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Microfilament-Rich Cells in the Toad Bladder Epithelium

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Summary. Basal cells of the bladder epithelium of *Bufo marinus* have been found heterogenous and consist of microfilament-rich cells (MFR-cell) and undifferentiated cells (Un-cell). The MFR-cell, which represents approximately 20% of the epithelial cell population, lies between the epithelial layer lining the urinary space and the basement membrane; it extends under several epithelial cells by processes of varying widths and lengths which contact, via desmosomes, other MFR-cells, as well as cells in the superficial layer, i.e., granular and mitochondria-rich cells. The cytoplasm of MFR-cell is filled with intermediate filaments arranged in bundles which run parallel to the plane of the epithelium and no dense granules, typical of granular cells, have been detected. Strong immunofluorescence for actin is associated with cells which occupy the same basal position as MFR-cells. Undifferentiated cells have no contact via desmosomes with adjacent cells and their cytoplasm is filled with free ribosomes; they lack bundles of intermediate filaments and possess no specialized organelles.

After a 4-hr pulse of ^{3}H-thymidine, 1.5% of epithelial cells incorporate thymidine into nuclear DNA, out of which $^3/_4$ are basally and $^1/_4$ are apically located. Identification of cell types by electron microscopy reveals that ~10% of undifferentiated basal cells are labeled, whereas less than 0.1% of granular cells and no MFR-cells incorporate ^{3}H-thymidine into DNA. When dissociated from the epithelium and separated by isopycnic centrifugation, MFR-cells possess a mean buoyant density of approximately 1.025, cosediment with mitochondria-rich cells and exhibit a strong immunofluorescence for actin. The function of MFR-cells remains unknown; however, they may play a role in cell coupling and responses to hormonal and physical factors.

The first histological description of the toad bladder, at the end of the last century, already indicated cell heterogeneity in the epithelium [10]. More recently, the cell composition of the epithelium was analyzed by electron microscopy [2, 13], and classically four cell types were described. The fine structure of the cells facing the urinary space, i.e.,

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mitochondria-rich, granular, and goblet cells, has been well documented. Extensive experimental work has been devoted to trying to correlate known functions (Na^+, H^+, H_2O transport) to these epithelial cells [6, 15, 17, 18, 20]. Little information is available on the underlying cells, termed basal cells. Choi described basal cells as small basophilic cells rich in intracellular filaments and located near the basement membrane [2]. He interpreted them as being young undifferentiated epithelial cells (stem cells). Later, Wade ascribed membrane specializations, i.e., desmosomes and gap junctions, to basal cells [21].

In the course of our study on dispersed toad bladder epithelial cells, we observed heterogeneity in basal cells, both in fine structure and in physical properties when following dissociation.

This paper describes in more detail the fine structure and some properties of basal cells which can be divided into microfilament-rich cells and undifferentiated cells. The role of MFR-cells as a potential target for tissue responses to hormonal, metabolic, and physical factors is discussed.

Materials and Methods

Toads

Adult male and female *Bufo marinus* (from Columbia) were killed by double pithing and perfused through a heart puncture with oxygenated Krebs Bicarbonate Ringer (KRB) medium.

Reagents

[6-^{3}H] thymidine (9.21 Ci/mmol) was obtained from New England Nuclear, Boston, Mass., and Ilford L-4 emulsion from Ilford Ltd., Ilford, Essex, England. All other chemicals were reagent grade. Sheep $F(ab')_2$ anti-rabbit F $(ab')_2$ coupled to fluorescein isothiocyanate (FITC) was prepared according to published procedures [8]. Rabbit serum directed against chicken G actin and anti-chicken G actin IgG, purified on an actin immunoadsorbent, were gifts from Dr. B. Jockusch, European Molecular Biology Laboratory, Heidelberg [5].

Dissociation Protocol and Isopycnic Centrifugation

The bladder epithelial cells were dispersed by collagenase digestion, divalent cation chelation, and mechanical disruption as modified from Kraehenbuhl [7]. The detailed procedure of dissociation and separation and the recoveries are reported in the accompanying paper [14].

Morphological Methods

Pieces of intact bladder were fixed for 2 hr at room temperature in 2% formaldehyde-2% glutaraldehyde buffered with 0.1 n Na cacodylate, pH 7.4. Some bladders were stretched and immediately fixed. Dissociated cells were fixed by mixing 1 vol of cell suspension with 2 ml of the above fixative. Tissue blocks and cells were postfixed in 1% OsO_4, stained in block with 0.5% uranyl acetate, dehydrated in ethanol and propylene oxide, and embedded in Epon.

[^3H]-Thymidine Incorporation

Hemibladders were mounted as sacs on a glass canula and filled with 5 ml of KRB medium containing 1 μCi/ml [^{3}H]-thymidine (10^{-4} mM) and incubated for 4 hr in 100 ml of KRB medium at 25 °C. The tissue was then processed for light and EM autoradiography using Ilford L_4 emulsion.

Immunofluorescence

Frozen sections (5 μm) were prepared from unfixed stretched bladders and collected on coverslips. Unfixed, dispersed cells (2.5×10^4) were cytocentrifuged at $100 \times g$ for 18 min on microscope slides. Sections and cells were fixed in ethanol-ether (1:1, vol/vol) for 5 min, rehydrated in phosphate saline buffer and exposed to immunological reagents, by incubation for 30 min with rabbit antiserum directed against chicken G actin (diluted 1:10) or anti-chicken G actin IgG (0.4 mg/ml). The sections and cells were rinsed, reacted for 30 min with sheep $F(ab')_2$ fragments directed against rabbit $F(ab')_2$ and coupled to FITC (0.4 ml/ml; F/P: 1,3), rinsed and mounted in buffered glycerin pH 9.0. The following controls were done: (i) to examine autofluorescence, fixed but unstained sections or cells were observed; (ii) to test for the presence of nonspecific staining, sections or cells were incubated with nonimmune rabbit serum; and (iii) for immunological specificity, the anti-actin reagents were absorbed with chicken G actin [5]. The sections and cells were examined in a Zeiss photomicroscope II with a fluorescence attachment, equipped with an Osram HBO 50-watt high pressure mercury vapor light source, BG 12 excitation filter and BG 38 suppression filter.

Results

Light and Electron Microscopy

Cells usually described as basal cells which underlie the epithelial sheet facing the urinary space and are adjacent to the basement membrane, are heterogeneous in morphology and constitute two cell types: (i) microfilament-rich cells (MFR-cell), and (ii) undifferentiated cells (Un-cells).

MFR-cells represent $20 \pm 3\%$ ($n = 10$) of the epithelial cell population (Table 3 in ref. [14]), and extend under several granular and mitochon-

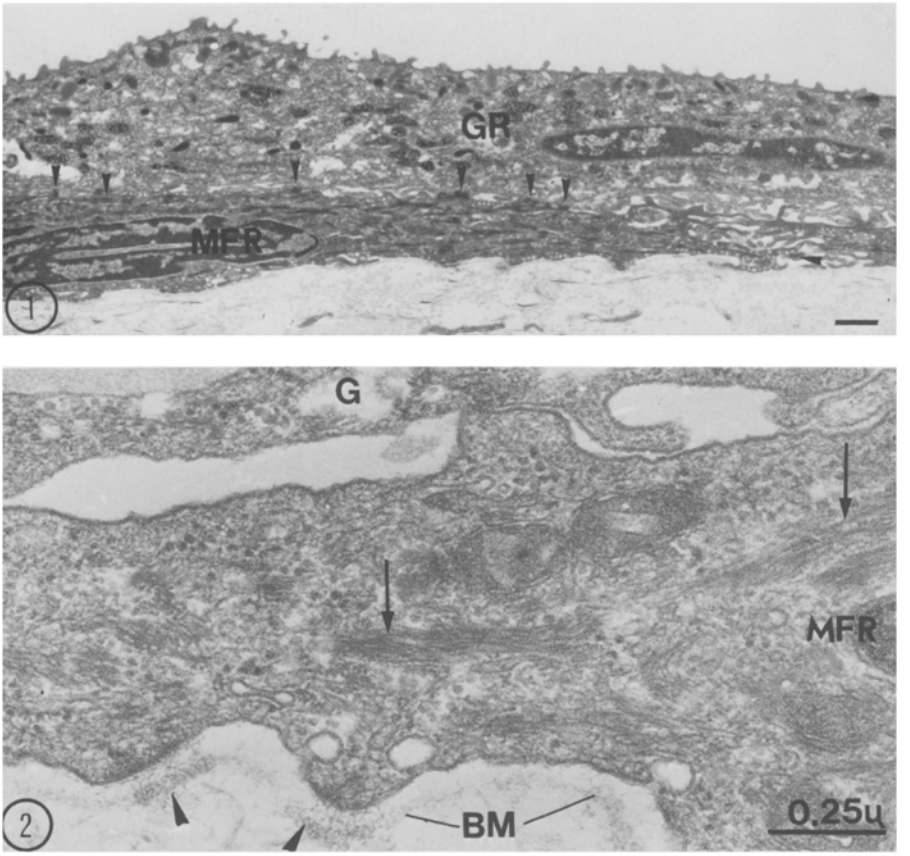


Fig. 1. Micrograph of a thin section of extended toad bladder. A microfilament-rich cell (*MFR*) extends under a granular cell (*GR*) which lines the urinary lumen. Several desmosomes between the two cells are indicated by arrowheads. Bundles of microfilaments run parallel to the plane of the epithelium. Numerous vesicles are seen in close contact with the plasma membrane. 7,100 ×

Fig. 2. Cytoplasmic process of microfilament-rich cell (*MFR*). Arrows indicate intermediate filaments (10 nm). Caveolae and hemidesmosomes (arrowheads) are associated with the plasma membrane facing the basement membrane (*BM*). 73,500 ×

dria-rich cells by processes which may reach 20 μm (Fig. 1). Microvillous-like extensions project from their surface and contact, via desmosomes, other MFR-cells as well as granular and mitochondria-rich cells (Fig. 1). Hemidesmosomes between MFR-cells and the basement membrane are abundant (Fig. 2). The nucleus is flat in stretched preparations and contorted with deep indentations in relaxed bladders. The cytoplasm never contains dense granules which occur in granular cells, but is filled with numerous microfilaments arranged in bundles which run parallel

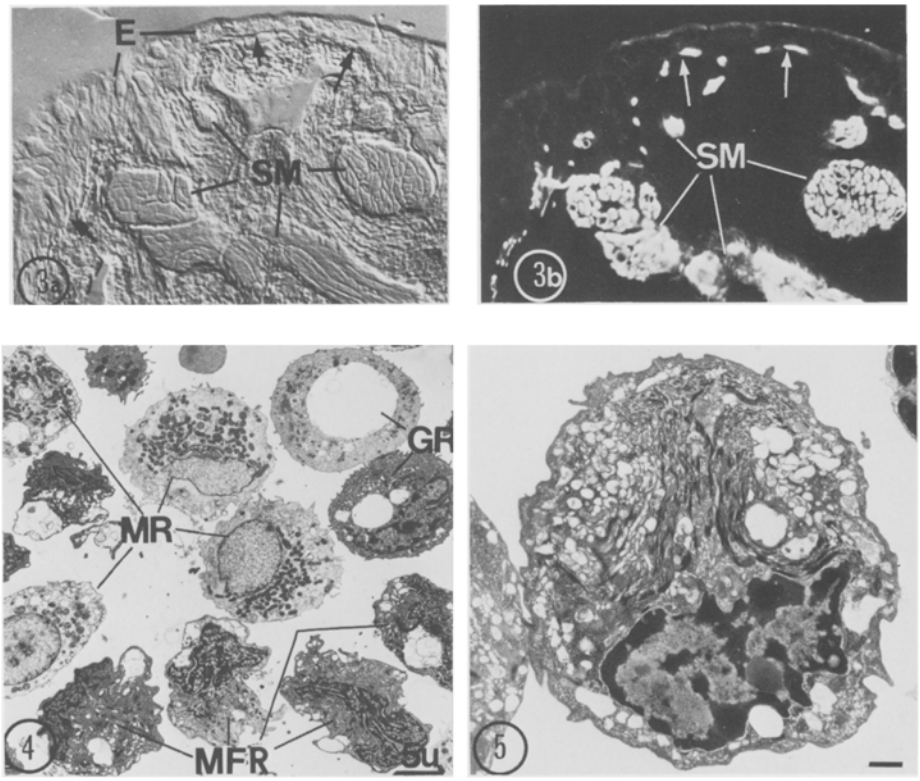


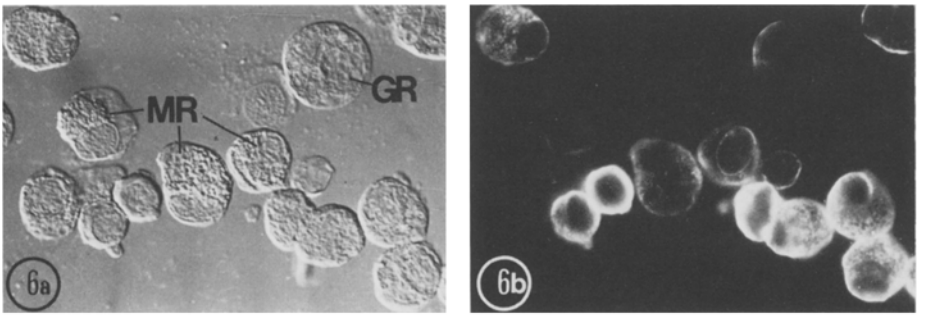
Fig. 3. Frozen section of extended bladder. (*a*): Interference contrast micrograph indicating the epithelium (*E*) lining the urinary space. The junction between the epithelium and the submucosa is indicated by arrows. Smooth muscle bundles (*SM*) are located in the submucosa. (*b*): Same section stained with anti-actin serum (diluted 1:10) (*see Methods*). Bundles of smooth muscles are intensively stained. Positive individual cells (arrows) occupy a basal position in the epithelium. Immunofluorescence is also associated with the apical region of the epithelium

Fig. 4. Dispersed epithelial cells from the lightest cell fraction of BSA density gradient (1.025). Four mitochondria-rich cells (*MR*), 2 vacuolated granular cells (*GR*) and 4 microfilament-rich cells (*MFR*) are seen. 1,600 ×

Fig. 5. A typical microfilament-rich cell with microfilaments arranged in bundles which run from the nucleus to one pole of the cell

to the plane of the epithelium (Fig. 1). The diameter of these filaments is 10 nm, making them intermediate types (Fig. 2). Numerous caveolae and vesicles are lined up at the cytoplasmic face of the plasma membrane (Fig. 1 and 2).

Undifferentiated cells, which represent 10% ± 3 of the epithelial cells, are smaller cells. The nucleus is large, usually not contorted, and the



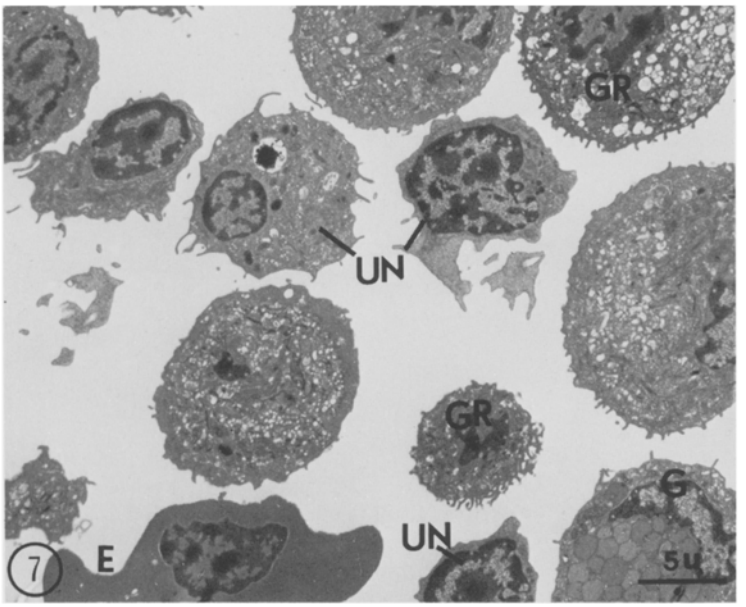


Fig. 6. Dispersed epithelial cells from the lightest cell fraction of BSA density gradient. (*a*): Interference contrast micrograph showing 3 mitochondria-rich cells (*MR*), 1 granular cell (*GR*) and smaller cells which cannot be identified. (*b*): Staining of the same cells with anti-actin serum (diluted 1:10). MR cells are weakly labeled. The granular cell exhibits immunofluorescence restricted to one pole of the cells. The other cells are strongly labeled with immunofluorescence concentrated at the periphery of the cells

Fig. 7. Electron micrograph of a section through cells from the pellet from BSA density gradient. Three undifferentiated cells (*UN*) are identified by the absence of specialized organelles present in other cell types, such as granular cells (*GR*) or goblet cells (*G*). Nucleated erythrocytes (*E*) are also present in the pellet fraction. 2,500 ×

cytoplasm contains numerous free ribosomes, a poorly developed secretory apparatus, and few mitochondria (Fig. 8). In contrast to MFR-cells, intermediate filaments, caveolae and vesicles are absent in these cells.

Moreover, neither desmosomes nor hemidesmosomes are associated with undifferentiated cells which are usually surrounded by cytoplasmic processes of MFR-cells.

Dissociation of the Bladder Epithelium and Separation of Basal Cells

After dissociation and isopycnic centrifugation on a continuous BSA gradient, MFR cells were enriched in the lightest fraction with a buoyant density of 1.020 to 1.030. The fraction which accounted for 0.5% of the cells recovered from the gradient contained 50% MFR-cells, 30% mitochondria-rich cells, and 20% vacuolated granular cells (Figs. 4 and 5). Undifferentiated cells were recovered mainly in the pellet (Fig. 7). As shown in Table 3 of ref. [14], selective loss of MFR-cells occurred after isopycnic centrifugation.

Thymidine Incorporation

Hemibladders incubated 4 hr with 1 μCi/ml of ^{3}H-thymidine exhibited a low labeling index: 1.5%. Labeled cells were identified by electron microscopy. A total of 50 labeled cells were examined. Three quarters of labeled cells were undifferentiated cells (Fig. 8), which represents ~10% of the total undifferentiated cell population. One quarter of labeled cells were granular cells (Fig. 9), which represents less than 0.1% of the total granular cell population. More than 500 MFR-cells were examined and no labeled cells were detected.

Immunofluorescence

The presence of caveolae and contorted nuclei suggests that MFR-cells are contractile cells. To detect the presence of actin in MFR-cells, sections of intact tissue and dispersed cells (lightest fraction) were incubated with anti-actin reagents. In intact tissue, smooth muscle cells present in the submucosa were intensively stained and served as an internal control; individual cells, underlying the epithelial monolayer, were also strongly labeled (Fig. 3*a* and *b*). The number of stained cells in the basal portion of the epithelium paralleled closely the number of MFR-cells identified by electron microscopy. Immunofluorescence was also

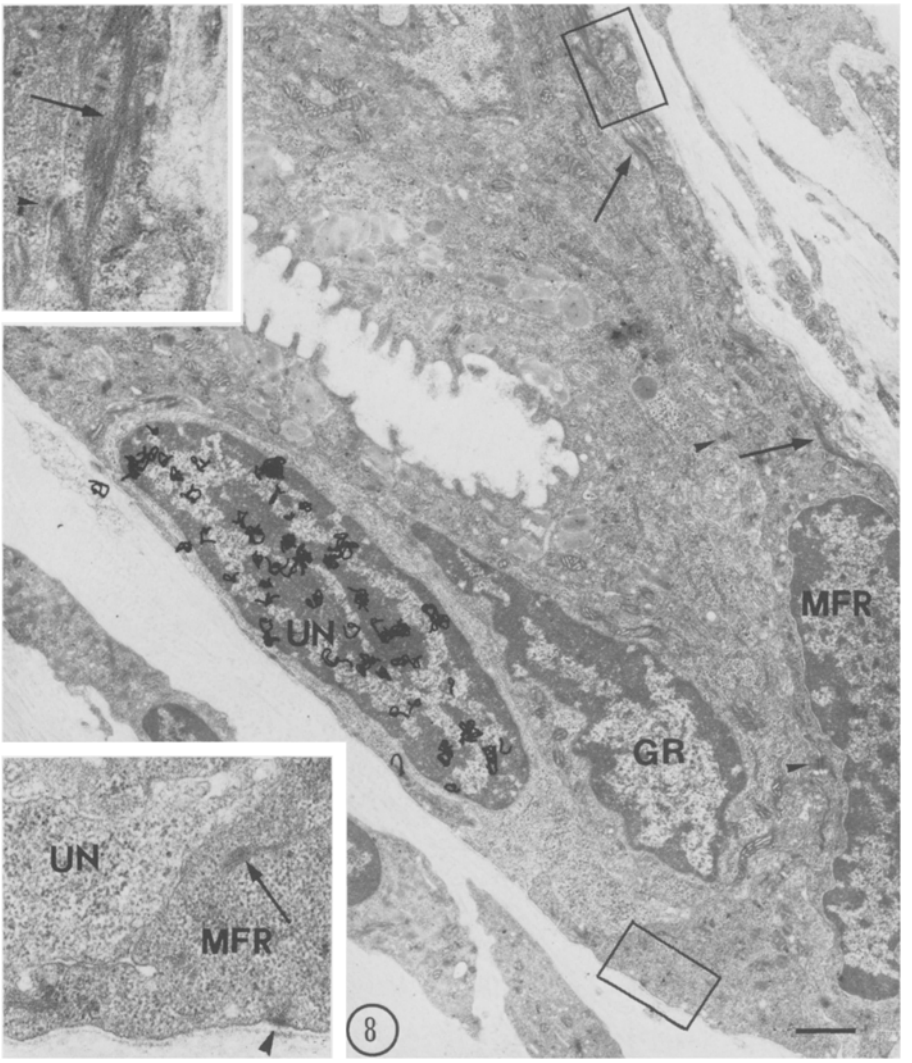


Fig. 8. Hemibladders incubated 4 hr with ^{3}H-thymidine and processed for EM autoradiography (*see Methods*). Grains are associated with the nucleus of an undifferentiated cell (*UN*), separated from the lumen by a granular cell (*GR*), surrounded by cytoplasmic processes of MFR-cells, and characterized by a flat nucleus, abundant free ribosomes, but no microfilaments, desmosomes and caveolae. 8,500×. *Upper inset:* high magnification of indicated area indicating a cytoplasmic process of a microfilament-rich cell (*MFR*) with bundles of microfilaments (arrows) and a desmosome (arrowhead). 27,500×. *Lower inset:* junction between an undifferentiated cell and a *MFR*-cell, containing microfilaments (arrow) and a hemidesmosomes (arrowhead). 27,500×

associated with the continuous epithelial monolayer, in which a narrow rim of fluorescence was outlining the apical region. In the very light cell fraction, approximately 50% of the total cells exhibited strong immu-

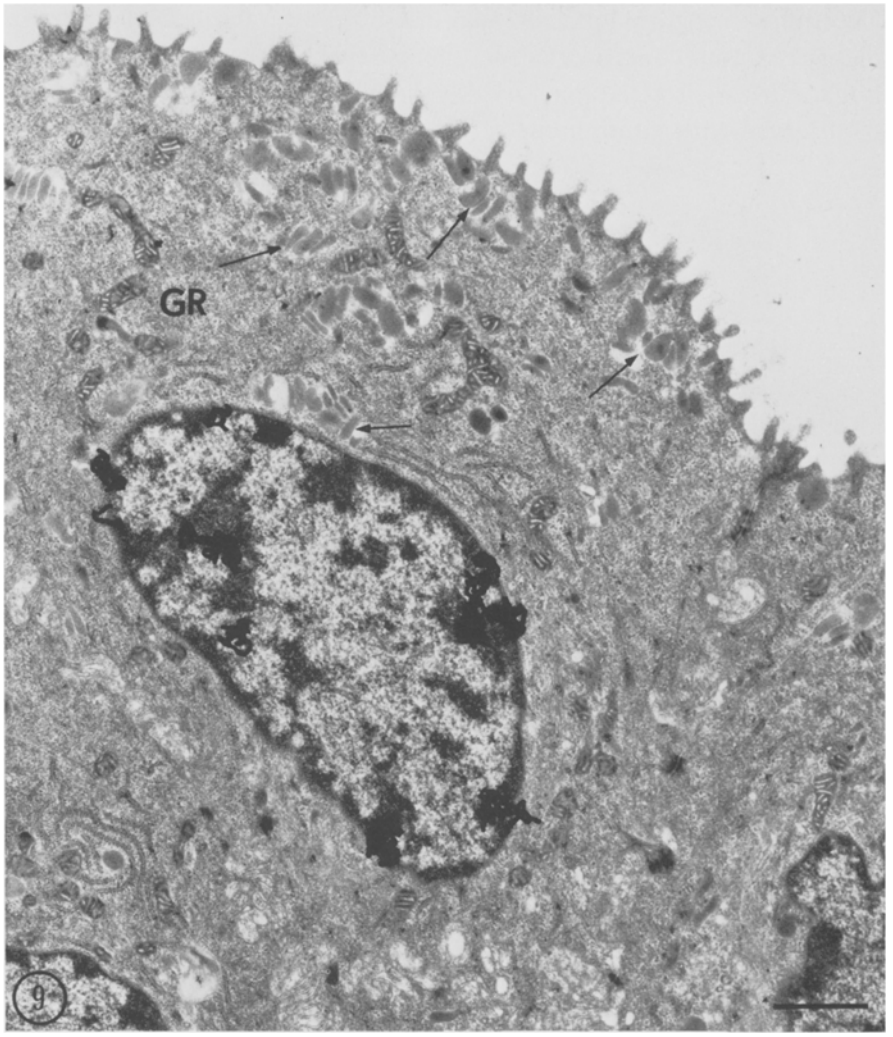


Fig. 9. Hemibladders incubated 4 hr with ^{3}H-thymidine and processed for EM autoradiography. Grains are associated with the nucleus of a differentiated granular cell (*GR*) rich in dense granules (arrows). 14,000 ×

nofluorescence frequently restricted to the periphery of cells. Immunofluorescence was punctate and no filamentous structures could be identified (Fig. 6*a* and *b*). In the light fraction, ~10% of the cells were intensely stained, whereas in the heavy fraction and the pellet less than 5% of the cells exhibited strong fluorescence. Thus, there was a strict parallelism between number of MFR-cells in each fraction and cells exhibiting a strong immunofluorescence for actin.

Within some granular cells, identified by the presence of large vacuoles, actin was concentrated at one pole of the cell (Fig. 6*a* and *b*). Mitochondria-rich cells were weakly labeled. No immunofluorescence was observed with nonimmune serum, or serum adsorbed with G actin.

Discussion

Heterogeneity of Basal Cells

The fine structure of the toad bladder epithelium was first analyzed by Choi [2] at the electron microscope. In its classical description, he described basal cells as small basophilic cells, basally located with numerous intracellular filaments. Based on these morphological criteria, he interpreted them as young undifferentiated epithelial cells. In the present study, the cell composition of the bladder epithelium was analyzed in seven different toads [14], giving an estimate of cell type variability in *Bufo marinus*. The cell distribution in the superficial layer was similar to that reported by others [2, 13], but heterogeneity of basal cells was observed. The basal cell described by Choi [2], which we term microfilament-rich cell, makes up approximately 20% of the epithelial cell population, whereas ~10% are represented by undifferentiated cells.

MFR-cells extend under several cells of the superficial monolayer, have no contact with the urinary lumen, and are adjacent to the basement membrane. MFR-cells are distinct from undifferentiated cells as reflected by the presence of (i) intermediate filaments (10 nm) which run in bundles from one extremity of the cell to the other, (ii) desmosomes between MFR-cells and granular and mitochondria-rich cells, (iii) caveolae and vesicles situated against the plasma membrane, and (iv) actin associated with the periphery of the cell. None of these features is shared by undifferentiated cells, characterized by a large nucleus, numerous free ribosomes, a poorly developed secretory apparatus and no plasma membrane specializations.

MFR-cells share several morphological features with smooth muscle cells, as well as myoepithelial cells from mammary, salivary, or sweat glands. In contrast to these cells, MFR-cells contain a majority of intermediate filaments (10 nm), which recently have been identified in epithelial cells, as prekeratin filaments [4]. In addition, large amounts of actin have been detected in the periphery of MFR-cells. The presence of con-

tractile elements and caveolae, together with membrane specializations, and intermediate filaments are strongly suggestive of differentiated epithelial cells, thus justifying the term of MFR-cell instead of young undifferentiated epithelial cells. This is further supported by pulse experiments using tritiated thymidine. MFR-cells do not incorporate thymidine into nuclear DNA, whereas some granular cells, considered as differentiated cells, are labeled. In contrast, 10% of undifferentiated basal cells incorporate thymidine, suggesting that they might be stem cells for the epithelium. However, no report in the literature nor our preliminary study allow us to conclude that these undifferentiated cells are stem cells. The possibility exists that some of these cells are lymphoid cells as described in the turtle urinary bladder [9]. To definitely distinguish true stem cells from lymphoid cells, additional experiments will be required using markers for T and B lymphocytes of *Bufo marinus* together with incorporation of thymidine in a pulse-chase format.

Possible Role of MFR-Cells as a Differentiated Epithelial Cell

The function of MFR-cells in the toad bladder remains unknown, but its distinct morphological features as well as experimental results described by others suggest that the cell may play a role in the following functions.

Cell coupling. The toad bladder was one of the first epithelia in which cell to cell ionic coupling was demonstrated [11]. As described recently by Wade [21], gap junctions, structures which have been clearly associated with cell coupling [12], occur only between basal cells or basal cells and granular cells, but not between granular cells or granular and mitochondria-rich cells. Wade identified basal cells by the presence of membrane specializations described as desmosomes and hemidesmosomes. In the present report, desmosomes and hemidesmosomes were associated with MFR-cells, but never with Un-cells. Since MFR-cells extend under several granular and mitochondria-rich cells and establish gap junctions with these cells, they may play a role in coordinating sodium and water transport activities in cells lining the urinary space.

Response to stretch. Walser *et al.* [22] described the effect of stretch in the toad bladder epithelium which can profoundly alter the basal rate of Na^+ transport. Increased stretch corresponded to a marked but reversible increase in net Na^+ reabsorption from the urinary space towards the capillary compartment. Walser *et al.* postulated the existence

of a biological "intrinsic" mechanism for this effect. MFR-cells could play a role in this response. The presence of intermediate filaments and (hemi)desmosomes with adjacent structures makes the MFR-cell an appropriate candidate for stretch sensing. The perturbation generated by stretch in MFR-cells could, in turn, be transferred to granular or mitochondria-rich cells through gap junctions which mediate ionic or metabolic coupling.

Control of intercellular spaces. Vasopressin increases the size of intercellular spaces of toad bladder epithelium by a mechanism independent of net transepithelial water flow as described by DiBona and Civan [3]. These authors postulated that epithelial geometry was not affected only by net water flow but also by changes in smooth muscle tone. Later, Strum and Danan [19] proposed alternative means to exert geometrical effects on the epithelium via smooth muscle cell-basal cell contacts. Our observations suggest a direct role of MFR-cells in the control of intercellular space. First, the cells are anchored by desmosomes to adjacent granular or mitochondria-rich cells and to the basement membrane by hemidesmosomes. Second, MFR-cells contain intermediate filaments and actin. We stress, however, that 7 nm filaments have not been visualized in MFR-cells. We have only a correlation between number of actin positive cells both in intact tissue or dispersed cells and numbers of MFR cells identified by electron microscopy. Studies are underway to localize actin in MFR-cells at the electron microscopic level.

Response to hormones. Recently Scott [16] described a technique for separating mitochondria-rich cells from the bulk of epithelial cells using a discontinuous Ficoll gradient. The composition of cell-enriched fractions and recoveries were not reported. Subsequently, Scott proposed that the mitochondria-rich cell was the exclusive target cell for aldosterone. According to Scott's finding, mitochondria-rich cells contained specific cytoplasmic and nuclear receptors for aldosterone, whereas the granular cells did not [15]. In addition, aldosterone induced RNA and protein synthesis only in the mitochondria-rich cells [1, 17].

In the present study, we find that MFR-cells cosediment with mitochondria-rich cells and represent 50% of the cells recovered in the lightest cell fraction ($\rho \sim 1.025$). Since both cell types have a similar buoyant density ($\rho \sim 1.025$), it is not possible to separate them by isopycnic centrifugation. Thus, the question of a unique target cell type for aldosterone is far from settled.

We realize that the various possibilities discussed above are speculative. The role of MFR-cells, however, can now be investigated since

MFR-cells have been isolated from the toad bladder and enriched by equilibrium density centrifugation. Additional separation procedures will be required to remove contaminating cells in order to analyze the effects of hormones and physical factors on purified MFR-cells.

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References

1. Brown, J.A., Jr., Scott, W.N. 1976. Aldosterone induces the synthesis of mRNA in mucosal cells of the toad's urinary bladder. *Physiologist (Abstr.)* **19**:141
2. Choi, J.K. 1963. The fine structure of the urinary bladder of the toad, *Bufo marinus*. *J. Cell Biol.* **16**:53
3. DiBona, D.R., Civan, M.M. 1972. Clarification of the intercellular space phenomenon in toad urinary bladder. *J. Membrane Biol.* **7**:267
4. Francke, W.W., Schmid, E., Osborn, M., Weber, K. 1978. Different intermediate-sized filaments distinguished by immunofluorescence microscopy. *Proc. Nat. Acad. Sci. USA* **75**:5034
5. Jockusch, B., Kellin, K.H., Meyer, R.M., Burger, M.M. 1978. An efficient method to produce specific anti-actin. *Histochemistry* **55**:177
6. Kachadorian, W.A., Wade, J.B., Discala, V.A. 1975. Vasopressin: induced structural change in toad bladder luminal membrane. *Science* **190**:67
7. Kraehenbuhl, J.P. 1977. Dispersed mammary gland epithelial cells. I. Isolation and separation procedures. *J. Cell Biol.* **72**:406
8. Kraehenbuhl, J.P., Jamieson, J.D. 1977. Enzyme-labeled antibody markers for electron microscopy. *In:* Methods in Immunolgy and Immunochemistry. C.A. Williams and M.W. Chase, editor.Vol. 5, pp. 482–495. Academic Press, New York
9. Lefèvre, M.E., Reincke, U., Arbas, R., Gennaro, J.F. 1973. Lymphoid cells in the turtle bladder. *Anat. Rec.* **176**:111
10. List, J.H. 1887. Ueber einzellige Drüsen (Becherzellen) im Blasenepithel der Amphibien. *Arch. Mikr. Anat.* **29**:147
11. Loewenstein, W.R., Socolar, S.J., Higashino, S., Kano, Y., Davidson, N. 1965. Intercellular communication: Renal, urinary, bladder, sensory and salivary gland cells. *Science* **149**:295
12. Payton, B.W., Bennet, M.V.L., Pappas, G.W. 1969. Permeability and structure of junctional membranes at an electronic synapse. *Science* **166**:1641
13. Peachey, L.D., Rasmussen, H. 1961. Structure of the toad's urinary bladder correlated to its physiology. *J. Biophys. Biochem. Cytol.* **10**:529
14. Rossier, M., Rossier, B., Pfeiffer, J., Kraehenbuhl, J.P. 1979. Isolation and separation of toad bladder epithelial cells. *J. Membrane Biol.* **48**:141
15. Sapirstein, V.S., Scott, W.N. 1975. Binding of aldosterone by mitochondria-rich cells of the toad urinary bladder. *Nature (London)* **257**:241
16. Scott, W.N., Sapirstein, V.S. 1974. Partition of tissue functions in epithelia: Localization of enzymes in "mitochondria-rich" cells of toad urinary bladder. *Science* **184**:797

17. Scott, W.N., Sapirstein, V.S. 1975. Identification of aldosterone induced proteins in the toad's urinary bladder. *Proc. Nat. Acad. Sci. USA* **72**:4056
18. Spinelli, F., Grosso, A., de Sousa, R.C. 1975. The hydroosmotic effect of vasopressin, a scanning electronmicroscope study. *J. Membrane Biol.* **23**:139
19. Strum, J.M., Danon, D. 1974. Fine structure of the urinary bladder of the bullfrog (*Rana catesbiana*) *Anat. Rec.* **178**:15
20. Voûte, C.L., Hänni, S., Ammann, E. 1972. Aldosterone induced morphological changes in amphibian epithelia *in vivo*. *J. Steroid Biochem.* **3**:161
21. Wade, J.B. 1978. Membrane structural specialization of the toad urinary bladder revealed by the freeze-fracture technique. III. Location, structure and vasopressin dependence of intramembrane particle arrays. *J. Membrane Biol. (in press)*
22. Walser, M., Butler, S.E., Hammond, V. 1969. Reversible stimulation of Na^+ transport in the toad bladder by stretch. *J. Clin. Invest.* **48**:1714