rapid fluid transport. The action of the Mt in terms of fluid secretion rate is under the influence of circulating levels of hormones each acting through its own second messenger system (Beyenbach, 1995). Of particular interest is the action of a member of the corticotropin releasing factor-related diuretic peptide family (CRF-DPs). The CRF-DP in *A. domesticus* activates the adenylate cyclase pathway and thereby increases the intracellular concentration of the second messenger, cAMP, that in turn activates various protein kinases. At present it is postulated that this cascade then effects the action of the Na^+, K^+, $2Cl^-$-cotransporter on the basolateral membrane (Spring, unpublished observation). Both Na^+ and K^+ exit the cell through a cation-H^+ exchanger coupled to a V-ATPase located in the apical membrane (Klein, 1992; Wieczorek et al., 1986, 1989, 1991). Whether the exchanger transports Na^+ or K^+ depends on the intracellular concentration of these ions. In cAMP-stimulated transport, the concentration of Na^+ within the cell increases, thus driving the expulsion of Na^+ into the lumen by the cation-H^+ exchanger, with water following the ionic gradient. The physiological response of the Mt to cAMP has been well documented (Maddrell, Pilcher & Gardiner, 1971; Nicolson, 1976; Spring & Clark, 1990; Spring & Kim, 1993; Coast et al., 1991; Hegarty et al., 1991).

The effect of the CRF-DP can be mimicked by adding the synthetic second messenger analog, db-cAMP. Hegarty et al., (1991) demonstrated that db-cAMP increases the Na^+ conductance of the basolateral membrane by acting on the Na^+, K^+, $2Cl^-$-cotransporter. Various researchers (Spring & Clark, 1990; Coast et al., 1991; Kim & Spring, 1992; Spring & Kim, 1993) have demonstrated that db-cAMP (1mM) added in vitro elicits an approximate doubling in Mt secretion rate. Concurrent with this increase in secretion rate, the cytoplasm undergoes extensive vesiculation (Spring & Felgenhauer, 1996; Hazelton et al., 2001a). The vesicles are of a variety of sizes and electron-lucent, suggesting an aqueous content. Vesiculation occurs extremely rapidly, beginning as early as 15 sec post-cAMP-stimulation, and with greater than 50% vesiculation occurring within 420 sec (Hazelton et al., 2001a).

Originally it was presumed that the vesicles were formed through fluid-phase endocytosis at the basolateral membrane and used to "shuttle" fluid across the cell to the lumen. However, experiments using fluorescent tracers and electron-dense markers to follow endocytosis failed to support this hypothesis (Hazelton, Spring & Felgenhauer, 2001b). Nonetheless, in view of the fact that the vesicles are formed concomitantly with stimulated fluid transport (Spring & Felgenhauer, 1996; Hazelton et al., 2001a), it is assumed that they do have functional significance. It is possible that the vesicles are functioning as volume control mechanisms to sequester water. A similar vesiculation of the cytoplasm occurs in vertebrate tissue (e.g., rat liver cells) in response to ouabain and functions in a volume-regulating capacity (reviewed by Van Rossum, Russo & Schisselbauer, 1987). The vesicles formed in the presence of ouabain are derived from internal membrane stores, specifically the Golgi apparatus and endoplasmic reticulum (ER). In comparison to liver cells, there appears to be a large quantity of internal membrane available in the midtubule of *A. domesticus* as revealed by a technique developed to examine internal membranes using cytosolic extraction and high resolution scanning electron microscopy (HR-SEM). Preliminary results exposed an impressive amount of endomembrane that was not evident in the standard transmission electron micrographs (Townsend et al., 2000).

Another prominent modification that occurred early in rapid fluid transport was the elongation of the basolateral infolds. Given that vesiculation and the expansion of the basolateral infolds occurs too rapidly for the membrane involved to be synthesized de novo, we hypothesize that internal membrane stores are recruited for this purpose. Based on the similarities in vesiculation pattern to that which oc-

curs in ouabain-sensitive transport and the speed at which the ultrastructural changes occur in conjunction with the endomembrane revealed by HR-SEM, the present study was designed to address the following questions:

1) Could the plasma membrane be differentially labeled to determine if it was being internalized or if vesicles were being inserted to expand the surface area?
2) Were the stores of internal membrane revealed by HR-SEM part of an extensive network of ER?
3) Could dilations in the internal membrane structure be responsible for the vesiculation?

Materials and Methods

Insect Material

Immature *Acheta domesticus* (Linnaeus) were obtained from Fluker Farms (Baton Rouge, LA) and maintained in the laboratory at 28 ± 2°C on a 14 hr light/10 hr dark photo cycle. Purina Cricket Chow®, oats and water were provided *ad libitum*. Only mature females with fully developed egg masses were used in these experiments.

Dissection Procedures

Acheta domesticus tubules are highly O_2-sensitive (Spring & Hazelton, 1987); therefore, during dissections, the insect Ringer solution was constantly oxygenated with water-saturated carbogen (95% O_2:5% CO_2). The pH of the saline did not change when bubbled with carbogen for 5 hr (Kim & Spring, 1992). The composition of the insect Ringer solution was (in mmol/l): NaCl, 95; K_2SO_4, 10; $MgSO_4.7H_2O$, 10; $CaCl_2.2H_2O$, 3.5; glucose, 10; glycine, 10; proline, 10; glutamine, 2; histidine, 2; leucine, 2; lysine, 4; tyrosine, 2; valine, 4; HEPES, 25; pH 7.2, final osmotic concentration 300–310 mOsmol/l. Crickets were dissected under oxygenated saline (Kim & Spring, 1992). The Mt-ampulla complex was removed and placed in oxygenated insect Ringer solution. The Mt were maintained in vitro for 60 min prior to treatment to remove the influence of any endogenous hormones that may have been present in the hemolymph.

Enzymatic Digestion of Basal Laminae

The basal lamina was removed by enzymatic digestion using elastase (Levinson & Bradley, 1984). For control treatments, Mt were immersed in oxygenated insect Ringer containing 0.4% elastase (Sigma, type III) for 30 min at room temperature. Stimulated Mt were treated in the same manner except that 1 mM db-cAMP (Sigma, St. Louis, MD) was included with the elastase solution. All manipulations occurred after the 60-min stabilization period. After 30 min the Mt were transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and fixed at 4°C for 18–19 hr. Following fixation, the Mt were rinsed in 0.1 M sodium cacodylate buffer and dehydrated in a graded alcohol series. Specimens were chemically dried using hexamethyldisilazane (HMDS; Nation, 1983), mounted on aluminum stubs with double-stick tape and sputter-coated with 20 nm gold for 90 sec. Samples were examined with a JEOL 6300-F field emission electron microscope at an accelerating voltage of 20 kV.

High Resolution Scanning Electron Microscopy

The internal membranes of the mid-tubule were revealed by using freeze-cleaving followed by cytosolic extraction (Townsend et al., 2000). Subsequent to the stabilization period, control tubules were immersed in 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at 4°C for approximately 18 hr. Following primary fixation, Mt were washed in sodium cacodylate buffer, postfixed in 1% OsO_4 for 1.5 hr, rinsed with distilled water, placed in 25% DMSO for 30 min and 50% DMSO for 60 min. DMSO was employed as a cryoprotectant to prevent ice crystal formation during rapid freezing (Haggis, 1992). A liquid nitrogen slush was used to rapidly freeze the tubules, which were then fractured using pre-cooled forceps and a razor blade. Specimens were thawed in 50% DMSO, washed in buffer, placed in 1% OsO_4 for 60 min, and then rinsed with buffer. To facilitate extraction of the cytosol, the fractured tubules were placed in 0.1% OsO_4 for 3–6 days. Following extraction, specimens were washed in buffer, placed in 1% tannic acid for 60 min, washed in buffer, placed in 1% OsO_4 for 60 min, washed in buffer, and dehydrated in a graded ethanol series. Stimulated tubules were treated in the same manner except that following the stabilization period the tubules were exposed to db-cAMP either for 15 sec, 30 sec or 30 min. These times were based on previous findings (Hazelton et al., 2001a; Spring & Felgenhauer, 1996), which showed major changes in membrane structure and vesiculation occurred in these time frames. HMDS was used to dry the specimens prior to mounting on aluminum stubs with double-stick tape and sputter-coating with gold for 90 sec. Specimens were observed with a JEOL 6300-F field emission scanning electron microscope at an accelerating voltage of 20 kV.

Confocal Laser Scanning Microscopy and Fluorescent Markers

Dye Staining — $DiOC_6(3)$ and Syto® 13

Endoplasmic reticulum was visualized with the short-chain carbocyanine dye $DiOC_6(3)$ (Molecular Probes, Eugene, OR) using a variation of the Terasaki (1998) method. Stock solutions of $DiOC_6(3)$ were prepared as 0.5 mg/ml in ethanol, stored at room temperature and diluted to 2.5 μg/ml in insect Ringer immediately before use. Syto®13 (Molecular Probes), a cell-permeant, green-fluorescent live-cell, nuclear stain, was stored as a 5 mM stock solution in DMSO. Working dye solution (5 μM in insect Ringer solution) was prepared immediately prior to use. Tubules were exposed to the dye solutions as described below. Control Mt were exposed to only the dyes in insect Ringer and the stimulated tubules were exposed to the dye solution containing db-cAMP (1 mM).

Whole Tubule Preparations. Following the 60-min stabilization period, the Mt were immersed in a bath containing the dye for 1–5 min. After 3 × 5-min washes with insect Ringer, 2–4 tubules from each mass were removed from the ampulla and attached to separate poly-L-lysine (300 kD: 10 mg/ml)-coated coverglasses. Tubules were mounted in insect Ringer solution. Spacers were positioned between the coverglass and the slide to prevent compression of the Mt. The coverglass was held in place using Flo-Texx®. Dye-loaded Mt were protected from light as much as possible to preserve the fluorescence.

Single Tubule Preparation. Following the 60-min stabilization period, 2–4 Mt from each mass were removed and attached to separate poly-L-lysine-coated coverglasses. From this point, there were two methods used to apply the dye to the Mt. The first method is referred to as the "direct" method. An excess of dye solution was added to the coverglass containing the Mt and al-

lowed to incubate for 1–2 min. The solution was gently poured off and the Mt rinsed 3× with oxygenated insect Ringer solution. The Mt were mounted in oxygenated insect Ringer solution and the coverglass attached to spacers as previously described. An alternate method involved loading the dye with the slides on the microscope and is referred to as the "flow through chamber" method. Utilizing this method, 2–4 Mt were removed from each ampulla and attached to separate poly-L-lysine-coated coverglasses. The Mt were mounted in oxygenated insect Ringer. The coverglass with attached tubules was then placed on spacers at the top and bottom edge of the glass microscope-slide base and secured with Flo-Texx® This arrangement left the sides open so that solutions could be added by "wicking" action to the Mt preparations without removing the preparation from the microscope stage, thus allowing real-time observation of dye uptake in the living Mt.

Alexa Fluor™-conjugated Concanavalin A (Con A)

Alexa Fluor™-conjugated Concanavalin A (Con A; Molecular Probes), a fluorescent lectin for detecting the endoplasmic reticulum, ER (Cottin, Van Linden & Riches, 1999), was dissolved in 0.1 M sodium bicarbonate, pH 8.3, containing 1 mM Mn^{2+} and 1 mM Ca^{2+}. Sodium azide was added to a final concentration of 2 mM. Final concentration of dye solution used was 50 μg/ml. Following the 60-min stabilization period, the Mt were exposed to db-cAMP (1 mM) for 30 min in oxygenated insect Ringer. The control treatments were exposed to oxygenated insect Ringer without the db-cAMP. Following the treatments, the Mt were immediately immersed in cold (4°C) fixative (4% paraformaldehyde) for 60 min. The Mt were then rinsed with three 5-min washes in insect Ringer. After rinsing, 2–4 Mt from each mass were removed and attached to separate poly-L-lysine (300 kD; 10 mg/ml)-coated coverglasses. Once attached the tubules were permeabilized in 1% Triton X (Sigma) for 30 min followed by three 5-min washes in insect Ringer. Mt were then stained with Con A for 30 min (50 μg/ml) and rinsed 5× for 5 min in insect Ringer. The preparations were mounted in insect Ringer and the coverglass attached to spacers on slides.

Plasma-Membrane Staining with FM4-64®

After the stabilization period, the Mt/ampulla complex was incubated in oxygenated insect Ringer containing 30 μM FM4-64® (Molecular Probes) at 4°C for 60 min. The FM4-64® was prepared from a stock solution of 16 mM in DMSO. After loading, the Mt were washed 3 × for 5 min in oxygenated insect Ringer solution at 4°C. Three to four Mt from each mass were attached to separate poly-L-lysine-coated coverglasses. The Mt were mounted in oxygenated insect Ringer on spacers attached to glass slides as previously described. After imaging the control Mt, insect Ringer containing 1 mM db-cAMP was added to the Mt while on the slide.

All fluorescent material was examined with a Biorad® MRC 1024 ES confocal laser scanning microscope.

Results

External Membranes

Surface Subsequent to Removal of the Basal Lamina

Malpighian tubules of *A. domesticus* are covered by an acellular basal lamina that is approximately 0.5 μm thick. Treatment of the Mt with elastase removed the basal lamina to reveal the complex infoldings of the basal membranes. These infoldings appeared as a series of ridges and deep slits that ran parallel to the long axis of the tubule (Fig. 1*A*, *B*). In the control Mt, the ridges appeared more uniform and closely apposed (Fig. 1*A*). In comparison, the basal infoldings of the db-cAMP-stimulated Mt appeared less organized with obvious dilated areas between the folds (Fig. 1*B*).

Internal Membrane Structure

HR-SEM

Using the freeze-cleaving cytosolic extraction method of Townsend et al. (2000), the three-dimensional arrangement of the basolateral membrane and various organelles (i.e., Golgi apparatus, ER, mitochondria, nuclei) was revealed (Fig.1*C–F*, Fig. 2*A–F*). These results showed an increased amount of internal membrane that was not evident using standard transmission electron microscopy.

Control (Unstimulated). A smooth basal lamina covered the tubule with shallow infoldings of the basolateral membrane extending into the cell for approximately 1/3 its depth (Fig. 1*C*, *D*). The binucleate condition of the cells of the mid-tubule was clearly evident (Fig. 1*C*). Numerous spherites were present and in spherites with intact lamellae (Fig. 1*E*), there was a definite space between the rings, suggesting a protein matrix had been extracted. By far the most striking aspect of the cells was the quantity of endomembrane that filled the interior of the cell (Fig. l*C–F*). Mitochondria were dispersed throughout the membrane system (Fig. 1*C–F*). The brush border appeared organized and dense (Fig. 1*F*).

30 sec. After a 30-sec db-cAMP exposure the basolateral infolds appeared more distinct, extending 2/3 the depth of the cell (Fig. 2*A*). The membranes were parallel in arrangement with numerous mitochondria moving into the basal area (Fig. 2*B*). The internal membranes were much more distinct, especially in the central portion of the cell (Fig. 2*C*). There were dilated areas between the folds in the perinuclear area. This membrane appeared to be SER and stacks of the Golgi apparatus (Fig. 2C).

30 min. The basolateral membranes were distinct, but due to the complexity of the internal membrane system, we were no longer able to distinguish the boundaries of the basolateral membrane from the membranous components in the central portion of the cell (Fig. 2*D*, *E*). There were numerous mitochondria concentrating in the basal region. Again, there was evidence of many spherites throughout the cytoplasm. The immense membrane system present

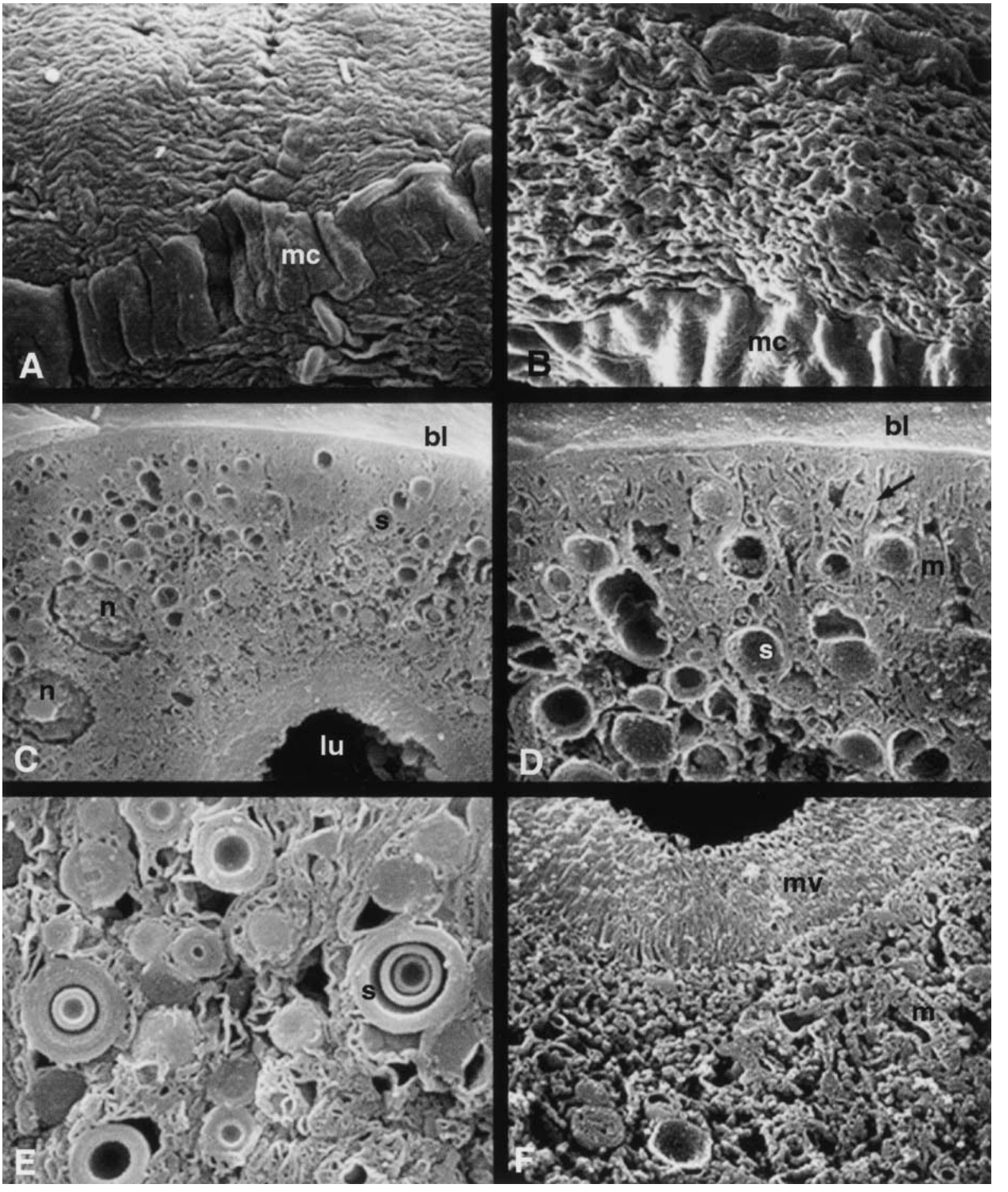

Fig. 1. Scanning electron micrograph of the mid Malpighian tubule (Mt) of *Acheta domesticus* following elastase digestion of the basal lamina (*A* and *B*) and following freeze-cleaving and cytosolic extraction (*C–F*) as observed by high resolution scanning electron microscopy (HR-SEM). *A*. *Control*. In the unstimulated Mt the infoldings are closely aligned (3000×). *B*. In the db-cAMP-stimulated Mt the infoldings are much more widely spaced (2000×). *C–F* represent unstimulated Mt. *C*. Low magnification showing the density of the internal membranes and the binucleate condition of the cells (3000×). *D*. Higher magnification of the basal region of the cell with shallow basolateral infolds (*arrow*) and evidence of numerous spherites (8500×). *E*. Calcium phosphate spherites, indicative of the mid-tubule, with concentric rings separated due to the extraction of the protein matrix (8500×). *F*. Apical region with dense brush border (9500×), *bl*, basal lamina; *lu*, lumen; *m*, mitochondria; *mc*, myoid cell; *mv*, microvilli; *n*, nucleus; *s*, spherites.

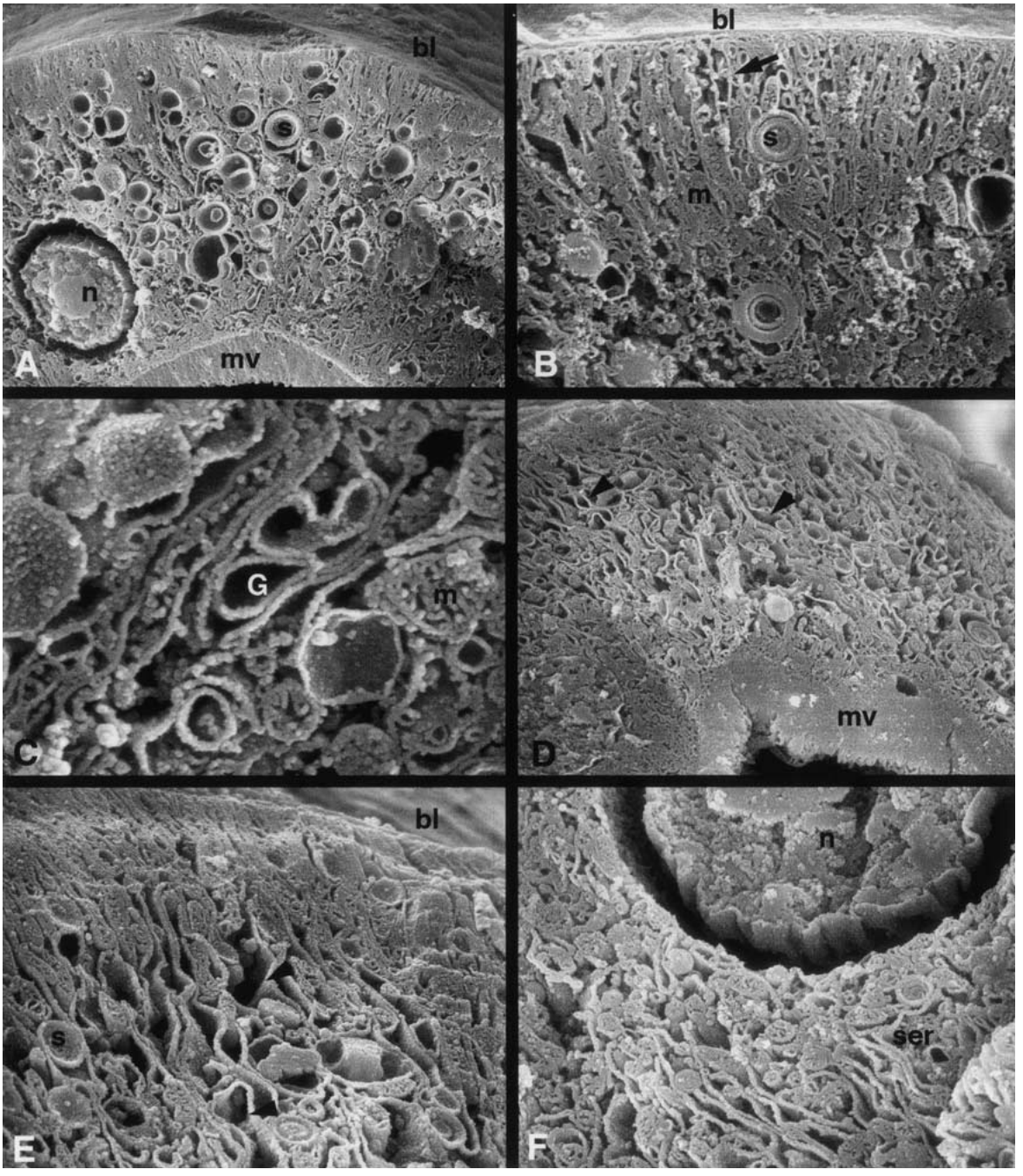

Fig. 2. Internal morphology of the mid Malpighian tubule (Mt) of *A. domesticus* as revealed by freeze-cleaving followed by cytosolic extraction as observed by HR-SEM. *A–C* represent the mid-Mt following 30 sec exposure to db-cAMP. *A*. Cross section of the mid-Mt showing more defined internal membrane structure (3000×). *B*. Higher magnification of the basal region. Note the elongation of the basolateral infolds (*arrow*) and the presence of many mitochondria (8000×). *C*. Golgi with dilated sacs in the perinuclear region (20000×). *D–F* represent the mid-Mt following 30-min exposure to db-cAMP. *D*. Overview of the internal arrangement. Membranes are much more pronounced with spaces appearing between them (*arrowhead*) (3500×). *E*. Higher magnification of the basal region with prominent dilated areas (*arrowhead*) between the membranes (8500×). *F*. Complexity of smooth endoplasmic reticulum in the perinuclear region (8000×). *bl*, basal lamina; *G*, Golgi; *m*, mitochondria; *mv*, microvilli; *n*, nucleus; *ser*, smooth endoplasmic reticulum; *s*, spherites).

within the cell was traversed with dilated areas evident (Fig. 2*D–F*). These dilations appeared between folds of membrane from the basal to the apical domains. However, no clear continuous conduit connecting one domain to the other was present. It was not possible to distinguish the Golgi apparatus but the majority of the membrane appeared to be SER as there were no visible ribosomes.

Fluorescent Labeling

Owing to the complexity and the amount of the internal membrane revealed by the freeze-cleaving cytosolic extraction study, our goal was to determine if the membranes were part of the ER. The overall result of the differential fluorescent labeling experiments was inconclusive. None of the markers behaved as expected based on the findings in the literature for other epithelial tissues as well as trial labeling experiments conducted on plant material (onion epidermis; unpublished observation) and the Mt of another arthropod (Chilean common tarantula, *Grammastola cala*) (Hazelton et al., 2001c).

To follow membrane movement, the plasma membrane was selectively labelled with the lipophilic marker, FM4-64® at 4°C and at room temperature. Again the results were atypical. The dye was incorporated not only into the plasma membranes as seen in the surface view of control tubules but also localized around the nuclei (Fig. 3*A*). Instead of preferentially labeling the plasma membrane, both the basal and apical domains were labelled (Fig. 3*C–E*). The overall intensity of staining was greater in the db-cAMP-stimulated tubules (Fig. 3*D*) and a distinct labeling of membrane occurred around vesicles (Fig. 3*E*). At low magnification there was a ''checkerboard'' pattern to the surface of the db-cAMP-stimulated, labeled membrane; Mt, possibly indicating preferential dye uptake by some cells and not by their neighboring cells (Fig. 3*F*).

The fluorescent marker, $DiOC_6(3)$, which is selective for ER at high concentrations, was unable to differentiate a definite subset of the internal membrane as being ER (Fig. 4*A–D*). In the control Mt, there appeared to be an accumulation of the label in the basal region of the cell with weak staining in the perinuclear region (Fig. 4*A*). In the stimulated Mt (Fig. 4*B–D*), the dye labeled an intricate web of internal membrane. There were numerous dark circular areas visible within the labeled membrane presumably these represent vesicles and spherites. In perinuclear areas a ''lace-like'' pattern was discernable (Fig. 4*D*).

Con A labeling revealed much the same complexity of internal membrane as $DiOC_6(3)$. In control Mt, the dye accumulated toward the exterior of the Mt (Fig. 5*A*, *B*) and a diffuse pattern of internal membrane labeling was noted around the nuclei (Fig. 5*B*). The label also reached the apical border in both control and db-cAMP-stimulated Mt (Fig. 5*A–D*). In stimulated tubules, a weak labeling of the outer nuclear envelope (Fig. 5*C*) was observed. A reticular pattern was evident from the label distribution but not as distinct as that noted in vertebrate tissue (da Silva, Torrisi & Kachar, 1981). In the db-cAMP-stimulated Mt, the marker was dispersed throughout the cytoplasm, localizing around the nuclei (Fig. 5*D*).

The nuclear stain provided a unique picture of organization of the Mt (Fig. 6*A–D*). The mid-tubule is composed of cuboidal cells arranged in a mosaic pattern. Each cell is dominated by the presence of two large nuclei, a condition that was not discovered in prior TEM studies describing the organization of the cells (Hazelton et al., 1988). The nuclei are in the lower half of the cell, often close to the microvillar border. There was diffuse staining of the cytoplasm, as can be expected with the Syto® dyes (Haugland, 1996). Cytoplasmic ''blebs'', which had been previously noted in TEM (Spring & Flegenhauer, 1996; Hazelton et al., 2001a), were visible in the Mt lumen (Fig. 6*D*).

Discussion

The Malpighian tubule, specifically the mid-tubule, of *A. domesticus* exhibits several extraordinary responses at the onset of db-cAMP-stimulated fluid transport. One response is a threefold increase in the surface area of the basolateral infolds within 15 sec of cAMP stimulation and the other a 50% vesiculation of the cytoplasm (Hazelton et al., 2001a); both occur too rapidly for the membrane to be created de novo. The amount and complexity of internal membrane structure revealed by cytosolic extraction and HR-SEM in the mid-tubule in preliminary investigations led to the application of this technique to follow changes in internal membrane structure in response to db-cAMP stimulation.

The initial objective of this project was to examine the changes in the basolateral membrane and vesiculation that accompany the onset of rapid fluid secretion in a three-dimensional representation. Based on TEM (Hazelton et al., 2001a) there is an increase in vesiculation seen within 30 sec of db-cAMP stimulation and with as much as 50% of the cytoplasm vesiculated by 420 sec. This extensive vesiculation is present as long as 30 min post db-cAMP stimulation, corresponding with the time for the doubling in secretion rate.

The removal of the basal lamina provided a unique opportunity to view the surface of the basal infoldings. It was not surprising that the control membranes were closely apposed, while the stimulated membranes were spaced further apart, thereby creating microenvironments for fluid transport. What was rather puzzling was the orientation of the infoldings. In studies of the Mt of *Rhodnius* and of the vertebrate proximal convoluted tubule, where the basal lamina had been removed, the orientation of the infolds was perpendicular to the long axis of the tubule (Levinson & Bradley, 1984; Evan et al., 1976). However, in *Acheta*, the folds were parallel to the long axis of the tubule.

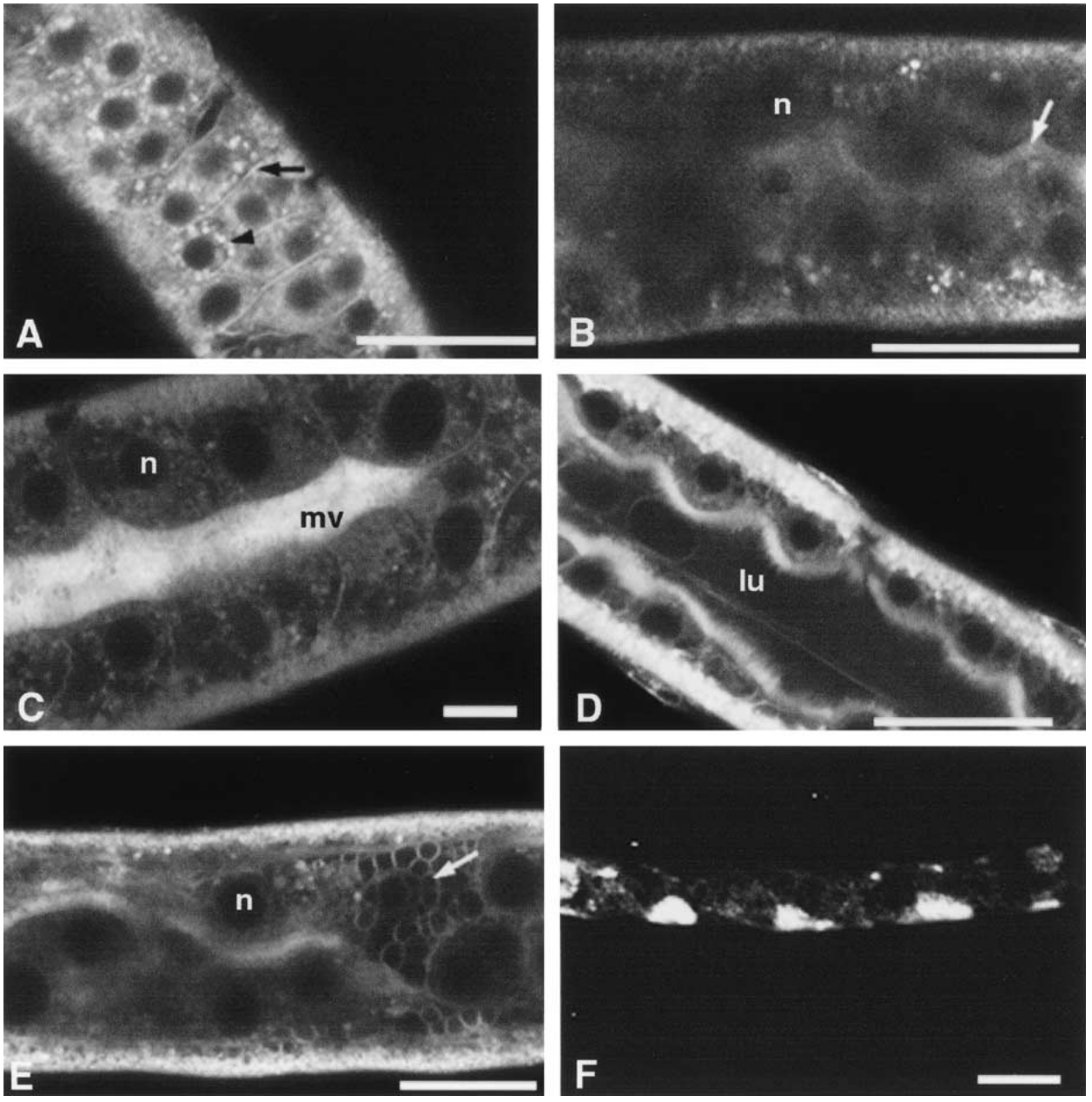

Fig. 3. Confocal images of the mid Malpighian tubule (Mt) of *A. domesticus* after staining with FM4-64® (30 μM) at room temperature (*A*, *B*, *D*, *F*) and at 4°C (*C*, *E*). *A*. Surface of an unstimulated Mt with dye marking cell boundaries (*arrow*) and in accumulations around the nuclei (*arrowhead*). Scale bar = 10 μm. *B*. Optical section near the level of the lumen in an unstimulated Mt. Note the presence of the marker in the apical membrane (*arrow*) and weakly staining the cytoplasm. Scale bar = 50 μm. *C*. Unstimulated Mt with the marker distributed in the basal region, but even more so in the apical region. Scale bar = 10 μm. *D*. Mt stimulated with db-cAMP (1 mM) showing clear distribution of the marker in both the basolateral membranes and the microvilli. Scale bar = 50 μm. *E*. Mt stimulated with db-cAMP (1 mM) with distinct labeling of vesicular areas (*arrow*). Scale bar = 50 μm. *F*. Low magnification of the surface of the mid-tubule indicating differential dye uptake by some cells. Scale bar = 100 μm. *lu*, lumen; *mv*, microvilli; *n*, nucleus.

When the sections of mid-tubule following freeze-cleaving and cytosolic extraction were examined, the same pattern of membrane elongation in the basal area was noted as had been in the earlier TEM studies (Spring & Felgenhauer, 1996; Hazelton et al., 2001a). This served to verify that the changes that precede rapid fluid transport occur almost immediately after stimulation with db-cAMP. What was surprising was the immense membrane area within the intermediate portion of the mid-tubule cell. The inner portion of the cells was packed with membranous elements, which in the control tissue were not identifiable as specific cellular organelles. The membranes became more distinct in the stimulated tissue

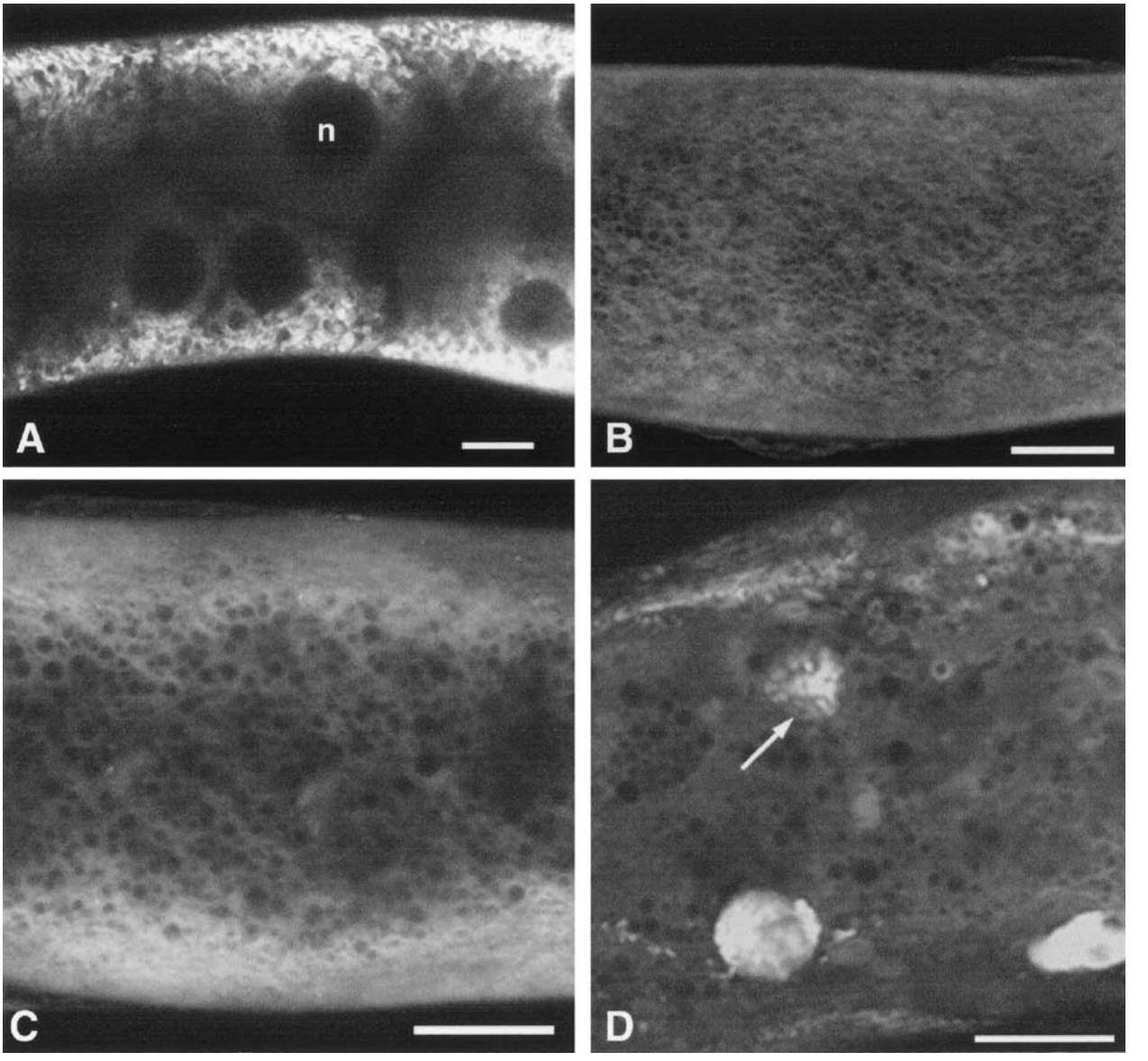

Fig. 4. Confocal images of the mid Malpighian tubule (Mt) of *A. domesticus* after staining with $DiOC_6(3)$ (2.5 μg/ml). Scale bars = 10 μm. *A*. Unstimulated Mt. The dye is concentrated in the basal region of the cells with the cytoplasm weakly staining. *B*. Mt stimulated with db-cAMP (1 mM). A web-like pattern of internal membrane is revealed. *C*. Higher magnification of the dye-distribution pattern in the db-cAMP-stimulated Mt with dark spherical areas appearing among the membranes. These are either spherites and/or vesicles. *D*. Optical section of the Mt showing the endoplasmic reticulum (*arrow*) in a lace-like pattern around a nucleus. Nuclei in this preparation were stained with propidium iodide (0.1% aqueous solution) for orientation. *n*, nucleus.

and appeared to be elements of either the ER or Golgi apparatus. The dilated areas seen in the interior of the cell at 30 min of cAMP stimulation possibly represent the vesicles and vacuoles depicted in the transmission electron micrographs. With this new information, it was logical to aim to identify these membranes and compartments.

The method of choice was to differentiate these membranes using fluorescent markers that would follow membrane movements in both living and fixed tissue. FM4-64® is a vital stain used to trace bulk membrane internalization and transport. In yeast (Vida & Emir, 1995), FM4-64® exclusively stained the plasma membrane at 4°C. When the yeast cells were warmed to 25°C, the fluorescence associated with the plasma membrane decreased and cytoplasmic punctate staining was evident, indicating the internalization of portions of the plasma membrane. Water-soluble styryl pyridinium dyes have also been used to follow vesicle traffic in endothelial cells (Niles & Malik, 1999). In order to restrict labeling to the cell surface plasma membrane, it is necessary to inhibit processes of membrane internalization such as endocytosis. The most widely used technique to accom-

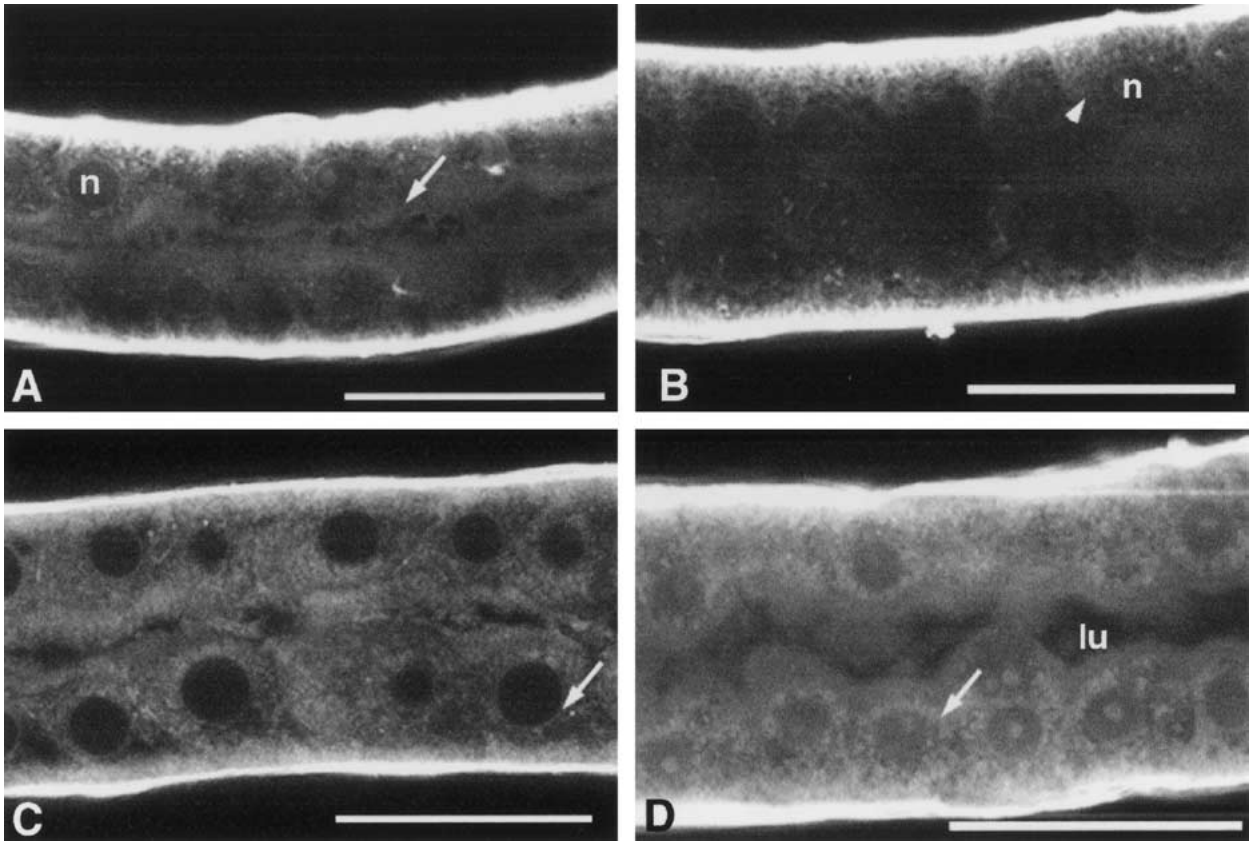

Fig. 5. Confocal images of the mid Malpighian tubule (Mt) of *A. domesticus* following exposure to Alexa Fluor™ conjugated Concanavalin A (50 μg/ml). Scale bars = 50 μm. *A*. Optical section of the control Mt at the level of the lumen. The basal and apical regions (*arrow*) as well as the cytoplasm are labeled. *B*. A more distinct web-like pattern of membrane (*arrowhead*) can be distinguished in the basal region and around nuclei. *C*. In the Mt stimulated with 1 mM db-cAMP, more of the lectin is distributed in the cytoplasm with weak labeling of the nuclear envelope (*arrow*). *D*. Optical section of the db-cAMP-stimulated tubule with the lumen clearly visible. Con A has accumulated in areas around the nuclei (*arrow*) and also in the brush border.

plish this is chilling the cells to 0–4°C or aldehyde fixation (reviewed by Steinman et al., 1983). Labeling of the Mt was performed at 4°C since this was the least invasive method to limit membrane movement and also allowed for warming the tissue gradually and following the corresponding membrane movements. However, the findings were unexpected. Malpighian tubules exposed to FM4-64® at 4°C labeled extensively from basal to apical region. One would think, based on the predicted behavior of membrane at 4°C, that the dye would distribute in the basolateral membranes and either remain there or be internalized, as warming occurred, as part of constitutive endocytosis. Alternatively, the basal membranes would fluoresce brightly initially and appear less bright when stimulated due to the incorporation of nonlabeled membrane sections inserted from internal stores. Significant labeling of the apical surface was not expected. It is not supported that the dye moved into the tubule from the lumenal surface since the Mt were intact and attached to the ampulla when exposed to the dye. To further verify that the dye was not reaching the apical surface from the lumen, the proximal portion of the Mt was anchored outside of the bathing droplet and mid and distal portions were selectively exposed to the dye. Again the apical region labeled intensely. Why the apical region would label so intensely with a dye targeted to the plasma membrane is unknown at this time.

Another unique situation revealed by the FM4-64® labeling was a "checker-board" pattern at the surface of the tubules. Although the region appears to be a homogeneous cell type, it may not be. Maddrell (1978) found that although the entire lower Mt in *Rodnius prolixus* appeared uniform with regard to gross morphology, the upper half of the lower Mt was more osmotically permeable than the lower half. The latter also exhibited more rapid KCl absorption. However, these two regions were functionally different. Dow, Davies & Sozen (1998), working with *Drosophila* Mt, proposed that each cell along the Mt may express a subset of transport genes consistent with its lineage and spatial position. Although segments within Mt may appear morphologically homogeneous, the cells composing them may represent a functionally heterogeneous epithelium.

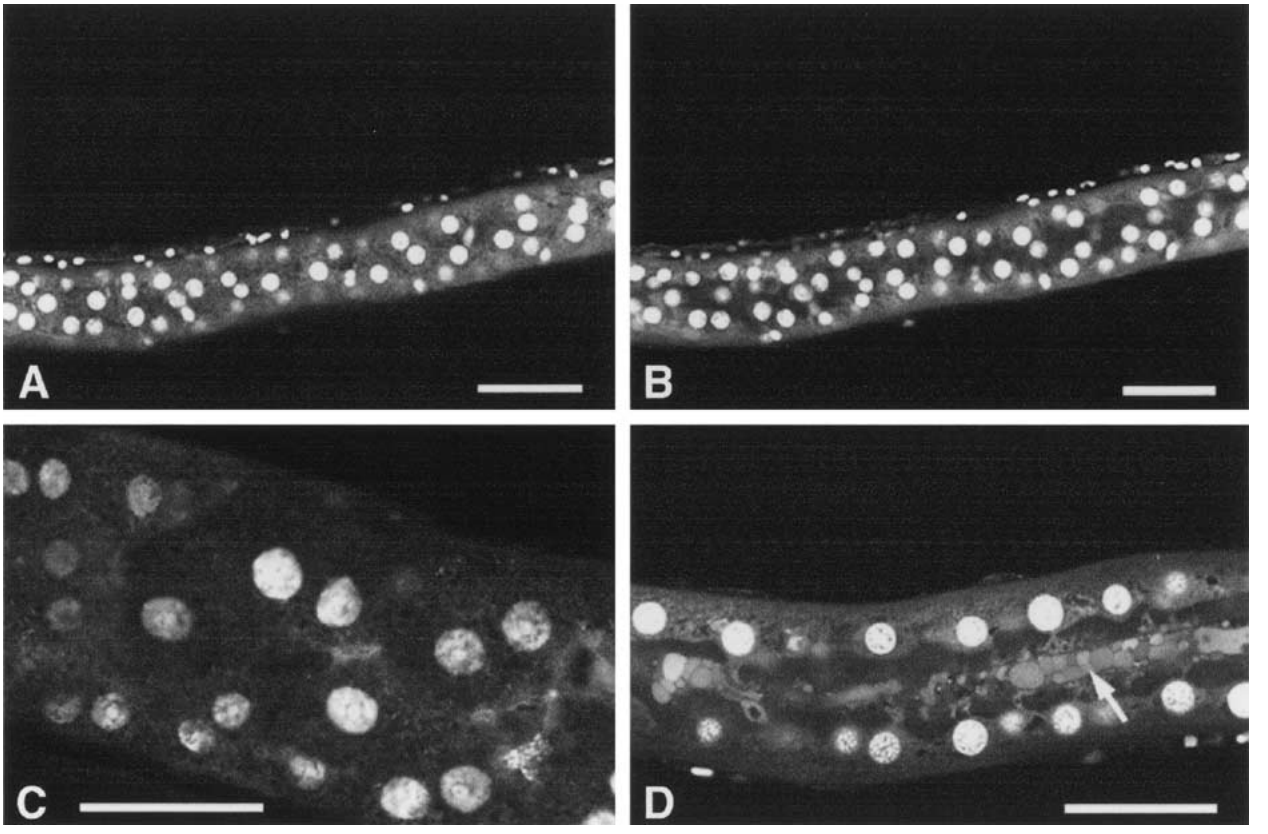

Fig. 6. Confocal images of the mid Malpighian tubule (Mt) of *A. domesticus* stained with the live-cell nuclear stain, Syto® 13. Scale bars = 50 μm. *A*. Optical section of the tubule several microns below the surface. Note the mosaic pattern of binucleate cells. *B*. Optical section closer to the lumen of the Mt. *C*. Higher magnification of the binucleate cells. Diffuse cytoplasmic staining is present. *D*. Optical section at the level of the lumen. The nuclei are located in the apical half of the cells. The lumen is filled with material and cytoplasmic "blebs" (*arrow*).

The next area of investigation was the store of endomembrane. Since approximately half of the total area of membrane in a cell encloses spaces of the ER (Alberts et al., 1989), the ER was targeted first. The major challenge with distinguishing ER in insect Mt is that there is no morphological feature, biochemical function or marker enzyme that has been identified to be indicative of insect ER. Therefore methods successful in labeling ER in vertebrate tissues were used. Since the ER forms an extensive network of tubular and cisternal membranes, its distribution is difficult to examine in two dimensions; therefore techniques have evolved to examine the arrangement of ER in living cells (reviewed by Terasaki, 1989). The dye of particular interest was $DiOC_6(3)$ which is a dicarbocyanine with both hydrophobic and hydrophilic characteristics. When $DiOC_6(3)$ is used in higher concentrations, as in this study, it should stain the ER. At lower concentrations it will also accumulate in mitochondria due to the negative membrane potential and the overall positive charge of the dye. At the higher concentration, there is very little to no staining observed in lysosomes, endosomes or the Golgi apparatus, and the plasma membrane should only be dimly visible (Terasaki et al., 1984), based on staining in African Green Monkey Kidney epithelia cell line, CV-1. When applied to epithelial tissue such as chick embryo cells, rat embryo fibroblast cells and smooth muscle cells, $DiOC_6(3)$ staining results in a distinctive "lace-like" pattern indicative of ER. For comparison, onion epidermal cells (unpublished observation) and the Mt of the Chilean common tarantula (Hazelton et al., 2001c) were stained with the dye, resulting in much the same reticular pattern noted for ER, with mitochondria dispersed among the membranes. However, when the same techniques were applied to the Mt of *A. domesticus*, the labeling pattern was less clear. The $DiOC_6(3)$ penetrated the living cells, becoming localized in the basolateral infoldings, but based on optical sections, membrane stained throughout the cytoplasm. The areas devoid of stain appeared as dark spheres representing a mixture of spherites and vesicles. There was an indistinct reticular pattern, but it was present throughout the cytoplasm.

Given that the $DiOC_6(3)$ did not label as we expected based on its performance in other tissues, including the Mt of another arthropod, we decided to

use Con A. This particular marker has widespread use in carbohydrate and cell surface research, specifically in the areas of agglutination (Moscona, 1971; Sharon & Lis, 1972) and carbohydrate labeling (da Silva et al., 1981). Con A is a lectin that selectively binds to α-glucopyranosyl and α-mannopyranosyl residues. Da Silva et al. (1981) examined the localization of Con A binding sites on freeze-fractured pancreatic cells. Although the Con A labeled the endoplasmic face of the plasma membrane, there was a higher density of label on the exoplasmic face of the freeze-fractured ER. It is thought that the plasma-membrane Con A label was associated with peripheral membrane components or integral membrane components that, upon fracture, partitioned with the exoplasmic half of the membrane. Da Silva et al. (1981) concluded that it is also likely that the Con A is binding to mannosyl residues associated with nascent peptide chains during the synthesis of membrane and secretory glycoproteins. The outer nuclear membrane labeled identically to the ER, probably due to the continuity of the outer envelope with the ER. The labeling experiments by da Silva et al. (1981) found Con A-binding sites uniformly distributed over the ER, suggesting widespread distribution of the mannosyl-bearing residues. Con A has also been found almost exclusively to localize in the lumen of the ER cisternae in rat epiphyseal chondrocytes (Velasco & Hidalgo, 1987), again in conjunction with biosynthesis of cartilage extracellular matrix components by chondrocytes.

However, when Con A is used to label the Mt of *A. domesticus* a different pattern emerges. Results indicate plasma membrane, ER and nuclear envelope labeling. There is a localization of the dye on the exoplasmic surface of the basolateral infolds, especially in the controls, suggesting binding to peripheral sugar groups. The more widespread labeling in the stimulated tissue suggests a possible dispersion and internalization of the marker. There does appear to be a pattern to the labeling and there is labeling around vesiculated areas. Although the marker labelled the apical membrane in the control Mt, it was not as uniformly distributed as in the stimulated Mt. The uniform basal-to-apical labeling suggests a more even distribution of the sugar groups in the membranes of the stimulated Mt. Although the assumption from previous investigators is that these groups exist on nascent proteins being manufactured by the ER, it is difficult to envision this to be the case in *A. domesticus* Mt. Based on the HR-SEM and TEM there is not a great deal of RER present, the majority of the internal membrane is SER. It could be that, since the crickets have reached the final stage of their life cycle with the adult molt and therefore have a limited life span, protein synthesis is reduced and quantities of RER to manufacture components are unnecessary. This could also explain why Golgi are not prevalent in the Mt. Since significant stores of endomembrane are present in the cells, it is feasible to suggest that this membrane could be premanufactured stores complete with the transport proteins and extracellular markers for interchange with both the apical and basal surfaces in response to endocrine signals.

An additional possibility is that portions of the internal membrane could vesiculate during rapid influx of fluid to compartmentalize water and result in electron-lucent vesicles. A similar phenomenon occurs in some vertebrate tissue in the presence of ouabain. In rat liver cells when the Na^+-K^+ pump is inhibited by ouabain, vesicles develop as a secondary volume control mechanism (Van Rossum et al., 1987). These vesicles, as those in Mt, appear in a variety of sizes. The clear vesicles have presumably aqueous contents and arise from vesiculation of the ER. In the ouabain-resistant volume regulation, the Golgi apparatus is also distinguishable and its elements are seen in communication with either the swollen terminal cisternae of the ER or with large rounded vesicles. Hazelton et al., (2001a) described an association of Golgi with vesicles in their TEM study of vesiculation in *A. domesticus*. It is possible that vesiculation exists in Mt as a method to deal with the rapid influx of water into the cytoplasm in much the same way that vesicles function in volume regulation in the presence of ouabain in some vertebrate systems (Van Rossum et al., 1987). The transport mechanisms necessary to move ions into the vesiculated areas would be present and activated by the surge in internal cAMP.

The labeling of the microvilli, although unusual, is indicative of ER labeling. The microvilli of insects differ from those of other animals in that they contain ER and often mitochondria (Bradley, 1985; Hazelton et al., 1988). Mitochondria and ER can move into and out of the microvilli under a variety of stimuli, due to the presence of core microfilaments (reviewed by Bradley, 1985). In *Rodnius prolixus*, Berthelet et al., (1987) found the ER possessed a transepithelial orientation extending from the basal pole to the tip of the microvilli. The presence of both mitochondria and ER within the microvilli would provide a ready source of the transport proteins necessary to drive fluid movement (i.e., cation exchangers) and the ATP (V-ATPases) to fuel them at the destination where they would facilitate stimulated transport.

At this point we have determined that the membrane system found in *A. domesticus* Mt is unique. We can only speculate on the reasons behind the lack of conformity to established labeling techniques, even techniques that provide clear results in other arthropods. It could be that there is a continuous complex network of membranes extending from the basal to

the apical domains. Normally one would assume barriers to keep these domains segregated, but it appears that this is not the case in *A. domesticus* Mt. What could be the possible benefit of such interchange and fluidity? The findings of this project open the way for a plethora of unanswered questions. What are the contents of the vesicles in terms of ions? Are V-ATPases coupled with ion exchangers present in the vesicle membranes? Do the vesicle membranes contain stores of aquaporins to facilitate movement of water into the lumen? If aquaporins are present, are they inserted into the apical membrane in response to rapid fluid secretion? What happens to the vesiculation as rapid fluid transport wanes? Given the complexity and fluidity of the membrane systems in the mid-tubule, what is the involvement of elements of the cytoskeleton? It is time now to narrow the focus and begin examining the internal membranes in greater detail to determine the nature of the transport processes occurring in *A. domesticus*.

This work was supported in part by a grant from the National Science Foundation (IBN 9807948) to J.H.S. and B.E.F and a Board of Regents Doctoral Fellowship to S.R.H. We wish to thank Dr. Tom Pesacreta for the use of the University of Louisiana at Lafayette Microscopy Center, and Dr. Roy Brown, Dr. Betty Lemmon, Dr. Regina McClinton, Hong Nguyen and Linda LeBlanc for their advice and assistance in preparing this manuscript.

References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J.D. 1989. *Molecular Biology of the Cell,* 2nd edition. Garland Publishing, Inc., New York

Berthelet, F., Beaudry-Lonergan, M., Linares, H., Whittembury, G., Bergeron, M. 1987. Polymorphic organization of the endoplasmic reticulum of the Malpighian tubule. Evidence for a transcellular route. *La Cellule* **74:**283–290

Beyenbach, K.W. 1995. Mechanism and regulation of electrolyte transport in Malpighian tubules. *J. Insect Physiol.* **41:**197–207

Bradley, T.J. 1985. The excretory system: Structure and function. *In:* Comprehensive Insect Physiology, Biochemistry, and Pharmacology. G.A. Kerkut and L.I. Gilbert, editors, pp. 421–465. Pergamon Press, New York

Clark, T.M., Spring, J.H. 1992. Excretion in the house cricket: Role of second messengers in Malpighian tubule function. *Can. J. Zool.* **70:**1347–1353

Coast, G.M., Cuusinato, O., Kay, I., Goldsworthy, G.J. 1991. An evaluation of the role of cyclic AMP as an intracellular second messenger in Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Insect Physiol.* **37:**563–573

Coast, G.M., Kay, I., Wheeler, C.H. 1993. Diuretic peptides in the house cricket, *Acheta domesticus* (L.): A possible dual control of Malpighian tubules. *In:* Structure and Function of Primary Messengers in Invertebrates: Insect Diuretic and Antidiuretic Peptides. Molecular Comparative Physiology, vol 12. K.W. Beyenbach, editor. pp. 38–66. Karger, New York

Cottin, V., Van Linden, A., Riches, D.W.H. 1999. Phosphorylation of tumor necrosis factor receptor CD120a (p55) by $p42^{mapk/erk2}$ induces changes in its subcellular localization. *J. Biol. Chem.* **374:**32975–32987

da Silva, P.P., Torrisi, M.R., Kachar, B. 1981. Freeze-fracture cytochemistry; localization of wheat-germ agglutinin and concanavalin A binding sites on freeze-fractured pancreatic cells. *J. Cell Biol.* **91:**361–372

Dow, J.A.T., Davies, S.A., Sozen, M.A. 1998. Fluid secretion by the *Drosophila* Malpighian tubule. *Amer. Zool.* **38:**450–460

Evan, A.P., Dail, W.G., Dammrose, D., Palmer, C. 1976. Scanning electron microscopy of cell surfaces following removal of extracellular material. *Anat. Rec.* **185:**433–446

Haggis, G.H. 1992. Sample preparation for electron microscopy of internal cell structure. *Microsc. Res. Tech.* **22:**151–159

Haugland, R.P. 1996. *Handbook of Fluorescent Probes and Research Chemicals,* 6th edition. Molecular Probes, Inc., Eugene, Oregon

Hazelton, S.R., Felgenhauer, B.E., Spring, J.H. 2001a. Ultrastructural changes in the Malpighian tubule of the house cricket, *Acheta domesticus*, at the onset of diuresis: A time study. *J. Morphol.* **247:**80–92

Hazelton, S.R., Parker, S.W., Spring, J.H. 1988. Excretion in the house cricket (*Acheta domesticus*). Fine structure of the Malpighian tubules. *Tissue Cell* **20:**443–460

Hazelton, S.R., Spring, J.H., Felgenhauer, B.E. 2001b. Fluid-phase endocytosis does not contribute to rapid fluid secretion in the Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Exp. Zool.* (in press)

Hazelton, S.R., Townsend, Jr. V.R., Richter, C., Ritter, M., Felgenhauer, B.E., Spring, J.H. 2001c. Morphology and Ultrastructure of the Malpighian Tubules of the Chilean Common Tarantula, *Grammostola cala* (Araneae, Theraphosidae). *J. Morphol.* (in press)

Hegarty, J.L., Zhang, B., Pannabecker, T.L., Petzel, D.H., Baustain, M.D., Beyenbach, K.W. 1991. Dibutyryl cAMP activates bumetanide-sensitive electrolyte transport in Malpighian tubules. *Am. J. Physiol* **261:**C521–C529

Kim, I.S., Spring, J.H. 1992. Excretion in the house cricket: Relative contribution of distal and mid-tubule to diuresis. *J. Insect Physiol.* **38:**373–381

Klein, U. 1992. The insect V-ATPase, a plasma membrane proton pump energizing secondary active transport: immunological evidence for the occurrence of a V-ATPase in insect ion-transporting epithelia. *J. Exp. Biol.* **172:**345–354

Levinson, G., Bradley, T.J. 1984. Removal of insect basal laminae using elastase. *Tissue Cell* **16:**367–375

Maddrell, S.H.P. 1978. Physiological discontinuity in an epithelium with an apparently uniform structure. *J. Exp. Biol.* **75:**133–145

Maddrell, S.H.P., Pilcher, D.E., Gardiner, B.O.C. 1971. Pharmacology of the Malpighian tubules of *Rhodnius* and *Carausius*: the structure-activity relationship of tryptamine analogues and the role of cyclic AMP. J. *Exp. Biol.* **54:**779–804

Moscona, A.A. 1971. Embryonic and neoplastic cell surfaces: Availability of receptors for concanavalin A and wheat germ agglutinin. *Science* **171:**905–907

Nicolson, S.W. 1976. Diuresis in the cabbage white butterfly, *Pieris brassicae*: fluid secretion by the Malpighian tubules. *J. Insect Physiol.* **22:**1347–1356

Niles, W.D., Malik, A.B. 1999. Endocytosis and exocytosis events regulate vesicle traffic in endothelial cells. *J. Membrane Biol.* **167:**85–101

Nation, J.L. 1983. A new method for using hexamethyldisilizane for preparation of soft insect tissue for scanning electron microscopy. *Stain Technol.* **58:**347–351

Pannabecker, T. 1995. Physiology of the Malpighian tubule. *Annu. Rev. Entomol.* **40:**493–510

Sharon, N., Lis, H. 1972. Lectins: Cell-agglutinating and sugar specific proteins. *Science* **177:**949–959

Spring, J.H., Clark, T.M. 1990. Diuretic and antidiuretic factors which act on the Malpighian tubules of the house cricket, *Acheta domesticus*. *In:* Progress in Comparative Endocrinology. A. Epple, C.G. Scanes, and M.H. Stetson, editors, pp. 559–564. Wiley-Liss, New York

Spring, J.H., Felgenhauer, B.E. 1996. Excretion in the house cricket, *Acheta domesticus*: Effects of diuretics on the structure of the mid-tubule. *J. Morphol.* **230:**43–53

Spring, J.H., Hazelton, S.R. 1987. Excretion in the house cricket (*Acheta domesticus*): Stimulation of diuresis by tissue homogenates. *J. Exp. Biol.* **129:**63–81

Spring, J.H., Kim, I.S. 1993. Excretion in the house cricket: Structural and functional differences between distal and mid-tubules. *In:* Mol. Comp. Physiol., Vol. 12: Structure and Function of Primary Messengers in Invertebrates: Insect Diuretic and Antidiuretic Peptides. K.W. Beyenbach, editor, pp. 130–145. Karger, New York

Spring, J.H., Kim, I.S. 1995. Differential effects of neuropeptides on the distal and mid-tubules of the house cricket. *Arch. Insect Biochem. Physiol.* **29:**11–23

Steinman, R.M., Mellman, I.S., Muller, W.A., Cohn, Z.A. 1983. Endocytosis and the recycling of plasma membrane. *J. Cell Biol.* **96:**1–27

Terasaki, M. 1989. Fluorescent labeling of endoplasmic reticulum. *In:* Methods in Cell Biology, Y.L. Wang and D.L. Taylor, editors. Vol. 29, pp. 125–135. Academic Press, San Diego

Terasaki, M. 1998. Labeling of the endoplasmic reticulum with $DiOC_6(3)$. *In:* Cell Biology: A Laboratory Handbook, 2^{nd} edition. J.E. Celis, editor. Vol 2, pp. 501–506. Academic Press, San Diego

Terasaki, M., Song, J., Wong, J.R., Weiss, M.J., Chen, L.B. 1984. Localization of endoplasmic reticulum in living and glutaraldehyde-fixed cells with fluorescent dyes. *Cell* **38:**101–108

Townsend, Jr. V.R., Hazelton, S.R,, Felgenhauer, B.E., Spring, J.H. 2000. New technique for examining invertebrate membranes using high resolution scanning electron microscopy. *Microsc. Res. Tech.* **49:**209–211

Van Rossum, G.D.V., Russo, M.A., Schisselbauer, J.C. 1987. Role of cytoplasmic vesicles in volume maintenance. *In:* Current Topics in Membranes and Transport, Vol 30, Cell Volume Control: Fundamental and Comparative Aspects in Animal Cells. R. Gilles, A. Kleinzeller and L. Bolis, editors, pp. 45–74. Academic Press, San Diego

Velasco, A., Hidalgo, J. 1987. Light and electron microscopical localization of concanavalin A lectin binding sites in rat epiphyseal chondrocytes. *Histochem. J.* **1:**7–14

Vida, T.A., Emr, S.D. 1995. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* **128:**779–792

Wieczorek, H., Putzenlechner, M., Zeiske, W., U. Klein. 1991. A vacuolar-type proton pump energizes K^+/H^+ antiport in an animal plasma membrane. *J. Biol. Chem.* **266:**15340–15347

Wieczorek, H., Weerth, S., Schindlbeck, M., Klein, U. 1989. A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes from tobacco hornworm midgut. *J. Biol. Chem.* **264:**11143–11148

Wieczorek, H., Wolfersberger, M.G., Cioffi, M., Harvey, W.R. 1986. Cation-stimulated ATPase in purified plasma membranes from tobacco hornworm midgut. *Biochim. Biophys. Acta* **857:**271–281

Wigglesworth, V.B. 1972. *The Principles of Insect Physiology*, 7^{th} edition. Halsted Press, New York