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The Volume of Mitochondria-Rich Cells of Frog Skin Epithelium

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Summary. The pathway for movement of chloride ions across frog skin is not well understood. Mitochondria-rich (MR) cells have been proposed as the route for chloride across the skin. To test this hypothesis we studied the MR cells of the skin of the frog, *Rana pipiens*, by quantitative light microscopic determination of cell volume. MR cell volume was influenced by changes in the chloride concentration or osmolality of the outside bathing solution. MR cells shrank about 23% when all chloride was removed from the outside (mucosal) bathing solution. MR cells were also shown to be responsive to changes in the osmolality of either the mucosal or serosal bath. Osmotically-induced swelling caused by dilution of the serosal bath resulted in volume regulatory decrease. These results are consistent with the hypothesis that MR cells constitute the pathway for chloride movement across frog skin.

Key Words cell volume · chloride transport · quantitative light microscopy · volume regulation

Introduction

The transepithelial movement of chloride ions across frog skin epithelium may be adequately described by the flux ratio equation for passive movements, but the pathways taken by the ions are unknown [3–7, 13]. Two pathways for Cl have been proposed: (i) a paracellular leak across the tight junctions and (ii) transcellular movement across the mitochondria-rich cells (MR) of the skin [5, 13]. A striking correlation between the number of MR cells and the magnitude of the Cl conductance across the skin was observed [3, 12]. Furthermore, it was noted that the Cl conductance of toad skin epithelium exhibited voltage-dependent gating: depolarization reduced Cl conductance, and hyperpolarization greatly increased the Cl conductance [4–7]. The Cl conductance of toad skin was found to be greatly reduced in salt-adapted animals, circumstances associated with a reduction in the number of MR cells [3].

Mitochondria-rich cells are often considered to be a minor cell type in frog skin and have been accorded little attention in the past [1, 13]. Although these cells occupy only a fraction of the volume of the epithelium, their numbers can be very great. Ehrenfeld et al. (1) determined that at room temperature the number of MR cells equaled that of the number of functional granular cells in the frog, *Rana esculenta*. Thus it seems feasible that the MR cells could constitute a pathway for transepithelial Cl movement.

It has been difficult in the past to study the function of MR cells because of their small size and distribution. In the present and previous papers [2] we utilized optical microscopic techniques to determine the volume of MR cells and to evaluate the MR cells as the possible site of Cl ions to cross the skin. Our results show that MR cells change their volume in response to alterations in the Cl concentration or osmolality of the outside (mucosal) bathing solution. This behavior is distinctly different from that of the granular cells which do not change their volume in response to alterations in the composition of the outside bath [8, 11].

Materials and Methods

Adult frogs (*Rana pipiens*) were maintained unfed, in tap water at room temperature for two to three weeks prior to the experiments. The animals were killed by decapitation and the abdominal skin removed. The inside surface of the skin was lightly scraped to remove the tela subcutanea, and the tissue was immersed in a Ringer solution containing collagenase (1 mg/ml, Worthington Biochemicals, St. Louis, MO). The tissue was allowed to incubate for 1 hr, and the epithelium was separated from the underlying connective tissue by gentle dissection. This "split skin" was mounted in a miniature Ussing chamber as a flat

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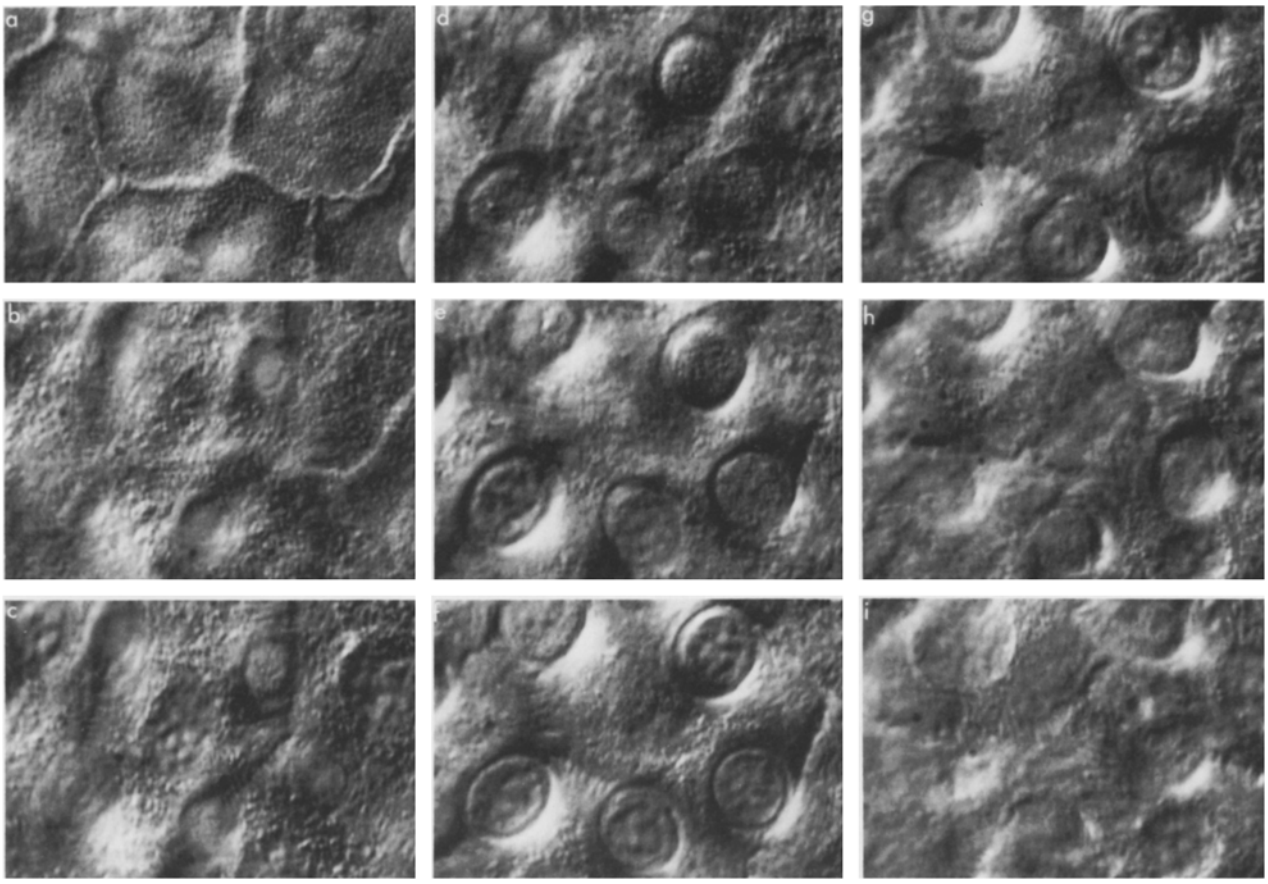

Fig. 1. Optical sections of a typical region of split frog skin epithelium. Each image (*a–i*) is taken at a plane 2.7 μm deeper than the preceding one. In section *a*, the stratum corneum cells are clearly visible. In sections *b* and *c*, the necks of five MR cells may be visualized. In *d* through *i* the sections pass through the basal portions of the MR cells and cells of the stratum spinosum. These pictures were photographed from a video monitor from records stored on video disc. The MR cells have a maximum dimension of 8–9 μm

sheet separating two flowing bathing solutions. Transepithelial electrical measurements were not made in these experiments; all preparations were studied at open circuit. The volume of the MR cells was measured by optical sectioning techniques described below. The tissue was equilibrated with control solution for 30 min prior to the experimental period.

Solutions

Control frog Ringer had the following composition in mM: NaCl 108, KCl 2, $CaCl_2$ 0.9, $NaHCO_3$ 2.5. The solution was gassed with air, had an osmolality of 194 mOsm/kg and a pH of about 8.2. This solution was diluted one-half by the addition of distilled water for experiments involving changes in the osmolality of the serosal bath. The mucosal bath was perfused with Ringer diluted 1/20 with distilled water where indicated.

Chloride-free Ringer had the following composition in mM: Na gluconate 108, K gluconate 2, Ca lactate 1, $NaHCO_3$ 2.5. This solution was also gassed with air and had a pH of about 8.2.

Microscopy

The preparation was mounted in a miniature Ussing chamber as previously described for gallbladder epithelium [9]. Both baths were perfused from reservoirs suspended above the preparation. The preparation was observed with an oil immersion objective lens (100 × 1.25 NA) on a microscope equipped with differential interference contrast optics (Diavert, E. Leitz Inc., Rockleigh, NJ). The preparation was illuminated with monochromatic light (540 nm) from a tungsten halogen lamp equipped with interference and neutral density filters (Ditric Optics, Hudson, MA). The resultant image was detected with a television camera attached to the microscope. The camera utilized a newvicon tube and electronic magnification such that the image magnification was increased 2.5 times (Model 65MKII, Dage-MTI, Michigan City, IN). The images were recorded on video disc and later analyzed by planimetry for determination of the cross-sectional area of the MR cells. Focus of the microscope was controlled by a stepping motor drive attached to a computer (Model 4051, Tektronix Inc., Beaverton, OR) as previously described [9].

MR cells were readily visualized as flask-shaped objects on the more superficial layers of the epithelium. Shown in Fig. 1 are optical sections of the typical region of frog skin as viewed on the television monitor. The images were obtained by focal displacement of 2.7 μm from the preceding plane. Such a sequence of images could be captured in about 1 to 1.5 seconds. At a later time the images were replayed and analyzed by planimetry for determination of cell volume from the area and focal displacements of each section. Figure 1 illustrates the fact the MR cells are present in great abundance in frogs stored in tap water at room temperature.

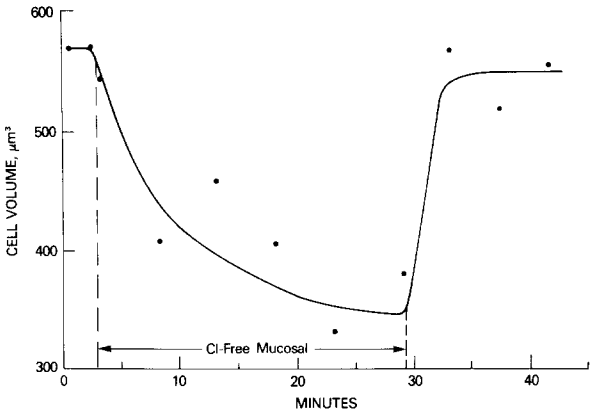

Fig. 2. MR cell volume is plotted on the ordinate as a function of time on the abscissa. After a control period in which both bathing solutions were normal frog Ringer, the mucosal bath was switched to Cl-free Ringer. All Cl was replaced by gluconate. After approximately 25 min the mucosal bath was again returned to normal Ringer at the second dashed vertical line

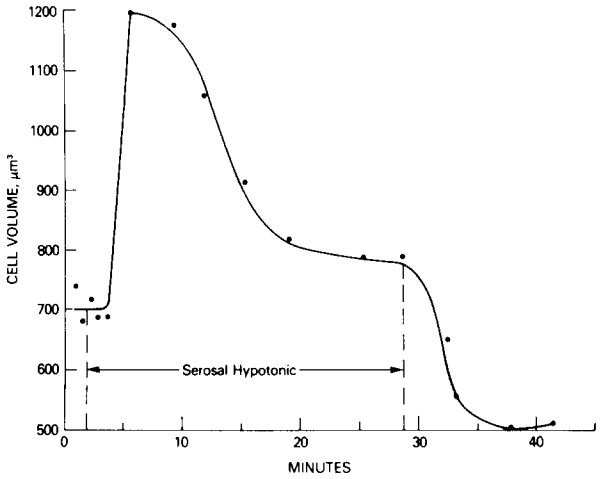

Fig. 3. MR cell volume as a function of time is shown for an experiment in which the serosal bath was diluted to one-half osmolality. After a control period, the serosal bathing solution was replaced by one that had been diluted 50% by the addition of distilled water. Cell volume increased suddenly due to osmotic water flow and then returned toward control despite the continued presence of the osmotic gradient. When the serosoal bath was returned to control osmolality the cell shrank below its control volume, indicating that the intracellular osmolality had decreased as a result of exposure to the dilute serosal bath

Statistics

All data are expressed as mean ± standard error (SE). Results were compared by the *t* test for paired and unpaired observations as indicated.

Results

Three series of experiments were undertaken to learn something about the determinants of MR cell volume and to ascertain whether MR cells were involved in the movement of Cl across frog skin. In the first series of experiments, Cl was suddenly removed from the mucosal bath and the effect on MR cell volume determined. The second series involved the effects of serosal bath hypotonicity on the volume of MR cells. The third series involved the alterations in MR cell volume produced by reduction of the osmolality of the mucosal bath.

Chloride Removal

As shown in Fig. 2, the sudden removal of all Cl from the mucosal bath, while Cl was still present in the serosal bath, caused the MR cells to shrink. Control cell volume averaged 517.8 ± 17.3 μm^3 (n = 16 cells, 12 tissues). Chloride removal from the mucosal bath caused these MR cells to shrink to 398.6 ± 19.7 μm^3 in an average time of 17 ± 1.25 min. The final cell volume was 77 ± 2.5% of control. The observed shrinkage was statistically significant at $P < 0.001$.

Shrinkage of the MR cells upon removal of Cl from the mucosal bath is consistent with the conclusion that Cl ions normally enter these cells across the apical membrane. Chloride removal could cause the loss of cell Cl across the apical membrane. On the basis of model calculations [6, 7] Cl efflux across the basolateral membrane might also be expected to continue for some time after removal of Cl from the mucosal perfusate. The loss of Cl and an accompanying cation, possibly K, would result in the observed cell shrinkage.

Serosal Bath Hypotonicity

Some insight into the regulation of MR cell volume as well as into the mechanism of Cl movement across the basolateral membrane of MR cells can be obtained by alterations in the serosal bath osmolality. A reduction of the serosal bath osmolality should produce cell swelling and dilution of intracellular KCl. If both K and Cl are distributed passively across the basolateral membrane as has been suggested by a mathematical model [6, 7], swelling the cell should not initiate a volume regulatory decrease. To prevent significant ion flow across the apical membrane during this experiment the mucosal bathing solution was 1/20 Ringer. The serosal bath was initially control Ringer, which was replaced with 1/2 Ringer.

As shown in Fig. 3, serosal hypotonicity caused initial cell swelling, which was followed by a volume decrease back to near control volume. Control cell volume was 707.2 ± 37.9 μm (n = 10 cells, 6 tissues). When the serosal osmolality was reduced by 50% the cells swelled significantly ($P < 0.001$) to

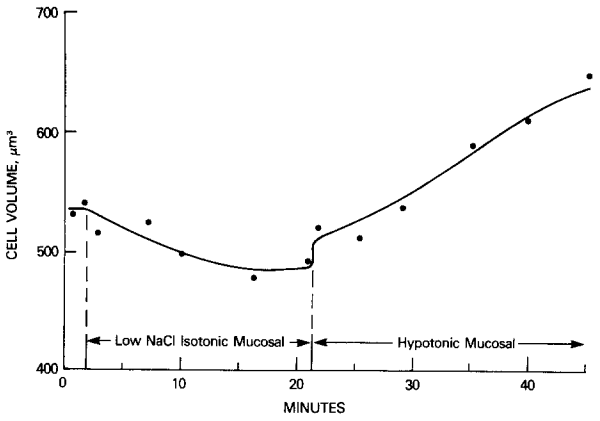

Fig. 4. MR cell volume as a function of time is shown for an experiment in which the mucosal bath osmolality and NaCl composition were altered. After a control period, shown at the left side, the mucosal bath was replaced by a low NaCl solution containing 5 mM NaCl plus enough mannitol to increase the osmolality to about 200 mOsm. In this experiment a small shrinkage occurred. After approximately 20 min the mucosal bath was switched to a solution containing only low NaCl, with an osmolality of about 10 mOsm. Over the next 20 min cell volume increased toward 700 μm^3

961.7 ± 55.7 μm^3, an increase of 36 ± 5%. This increased volume was followed by a spontaneous shrinkage to 752.7 ± 27.8 μm^3 in 29.2 ± 2.1 minutes. Paired analysis showed that the final volume was 100 ± 11% of the control value, not significantly different from control.

Clearly these MR cells were capable of volume regulation in hypotonic serosal bathing solution, an observation not predicted by the model calculations. Further, they were capable of a substantial cell shrinkage of 36%.

Mucosal Bath Hypotonicity

Reduction of mucosal solution osmolality has no effect on the volume of the granular cells of the frog skin [8, 12]. The results of the preceding experiments suggested to us that the MR cells may also respond to the osmolality of the mucosal bath. The MR cells exposed to normal Ringer under control conditions had a volume of about 518 μm^3, while the cells in the osmotic experiments, exposed to 1/20 Ringer on the mucosal surface, had a control volume of 707 μm^3. The difference between these values is significant at the 0.001 level. These results are consistent with the possibility that MR cells change their volume in response to the mucosal bath osmolality. We directly tested this hypothesis in the following experiments (*see* Fig. 4 and the Table).

The tissue was perfused continuously with Ringer solution in the serosal bath throughout the experiment. Initially the mucosal perfusate was frog Ringer. Cell volume averaged 577.1 ± 28 μm^3, not significantly different from the previous values. The mucosal perfusate was then switched to a solution of 1/20 Ringer with enough mannitol added to maintain the osmolality at about 200 mOsm. This solution was used to distinguish between the effects of NaCl removal on cell volume, which would tend to cause cell shrinkage, and the osmotic effect which would tend to cause cell swelling. Cell volume with this low NaCl, isosmotic, mucosal perfusate averaged 95.4 ± 2.6% of control, not significantly different from control Ringer. The mucosal perfusate was next changed to 1/20 Ringer without mannitol. Prompt swelling of the MR cells occurred to a value of 121 ± 4.9% of control. This was significant at the 0.01 level by paired analysis. As shown in the Table, the MR cell volume in 1/20 Ringer in these experiments averaged 698.3 ± 28.3 μm^3, not significantly different from the previous values in 1/20 Ringer. Thus the steady-state volume of MR cells is influenced by the osmolality of the mucosal bath as well as by the chloride concentration of that bathing solution.

Discussion

Our results show that MR cells respond to alterations in the concentration of chloride or osmolality of the outside bathing solution. Although our observations were limited to simple ion removal and osmotic experiments, the results obtained offer support for the hypothesis that chloride ions cross the frog skin epithelium by traversing MR cells.

Measurement of MR Cell Volume

MR cells were readily visualized and the boundaries easily detected by the techniques utilized in these studies. The split frog skin is transparent and easily mounted in a chamber for optical studies. Measurement of the volume of the cells involved some errors due to difficulty in determination of the exact outline of the neck region of these flask-shaped cells. This error was probably small since most of the volume is contained in the bulbous basal portion of the cells. Although an analysis of measurement errors was not made in this study, we can calculate from the control determinations of cell volume that the coefficient of variation was less than 5%. Thus the changes in cell volume, which occurred in response to chloride removal or hypotonicity, were substantially larger than the measurement errors.

MR Cell Volume and Mucosal Bath Chloride

When all of the chloride was removed from the mucosal bath, the MR cells shrank about 23% (*see* Fig. 1). This response is consistent with, but does not prove, the hypothesis that chloride enters the MR cell across the apcial membrane and leaves across the basolateral membrane. When chloride entry is prohibited, by the removal of all mucosal bath chloride, shrinkage of MR cells occurs presumably because chloride exits across both cell membranes. The nature of the cation movements, which must occur as the cell loses chloride, are unknown at this time. It is possible to estimate the intracellular chloride pool from the magnitude of the shrinkage caused by chloride removal. If all intracellular chloride is lost when the mucosal bath is chloride-free, the shrinkage observed corresponds to a cell chloride of about 23 mM. This is about twice as large as the measurements of MR cell chloride in electron microprobe studies of the skin of the frogs, *Rana tempararia* and *Rana esculenta* [10]. We do not know of measurements of the composition of MR cells from *R. pipiens* and are not able to determine whether cell shrinkage is a good estimate of intracellular chloride.

It has previously been shown, by a somewhat different quantitative light microscopic technique, that the stratum granulosum cells, which make up the bulk of the epithelium, do not change their volume in response to alterations in the composition of the outer bathing solution [8, 11]. Therefore the MR cells constitute the only known cell type responsive to alterations in extracellular Cl concentration.

MR Cell Volume and Bathing Solution Osmolality

As expected, MR cells swell when the serosal bath is made hypotonic (*see* Fig. 2). Then they exhibit a remarkably effective volume regulatory decrease of such a magnitude that it may involve more than loss of all intracellular chloride and an accompanying cation. Further study will be needed to determine the mechanism and sidedness of this volume regulatory decrease.

The cell swelling that accompanies reduction of the mucosal bath osmolality is also of interest. It shows that MR cells in their native state in pond water are swollen to about 700 μm^3 volume. If this increased steady-state volume is achieved without significant solute movements, MR cell osmolality can be calculated to be about 166 mOsm. If this new steady-state volume is solely due to an adjustment of osmotic water flow across the two cell membranes, the apical membrane water permeability is calculated to be about 22% of that of the basolateral membrane.

Table. Effect of mucosal hypotonicity on MR cell volume

Mucosal bath	Cell volume (μm^3)	Number of cells, tissues
Control (approx. 200 mOsm)	577.1 ± 28	11, 3
1/20 Ringer + mannitol (approx. 200 mOsm)	550.5 ± 15	11, 3
1/20 Ringer (approx. 10 mOsm)	698.3 ± 28.3[a]	11, 3

[a] $P < 0.01$.

MR Cells and Transepithelial Chloride Transport

Our results and those described in the previous paper [2] support the hypothesis that MR cells are involved in the transepithelial movement of chloride ions across frog skin. Clearly, additional experiments must be done to elucidate the exact nature and magnitude of this movement. Quantitative light microscopy makes a number of experiments possible, which should allow us to analyze the role of the MR cell in this tissue.

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