ABSENCE OF DILATED LATERAL INTERCELLULAR SPACES IN FLUID-TRANSPORTING FROG GALLBLADDER EPITHELIUM

Direct Microscopy Observations

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INTRODUCTION

Isosmotic transport of solutes and water from the mucosal to the serosal side in the absence of external physicochemical gradients is a characteristic feature of single-layered epithelia such as renal proximal tubule, small intestine, and gallbladder. In recent years the idea has developed that water transport by these epithelia is a secondary result of active solute (Na^+ or $NaCl$) transport (1–3). Coupling between salt and water transfer is held to occur in the epithelial layer in a separate compartment where the accumulation of solute establishes an osmotic gradient responsible for water movement. This scheme forms the basis of the three-compartment theory (4, 5), the local osmosis theory (6), and the standing gradient model (7). Correlated studies of function and micromorphology in gallbladder, proximal tubule, and small intestine have revealed that the lateral intercellular spaces between neighboring mucosal cells are dilated when the cell layers are functioning, but are occluded when fluid transport is inhibited (8–12). These morphological findings have been claimed as proof of the existence of an epithelial compartment in which the coupling between solute and water fluxes takes place. In these investigations, the morphology of the epithelia was studied in sections after the tissues had been fixed, dehydrated, and embedded. This raises the question of whether the preparative procedures interfere with the geometry of the epithelial tissues.

To answer this question we have studied the morphology of the frog gallbladder epithelium by direct observation of the living tissue and followed directly the effects upon the tissue of each step in the conventional electron microscopy preparative technique. The frog gallbladder was chosen because of its small wall thickness (0.2 mm), which offers the possibility of direct microscopy observation. In addition, the ability of frog gallbladders to generate transepithelial fluid transfer was studied in vitro.

MATERIALS AND METHODS

Frogs (*Rana esculenta*) of either sex, weighing about 100 g, were decapitated and pithed. The gallbladders were dissected free and cannulated by a method modified slightly from that described earlier in studies on fish (13) and rabbit (14) gallbladders. The sac preparation was incubated at room temperature (22–24°C) with identical Ringer's solution on both sides. The solution had the following composition: 112.5 mM NaCl, 2.5 mM $NaHCO_3$, 2.5 mM KCl, 1.0 mM $CaCl_2$, and 10.0 mM glucose, and was continuously stirred and oxygenated on the serosal side of the tissue. The rate of fluid transport was followed by weighing the Ringer's-filled sac preparation at time-intervals (14). By this procedure it was found that gallbladders were able to transfer fluid from the mucosal to the serosal side at constant rates for at least 4 h. The average rate for four gallbladders was 11 mg · h^{-1} · cm^{-2}.

For direct observation of the living epithelium, pieces of tissue (about 2–3 mm^2) from three gallbladders were placed with the mucosal side upwards in a Poyton and Branton microperfusion chamber (15) (chamber volume used, 0.3 ml; chamber depth, 400–500 μm; gallbladder wall thickness, about 200 μm). The chamber was perfused with Ringer's solution at room temperature at a rate of 0.5 ml/min. This stream of fluid did not cause the piece of gallbladder to move since a corner of the flat tissue specimen was bent over to achieve a slight contact with the top and bottom glass surfaces. This double layer of the tissue was not used for investigation. Microscopy was performed with a Carl Zeiss photomicroscope with Zeiss/Nomarski differential interference equipment for transmitted light (Carl Zeiss, Inc., N.Y.) (16). An oil immersion objective planchromat, 100/1.25 and an achromatic-aplanatic interference contrast condensor (numerical aperture 1.4) were used. The morphology of the mucosal epithelium was recorded by photomicrographs. The magnification was determined by photographing an object micrometer. After 1 h of Ringer's perfusion, the specimen was fixed for 1 h by perfusing the chamber with cold 1% OsO_4 in 0.15 M Sorensen phosphate buffer (pH 7.4, osmolality 300–310 mosmol). Photomicrographs were taken at the end of this period. Dehydration was performed by perfusion with increasing concentrations of alcohol (100% was achieved after 15 min). Photomicrographs were taken of the fully dehydrated specimen. The tissue was then removed from the

chamber, placed in propylene oxide, and infiltrated with Epon (17). The specimen was bisected. One-half was flat embedded and polymerized by heat (60°C for 2 days) (17). Nomarski transmitted-light microscopy of this nonsectioned preparation of gallbladder ("whole mount") was performed. The other Epon-infiltrated half was polymerized and 1-μm sections were cut parallel to the mucosal surface and photographed. Numbers of cells per 2920 μm^2 were counted in all micrographs.

Photomicrographs of 1-μm sections were also obtained from two gallbladders fixed directly after removal from the animals and from three of the four gallbladders used for the fluid transport experiments. These bladders were OsO$_4$ fixed, alcohol dehydrated, and Epon embedded for the same periods of time as stated above. Electron micrographs were routinely taken of all gallbladder preparations.

To minimize methodological errors, all quantitative data were obtained from light photomicrographs taken at the cell nuclear level.

RESULTS AND DISCUSSION

The results of these histological observations clearly demonstrated two phenomena: (a) during continuous direct observation of the mucosa with the Nomarski technique, a considerable shrinkage of the whole mucosal surface area and of the individual epithelial cells was observed. This was revealed by an increase in the number of cells per unit mucosal area from the Ringer's-incubated state to the Epon-embedded state (Table I). From the values in Table I it was calculated that the number of square micrometers per cell in the mucosal epithelial layer decreased by an average of 34% from 70 μm^2 to 46 μm^2. Note that the last

value includes the cell plus the lateral intercellular space belonging to it, (see below). Further, Table I demonstrates that the step involving Epon embedding produced the greatest relative shrinkage. To ensure that the degree of shrinkage was not a result of special conditions during Ringer's incubation in this type of experiment, the number of cells per 2,920-μm^2 mucosal surface in tangential 1-μm sections in the present experimental series was compared with the corresponding value from three of the gallbladders used for studying fluid transport rate. The average numbers of cells per 2,920 μm^2 were 63 and 61, respectively. Thus, there was no difference between the two groups.

(b) Direct observations by the Nomarski technique of the mucosal cell layer in Ringer's-incubated gallbladders (Fig. 1 a) showed no lateral intercellular spaces between the cells at any level between the apical junctional complex and the cellular base. This applied to all three gallbladders studied. By continuous direct observation during the steps of histological procedure the following were observed: while causing visible darkening of the tissue, osmium fixation did not significantly alter the geometry of the mucosal cell layer (Fig. 1 b). Alcohol dehydration, but particularly the Epon embedding, resulted in the production of clearly visible intercellular spaces (Fig. 1 c, and d). The width of these spaces measured at the nuclear level varied generally between 0.5 and 3 μm, and increased toward the basal surface of the epithelium. It is noteworthy that similar intercellular spaces were seen in the 1-μm sections of gallbladders used for fluid transport measurements.

TABLE I

Number of Absorptive Epithelial Cells per 2920 μm^2 Mucosal Surface of Gallbladder

| Frog no. | In Ringer's solution | In OsO$_4$ | In alcohol | Epon embedded | | Increase in number of cells (1-μm sections compared with Ringer's incubation) |
				Whole mount (nonsectioned)	1-μm sections	
1	38.8 ± 3.1* (n = 3)‡	44.8 ± 0.9 (n = 4)	42.8 ± 3.8 (n = 4)	59.3 ± 1.3 (n = 4)	59.4 ± 2.6 (n = 7)	53%
2	43.2 ± 1.4 (n = 12)	55.0 ± 2.4 (n = 4)	55.7 ± 3.2 (n = 7)	69.6 ± 1.6 (n = 9)	67.9 ± 3.3 (n = 15)	57%
3	43.4 ± 1.8 (n = 8)	41.9 ± 1.5 (n = 9)	46.8 ± 1.7 (n = 15)	64.7 ± 2.8 (n = 7)	62.6 ± 3.0 (n = 7)	44%

* Mean ± SEM
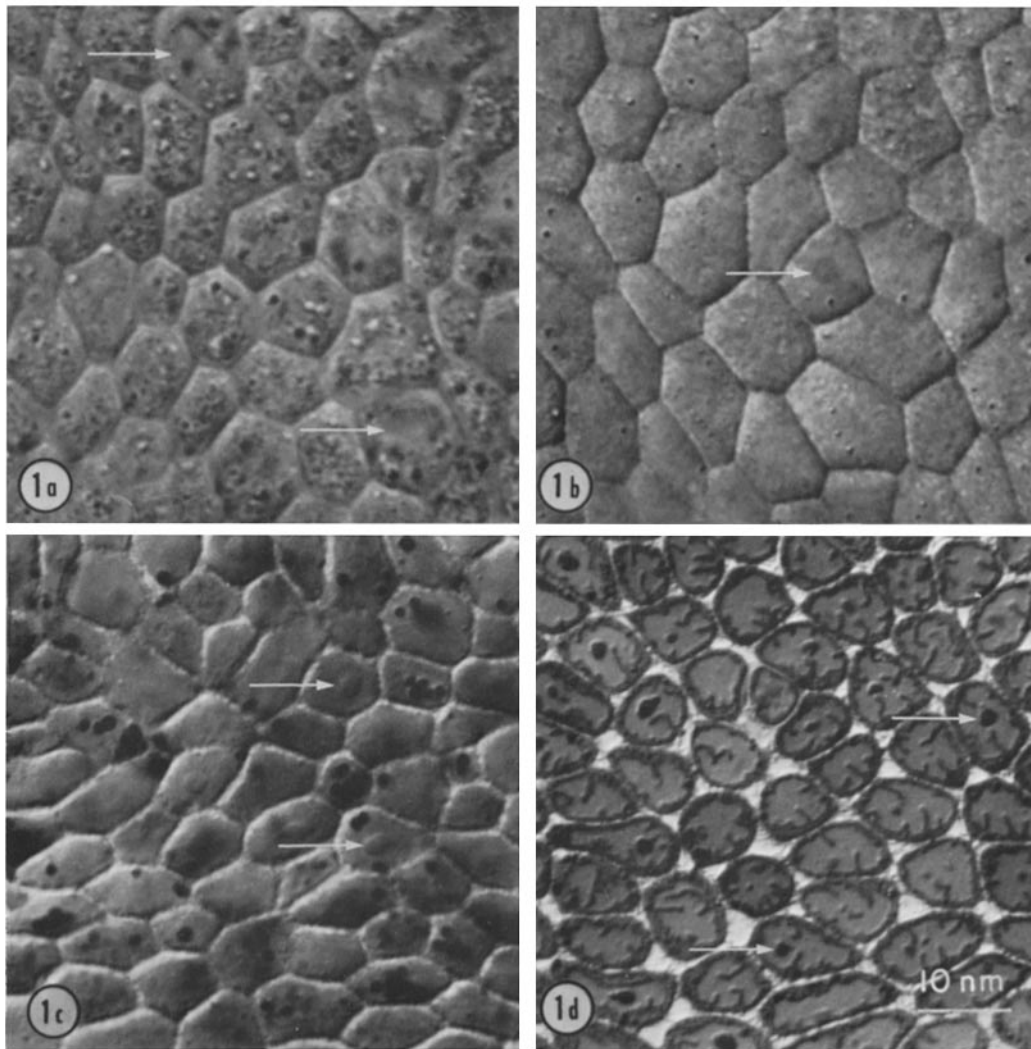‡ Number of fields (2920 μm^2) counted.

FIGURE 1 Photomicrographs of frog gallbladder epithelial cells obtained by Nomarski differential interference transmitted-light microscopy. Morphological changes occurring along the main steps of preparative procedures used in conventional electron microscopy are depicted. (A) Ringer's incubated. (B) After OsO_4 fixation. (C) Epon embedded ("whole mount"). (D) Semithick (1 μm) section. A–D were obtained from the same piece of gallbladder tissue and were all focused at the nuclear level (arrows indicate nucleoli). All photomicrographs $\times$ 1,250. Note the progressive cell shrinkage and the development of dilated intercellular spaces.

Thus, the effect of fixation, dehydration, and embedding can be described in the following way: a pronounced shrinkage of individual epithelial cells resulting in (a) an overall shrinkage of the mucosal cell layer which amounts to about a 34% decrease in mucosal area, and (b) appearance of open spaces between the cells. This indicates that individual cells shrink more than does the whole tissue.

Therefore it seems reasonable to conclude that dilated lateral intercellular spaces in frog gallbladder (and probably in other fluid-transporting epithelial tissues) can be an artificial product of a conventional preservation technique used for electron microscopy. The widened lateral intercellular spaces appear as a consequence of cell shrinkage. This observation has direct relevance to the evalua-

tion of the earlier reports of intercellular spaces in rabbit gallbladder epithelium (8, 9). The two epithelia are morphologically similar and display similar fluid transfer capacities (see reference 3 and this paper).

The fact that the functional state of the mucosal cells was not directly measured during the experiments with Nomarski microscopy cannot be raised as a serious objection to our interpretation. Gallbladders incubated in oxygenated Ringer's solution were able to maintain stable fluid absorption rates for 4 h and it seems reasonable to assume that vital function was preserved during periods of direct microscope observations in which the experimental protocol involved only 1 h of incubation in the same oxygenated Ringer's. Furthermore, electron and light microscopy of epithelial cells (not presented here) showed no obvious difference with respect to cytomorphology and lateral space formation whether gallbladders were primarily incubated for transport measurements for direct microscopic observation, or were fixed immediately after removal from the animal.

Earlier reports (18–24) have shown that other tissues exhibit whole tissue and cell shrinkage when exposed to fixation, dehydration, and embedding. Recently it has been reported that considerable cell shrinkage may occur during Epon embedding (possibly by an osmotic mechanism) (24), which is in accordance with the present observations. Furthermore, the extent of cell volume change seems to be determined by the cell type and the environmental milieu before histological preservation maneuvers (18, 25). In earlier reports on epithelial cell geometry in rabbit gallbladders (8, 9), it was stated that lateral intercellular spaces were not visible in sections from gallbladders in which fluid absorption was inhibited by various procedures (mucosal application of osmotic-active nonelectrolytes, low temperature, or addition of transport inhibitors). However, the applied experimental manipulations are all known to interfere with epithelial cell volume regulatory mechanisms (cell membrane permeability, ion pumps, and/or contractile mechanisms), and would probably also influence (diminish) the degree of preparative cell shrinkage. Preliminary results by the present authors indicate that this is the case when frog gallbladder epithelium is treated with ouabain.[1]

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[1] Rostgaard, J., and O. Frederiksen. Manuscript in preparation.

In our opinion, these facts indicate that supravital observation methods should always be included in correlated functional and morphological studies of transporting epithelia.

SUMMARY

The use of Zeiss/Nomarski differential interference equipment for transmitted-light microscopy in the study of a living epithelium is reported. The method offers the advantage of efficient optical sectioning of thick objects and absence of out-of-focus details. By use of this direct observation method it is demonstrated that visible lateral intercellular spaces between mucosal cells are not present in the living frog gallbladder. However, when the tissue is treated with the conventional preparative technique for electron microscopy, widened lateral spaces appear as a consequence of cell shrinkage.

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