Dissociation of Insect Malpighian Tubules into Single, Viable Cells

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SUMMARY

The Malpighian tubules of insects are generally composed of more than one cell type. In the hemipteran Rhodnius prolixus, the tubules are divided into two regions, termed the upper and lower tubules, each of which is composed of a distinct cell type. In the dipteran Aedes taeniorhynchus, primary and stellate cells are interspersed throughout the length of the tubules. We report here techniques for the dissociation of the Malpighian tubules of both of these species into single cells. Tubules are removed from the insect and placed for 1 h in insect Ringer containing elastase (Sigma, type III) at 4 mg/ml. This treatment fully removes the basal lamella. Mild agitation by hand produces a suspension of single cells, which remain viable as determined by Trypan Blue exclusion. Isolated cells have been maintained in cell culture for one week. Using light and scanning electron microscopy, upper and lower tubule cells of Rhodnius and primary and stellate cells of Aedes can be distinguished on the basis of size, shape, microvillar length, and the presence or absence of intracellular crystals.

INTRODUCTION

The Malpighian tubules of insects are tubular epithelia that lie in the haemocoel and are the site of haemolymph filtration and primary urine production. In all insect species examined to date, Malpighian tubule epithelia are composed of more than one cell type. In some species the tubules are separated into morphologically distinct regions, each homogeneous for a given cell type. In other species the different cell types are intermixed as a heterogeneous population of cells.

The hemipteran Rhodnius prolixus is an example of a species in which the Malpighian tubules are divided into regions. Each of the four tubules in this insect consists of an upper tubule and a lower tubule, composed of distinct cell types. The different cell types, and hence the different regions of the tubule, can be readily distinguished both morphologically and ultrastructurally. Cells of the upper tubule are larger than those in the lower tubule, their microvilli are clumped together, and they contain intracellular, spherical, mineralized concretions, which are not found in the cells of the lower tubule (Wigglesworth & Salpeter, 1962; Bradley, 1983). The two cell types also perform different physiological functions. For example, fluid is known to be secreted in the upper tubule, while cells of the lower tubule may secrete uric acid or resorb KCl.

In the Malpighian tubules of the dipteran Aedes taeniorhynchus there are no

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distinct regions within the tubule. Instead, a heterogeneous arrangement of two cell types is observed. The majority of cells within the tubule are primary cells, and a second cell type, the stellate cell, is interspersed at fairly regular intervals along the length of the tubule (Satmary & Bradley, 1984). Primary cells are larger than stellate cells, contain intracellular crystals and possess larger apical microvilli. The primary cells are thought to be the site of primary urine production. It has been proposed that the smaller stellate cells may be involved in Na\textsuperscript{+} resorption from the primary urine (Berridge & Oschman, 1969), although no direct experimental evidence regarding their function is available.

In the present study, Malpighian tubules of both *Rhodnius* and *Aedes* were dissociated using a technique that involves the enzymic removal of the basal lamella. To our knowledge, this is the first successful dissociation of any Malpighian tubule into single, viable cells. The dissociated cells were then examined using light and scanning electron microscopy.

**MATERIALS AND METHODS**

*Rhodnius prolixus* used for these studies were fifth instar larvae taken from a colony maintained as previously described (Bradley & Satir, 1981). Lengths of upper or lower Malpighian tubule were dissected from animals in an insect Ringer solution: 129 mM-NaCl, 8.6 mM-KCl, 8.5 mM-MgCl₂, 2 mM-CaCl₂, 10.2 mM-NaHCO₃, 4.3 mM-NaH₂PO₄, and 34 mM-glucose, buffered to pH 6.9 (Maddrell, 1969).

*Aedes taeniorhynchus* used for these studies were taken from a laboratory colony maintained as previously described (Bradley & Phillips, 1975). All *Aedes* used were fourth instar larvae reared in 100% artificial seawater (Instant Ocean). Malpighian tubules were dissected from these insects in the above Ringer solution to which 100 mM-sucrose had been added.

Malpighian tubules from 8-12 *Rhodnius* were separated into upper and lower tubules and transferred to separate vials using glass needles. Whole tubules from 15-30 *Aedes* were transferred to a third vial. Each vial contained 1 ml of the Ringer solution in which the tubules were dissected, to which 4 mg of porcine pancreatic elastase (elastase type III, Sigma) had been added. Treatment with elastase removes the basal lamella surrounding the tubule (Levinson & Bradley, 1984). Tubules were placed in the elastase solution for 1 to 1.5 h at room temperature with occasional mild agitation. Following this treatment the enzyme solution was replaced with three successive washes (1 ml each) of the appropriate Ringer solution. The vial was then shaken by hand for several seconds until a suspension of single cells and short sections of tubule was produced.

**Determination of viability**

A drop of cell suspension was added to a drop of 0.4 % Trypan Blue in insect Ringer a microscope slide. Small amounts of petroleum jelly were applied to the underside edges of a coverslip, which was placed over the specimen. The jelly raised the coverslip a short distance above the slide so that the cells would not be crushed. Slides were viewed and photographed using an Olympus BH-2 compound microscope.

**Preparation for scanning electron microscopy**

Samples from single cell suspensions were placed in 3-5 ml of fixative for 1 h. For *Rhodnius* the fixative was 4 % glutaraldehyde, 0.05 M-sodium cacodylate and 0.1 M-sucrose (pH 6.9). The fixative used for tubules of *Aedes* was identical to that used for *Rhodnius* with the addition of 0.2 M-sucrose. The suspension of fixed cells was placed on a 13 mm diameter polycarbonate membrane filter (Nuclepore Corp.), pore size 10-0 μm, held in a plastic Pop-Top filter holder (Nuclepore Corp.). Polyethylene tubing connected the outlet of the filter holder to a vacuum flask. This arrangement permitted cells to be held by the filter and washed with solutions, which flowed through
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the filter by gravity or with slight negative pressure. In this manner, fixed cells were rinsed with buffer solution and dehydrated in an ethanol series, followed by an ethanol-to-Freon 113 gradient series. At no time were the cells exposed to the fluid/air interface. The filter and holder were critical-point dried using Freon 13 (Bomar apparatus). The filter was removed from the holder and placed on a scanning electron microscope (SEM) stub covered with double-sided tape. Specimens were coated with gold/palladium using a Hummer II sputter coater (Technics). Scanning electron micrographs were taken on a Hitachi S-500.

Culture of dissociated cells

Samples of the single-cell suspension were added to sterile 60 mm × 15 mm Petri dishes containing membrane-filtered Schneider's Drosophila medium (KC Biological). Three different kinds of dishes were used: glass (Kimax), film-lined plastic (Falcon), and surface-modified plastic designed for primary cell culture (Primaria brand, Falcon). Garamycin (100 μg/ml) (Schering Corp.) was added as an antibiotic. Dishes were incubated at 26 °C under normal atmospheric conditions. Cultured cells were viewed using a Nikon Diaphot-TMD inverted microscope.

RESULTS

It has been shown that elastase fully removes the basal lamella from insect Malpighian tubules (Levinson & Bradley, 1984). Without a basal lamella the tubules are very fragile. Mild agitation breaks elastase-treated tubules into single cells and multicellular fragments. Cell lysis occurs if the agitation is too harsh, especially with Aedes tubules, which tend to be more delicate. Single cells and tubule fragments are suspended in the Ringer solution following agitation. As a suspension, the cells are easily transferred using a pipette.

Viability of cells

As viewed in the light microscope each suspension of dissociated Malpighian tubules contained short pieces of tubule, intact single cells and cell fragments. When examined in Ringer containing Trypan Blue, some cells and all the cell fragments stained intensely. Most of the cells excluded Trypan Blue, indicating that these were intact and viable.

SEM of dissociated tubules

In the scanning electron microscope all the isolated cells appear to be devoid of a basal lamella. Since the basal surface of these cells had never been observed, short segments of tubule were examined to find distinguishing features of apical and basal cell surfaces. Due to the zig-zag arrangement of cells in the epithelium it was possible to view simultaneously the basal surface of one cell and the apical surface of an adjacent cell in a short segment of tubule (Figs 1, 3).

Cells of Rhodnus. The basal surface of upper tubule cells of Rhodnus is marked by transverse furrows of the plasma membrane (Fig. 1). The organization of these furrows is consistent with the membrane infoldings that have been seen in transmission electron micrographs (Wigglesworth & Salpeter, 1962). The basal surface of individual, dissociated cells appears identical to the basal surface of cells in short or long sections of elastase-treated tubule.

In cells of the upper tubule the apical surface, which faces the lumen of the tubule,
is covered with microvilli (Figs 1, 2). Bradley (1983) used scanning electron microscopy to examine the cells of intact, *Rhodnius* Malpighian tubules. He found that the microvilli on the cells of the upper tubule are organized in clumps consisting of closely aligned microvilli. We find that in elastase-treated, intact tubules the microvilli remain clumped. In upper tubule cells that have been dissociated, however, the
microvilli are usually freestanding (Fig. 2), although the microvilli of some dissociated cells do retain their clumped configuration.

The plasma membrane between the apical and basal surfaces is smooth (Fig. 2). This smooth region is presumably the area of the cell surface that was adjacent to a neighbouring cell before dissociation. This intercellular region forms a continuous ring around the edge of the cell.

Apical and basal surfaces of lower tubule cells can also be clearly differentiated when viewing short pieces of tubule (Fig. 3). The basal surface of lower tubule cells is rough in appearance. The infoldings of the plasma membrane lack the furrowed organization seen in cells of the upper tubule. The apical surface of lower tubule cells is covered with microvilli. Microvilli are free-standing both in dissociated lower tubule cells (Figs 3, 4) and in intact lower tubules (Bradley, 1983). A smooth intercellular region is also observed between apical and basal surfaces in dissociated cells of the lower tubule (Fig. 4).

Interestingly, isolated cells viewed in the SEM appear to have a greater volume than similar cells retaining the configuration of the tubule (compare Figs 1 and 2; and 3 and 4). This may be a result of the initiation of cell swelling and membrane unfolding that occurs following cell isolation (see Discussion).

**Cells of Aedes.** Primary cells in Malpighian tubules of *Aedes* have a basal surface that is covered with bumps (Figs 5, 6, 7). These protrusions are easily distinguished from the free-standing microvilli observed on the apical side of the cell (Figs 5, 6). A region of smooth membrane, often bordered by a ridge, lies between apical and basal surfaces (Fig. 6).

Stellate cells are interspersed between the larger primary cells in Malpighian tubules of *Aedes*. This arrangement is clearly observed in tubules where the basal lamella has been removed using elastase (Fig. 7). Stellate cells can be identified by their smooth basal surface. The narrow arms of stellate cells extend between adjacent primary cells. Stellate cells retain their characteristic shape even after dissociation (Fig. 8). The lack of intracellular crystals is an important identifying feature of stellate cells viewed with the light microscope.

**Culture of dissociated cells**

Preliminary studies on the culture of dissociated cells *in vitro* have been conducted

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**Figs 1-4.** Scanning electron micrographs of dissociated Malpighian tubule cells of *Rhodnius*.

Fig. 1. A short piece of upper tubule, consisting of two cells. Removal of the basal lamella reveals transverse infoldings of the plasma membrane on the basal (b) surface of the cell. Microvilli are seen on the apical surface (a), facing the lumen (l). ×1040.

Fig. 2. A single cell from the upper tubule. Microvilli are found on the apical side (a) of the cell. A smooth intercellular region (i) is observed between the apical and basal membrane surfaces. ×1040.

Fig. 3. Two adjacent lower tubule cells. The basal surface (b) has a characteristic rough appearance. The apical surface of the cell (a) is covered with microvilli. Lumen, (l). ×1360.

Fig. 4. A single cell from the lower tubule. A smooth intercellular region (i) lies between the apical (a) and basal (b) surfaces. ×1360.
Figs 5–7. Scanning electron micrographs of elastase-treated Malpighian tubules of *Aedes*.

Fig. 5. A single primary cell. The basal surface (b) is covered with large protrusions. Microvilli are found on the apical surface (a). ×990.

Fig. 6. A higher magnification of the cell shown in Fig. 5. A ridge borders the intercellular region (i) between the apical (a) and basal (b) surfaces. ×4140.

Fig. 7. An intact tubule, following removal of the basal lamella. A single stellate cell (s) lies between larger primary cells (p). ×820.

Fig. 8. Light micrograph of a single, isolated stellate cell of *Aedes*. ×1640.
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using Malpighian tubule cells from *Rhodnius*. Dissociated cells adhere weakly to glass, plastic, and surface-modified plastic (Primaria dishes). These cells remain viable in Schneider’s *Drosophila* medium for 7 days at which time our experiments were terminated. After 1–2 days in culture both upper tubule and lower tubule cells develop large blebs. The cells did not divide or become fully flattened over the time-course of our experiments.

**Discussion**

Identifiable, isolated cells can be obtained from Malpighian tubules with heterogeneous as well as homogeneous cellular distributions. Immediately after dissociation, the isolated cells retain identifiable plasma membrane domains including microvilli, intercellular membrane and basal membrane regions. Upon prolonged tissue culture (days) under non-confluent conditions the cells lose a substantial portion of their epithelial polarity, showing instead a spread morphology with large blebs.

The apical surface of all dissociated cells examined in the SEM is covered with microvilli. In intact upper tubules of *Rhodnius* the microvilli are arranged in clumps of closely aligned microvilli (Bradley, 1983). Occasionally, the microvilli on cells of dissociated upper tubules retain this clumped configuration. Usually, however, microvilli in dissociated cells are free-standing. The disappearance of the clumping pattern may be due to mechanical disruption during dissociation or to the removal of microvillar surface components by elastase treatment. Alternatively, changes in microvillar configuration may be spontaneously initiated by the cells following removal of the basal lamella as a preliminary event in the cell dedifferentiation observed at later stages.

The basal surface of cells composing a Malpighian tubule is overlaid with a relatively amorphous basal lamella. As a result, the basal surface of the tubule is smooth when viewed in the SEM. Treatment with elastase removes the basal lamella and reveals features of the plasma membrane of the basal surface.

The isolated cells of the upper tubule retain a pattern of basal folds very similar to that observed in intact tubules using transmission electron microscopy of thin sections (Wigglesworth & Salpeter, 1962). The basal folds on elastase-treated cell of the lower tubule are less regularly arranged (compare Figs 1 and 3). Based on transmission electron microscopy (TEM) of intact tubules (Bradley, unpublished observations) the basal plasma membrane of the lower tubule *in vivo* seems to have transverse folds resembling those of the upper tubule. The disorganization of the pattern of folding is probably a result of elastase treatment.

Both the primary and stellate cells of *Aedes* show non-folded basal surfaces following elastase treatment. TEM of intact tubules reveals that both these cell types have highly folded basal plasma membranes in untreated tubules (Bradley, Stuart & Satir, 1982). It would seem, therefore, that elastase treatment leads to a disorganization of the basal pattern of folding in these cells as well.

We believe that the disorganization of the basal pattern of folding upon elastase treatment of the lower tubule cells of *Rhodnius* and the cells of *Aedes* is a specific result
of the removal of the basal lamella. The lamella clearly serves to strengthen the tubule mechanically, as evidenced by the ease with which the cells can be dissociated upon its removal. The basal lamella may also serve as a structural template that maintains the integrity of the basal infoldings. The basal folds in insect Malpighian tubules have been shown to contain a highly differentiated cytoskeleton containing microfilaments, microtubules and hemidesmosomes (reviewed by Bradley, 1984). The retention of folds for a short period of time in the upper tubule of *Rhodnius* following removal of the basal lamella, may reflect a less-labile arrangement of the cytoskeletal elements found in the folds.

Dissociation of Malpighian tubules permits a unique view of the cells in the scanning electron microscope. Of particular interest is the area of the plasma membrane that was presumably adjacent to neighbouring cells before dissociation. On the basis of transmission electron micrographs it appears that this region is differentiated apically into smooth septate junction and basally into membrane resembling the basal folds (Wigglesworth & Salpeter, 1962; Bradley *et al.* 1982; Bradley, 1983). In the SEM, this region appears uniformly smooth. Structures associated with septate junctions may have been digested by the elastase used in dissociating the tissue. Alternatively, the structures may simply be smaller than the resolution of conventional scanning electron microscopy.

Cells from *Rhodnius* Malpighian tubules dissociated by our technique were viable in culture for several days at which time our preliminary experiments were terminated. Both upper tubule and lower tubule cells adhere weakly to the substrate. The cells behave identically on glass, plastic and Primaria dishes. After 1–2 days in culture a single, large bleb forms on each cell. We believe that this bleb is the result of a reorganization of the basal plasma membrane. Basal infoldings are probably unfolded, producing a great expansion of the surface membrane. The cells do not fully spread or divide. Future modifications to the culture medium or to the substrate on which cells are plated may enhance the culture capabilities of these cells.

At present the precise physiological role of each cell type, in heterogeneous Malpighian tubules such as those of *Aedes*, cannot be determined. Isolation and separate culture of primary and stellate cells may prove useful in differentiating the physiological and biochemical characteristics of these cell types.

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REFERENCES


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