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Ion Transport by Mitochondria-Rich Cells in Toad Skin

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Summary. The optical sectioning video imaging technique was used for measurements of the volume of mitochondria-rich (m.r.) cells of the isolated epithelium of toad skin. Under short-circuit conditions, cell volume decreased by about 14% in response to bilateral exposure to Cl-free (gluconate substitution) solutions, apical exposure to a sodium-free solution, or to amiloride. Serosal exposure to ouabain resulted in a large increase in volume, which could be prevented either by the simultaneous application of amiloride in the apical solution or by the exposure of the epithelium to bilateral Cl-free solutions. Unilateral exposure to a Cl-free solution did not prevent ouabain-induced cell swelling. It is concluded that m.r. cells have an amiloride-blockable Na conductance in the apical membrane, a ouabain-sensitive Na pump in the basolateral membrane, and a passive Cl permeability in both membranes. From the initial rate of ouabain-induced cell volume increase the active Na current carried by a single m.r. cell was estimated to be 9.9 ± 1.3 pA. Voltage clamping of the preparation in the physiological range of potentials (0 to −100 mV, serosa grounded) resulted in a cell volume increase with a time course similar to that of the stimulation of the voltage-dependent Cl conductance. Volume increase and conductance activation were prevented by exposure of the tissue to a Cl-free apical solution. The steady-state volume of the m.r. cells increased with the clamping voltage, and at −100 mV the volume was about 1.15 times that under short-circuit conditions. The rate of volume increase during current passage was significantly decreased by lowering the serosal K concentration (K_i) to 0.5 mM, but was independent of whether K_i was 2.4, 5, or 10 mM. This indicates that the K conductance of the serosal membrane becomes rate limiting for the uptake of KCl when K_i is significantly lower than its physiological value. It is concluded that the voltage-activated Cl currents flow through the m.r. cells and that swelling is caused by an uptake of Cl ions from the apical bath and K ions from the serosal bath. Bilateral exposure of the tissue to hypo- or hypertonic bathing solutions changed cell volume without detectable changes in the Cl conductance. The volume response to external osmotic perturbations followed that of an osmometer with an osmotically inactive volume of 21%. Using this value and the change in cell volume in response to bilateral Cl-free solutions, we calculated an intracellular steady-state Cl concentration of 19.8 ± 1.7 mM (n = 6) of the short-circuited cell.

Key Words sodium transport · chloride transport · quantitative light microscopy · cell volume · voltage-dependent chloride conductance · mitochondria-rich cell

Introduction

During the past years it has been recognized that the mitochondria-rich (m.r.) cells of the amphibian skin may have a specialized role for transepithelial ion transport. It was suggested that the transepithelial passive Cl flux is confined primarily to these cells (Voûte & Meier, 1978; Kristensen, 1981; Ussing, 1982*b*; Kristensen & Ussing, 1985; Larsen & Rasmussen, 1985). Evidence supporting the hypothesis that a cellular Cl pathway is localized in the m.r. cells, and not the principal cells, was obtained from the following type of studies. It was found that the Cl fluxes, but not the sulfate fluxes, are significantly reduced when the frogs are stored at low temperature, and that copper ions (Koefoed-Johnsen & Ussing, 1974) and inhibitors of cellular Cl transport systems, e.g. furosemide, diamox, and phloretin (Bruus, Kristensen & Larsen, 1976; Kristensen & Larsen, 1978) inhibit the passive Cl permeability of skins of room temperature adapted animals. From these observations it was suggested that the large Cl fluxes in skins of amphibians adapted to temperatures above 4°C are localized to a cellular pathway. Furthermore, experimentally induced changes of the cellular Na permeability were also found to affect the equilibrium fluxes of Cl in the short-circuited preparation (Macey & Meyers, 1963; Candia, 1978; Kristensen, 1978; 1981; Ques-von Petery, Rotunno & Cereijido, 1978). This observation, as well, indicated a cellular rather than a paracellular route for the passive Cl ion flow. It was

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found that the distribution of Cl transport systems in the large Na transporting principal cells is not consistent with passive Cl uptake into these cells (Ferreira & Ferreira, 1981; Nagel, Garcia-Diaz & Armstrong, 1981; Ussing, 1982*a*; Giraldez & Ferreira, 1984; Harvey & Kernan, 1984; Biber et al., 1985; Dörge, Rick & Thurau, 1985; Willumsen & Larsen, 1986). Even in skins in which the voltage-dependent Cl conductance was fully activated, the apical membrane of the principal cells was found to be tight to Cl (Willumsen & Larsen, 1986). Finally, it was observed that the density of m.r. cells, which shows no correlation with the active Na flux (Brown, Grosso & DeSousa, 1981), is proportional to the passive Cl conductance of the skin (Voûte & Meier, 1978; Willumsen & Larsen, 1985).

The bodies of the flask-shaped m.r. cells are located in the second and third layer of principal cells, and the necks protrude up between the outermost cells with the apical membrane facing the subcorneal space. In toad skin, the m.r. cell density is about 50,000 to 100,000 cells per cm^2 (Willumsen & Larsen, 1985) and their apical membrane area and volume add up to about 1% of the total surface area and intracellular volume of the epithelium, respectively. These unfavorable dimensions impeded previous investigations by conventional techniques of the significance of the m.r. cells for the transport of ions through the skin.

Recently, it was demonstrated that two methods can be applied for the study of ion transport by the m.r. cells of amphibian skin. With the vibrating probe technique it was shown that the current carried by the inward flux of Cl ions was not uniformly distributed over the entire epithelial surface, but confined to areas above the m.r. cells (Foskett & Ussing, 1986; Katz & Scheffey, 1986). With the optical sectioning/video imaging technique it was shown that the volume of single m.r. cells responded to the passage of large Cl currents with a volume gain (Foskett & Ussing, 1986) and to removal of the Cl ions from the apical solution with a volume loss (Spring & Ussing, 1986). Thus, these studies provided the definitive proof that the m.r. cell is furnished with a Cl-selective permeability.

In the present study the method of quantitative light microscopy (Spring & Hope, 1978; Spring, 1985) was used for further characterizing the transport systems and the polarity of the m.r. cell. Some of the results of the study have been published in abstract form (Larsen, Ussing & Spring, 1986).

Materials and Methods

Preparation and Experimental Chamber

Female Colombian toads (National Reagent, Bridgeport, CT), *Bufo marinus*, were kept at room temperature on a dry substrate with free access to a pool of tap water. The epithelium was isolated by a 3 to 4 hr exposure of the serosal side of the excised belly skin to Ringer's solution containing 2 mg/ml of collagenase (103586, Boehringer-Mannheim GmbH). The epithelium was gently peeled off the corium by a pair of forceps and kept overnight at 4°C in well-aerated, acetate-enriched (5 mM) Ringer's solution. This procedure provided us with preparations of high specific Na and Cl conductances and low leakage conductance. The isolated epithelium was mounted with the apical side down, supported by a nylon mesh in a chamber with an exposed area of 0.25 cm^2 (Fig. 1). The tissue was kept in place by stretching it over small pins, sealed with silicone grease, and the two half chambers were held together in a modified Dvorak holder as previously described (Spring & Hope, 1978). For current passing, a pair of circular platinum wires were embedded along the rim of the two half chambers. The potential-measuring bridges,

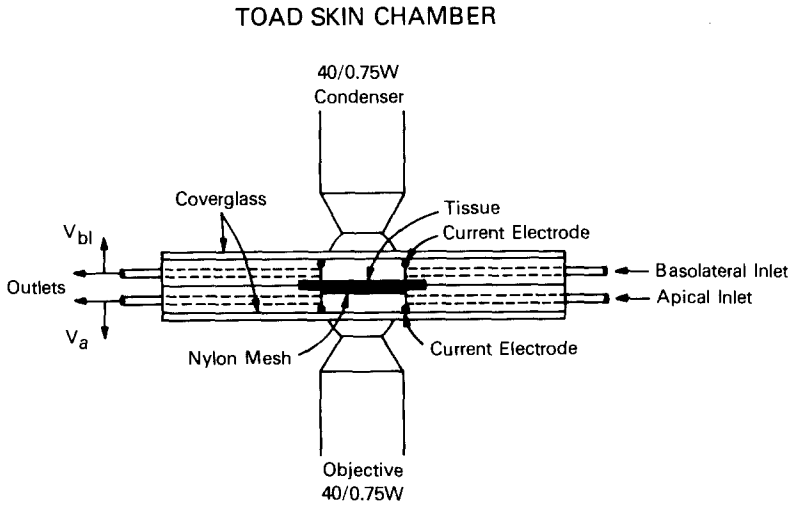

Fig. 1. Experimental chamber for video-microscopic determination of cell volume of m.r. cells of isolated voltage-clamped toad skin epithelium. The preparation is mounted with the apical side down, supported by a nylon mesh of which the center threads are cut to improve illumination of the tissue. Uniform current density is provided by the circular platinum electrodes along the contraepithelial edge of the two half chambers. The outlets for the perfusing solutions are also the potential-measuring bridges which are connected to the saturated KCl reservoirs of the calomel electrodes. The chamber is placed on the stage of an inverted microscope with Nomarski optics, and the tissue illuminated with 540 nm monochromatic light from a halogen lamp

which were the outlet tubes for the perfusing solutions, were connected to large saturated-KCl reservoirs containing calomel electrodes. Following mounting of the epithelium, the tissue was kept at open circuit for about an hour before the experimental protocol started. Then the tissue was clamped to the desired voltage. For monitoring of the tissue conductance, voltage pulses of 500 or 1000 msec duration and a preset frequency were delivered.

The preparations generated spontaneous potentials (V_{sp}) and short-circuit currents (I_{sc}) comparable to those of freshly mounted whole toad skin preparations, e.g., for 17 tissues we recorded $V_{sp} = -17.5 \pm 1.8$ mV and $I_{sc} = 23.3 \pm 2.7$ μA cm^{-2} (mean ± SE). These large short-circuit currents could be eliminated by 50 μM amiloride in the outer bath ($I_{sc} = 0.9 \pm 0.5$ μA cm^{-2}, $n = 12$). This result shows that passive components were not contained in the short-circuit current, i.e., the preparation was indeed clamped at the command potential.

Microscopy

The Dvorak holder with the experimental chamber was mounted on the stage of an inverted microscope (Diavert, E. Leitz, Rockleigh, NJ) and observed with differential interference contrast optics using strain-free water immersion lenses both as condensor and objective (working distance: 1.5 mm (Zeiss 40×/0.75 NA)), and eyepieces of 10 times magnification. The preparation was illuminated by 540 nm monochromatic light; the resultant image was magnified 2.5-fold and detected by a television camera equipped with a Newvicon tube (Model NC-65S, Dage-MTI, Michigan City, IN). The video image was displayed on a set of monitors and, for later planimetric analysis, recorded on a magnetic videodisk with a capacity of 400 frames (Model VDR-1RA, Echo Science, Mt. View, CA).

Determination of Cell Volume

Due to their flask-like shape and small apical membrane area, m.r. cells are easily detected. Often they occur in clusters so that several cells are observed within a single microscopic field. However, not all cells are well suited for quantitative microscopy. Cells were selected that had the neck strictly vertically positioned, a necessary condition for easy tracing of the cell boundary at the level where the neck merges with the cell body. The best optical resolution was obtained with m.r. cells that were not positioned beneath pigmented cornified cells. Therefore, often a single cell only, out of several within a field, could be analyzed.

The microscope was focused on the apical area of a m.r. cell and in order to make returning easy to this plane in subsequent volume measurements, the frame was stored in a digital frame memory (Model FM-60, FORA, W. Newton, MA). The focus of the microscope was controlled by a stepping motor drive attached to a computer programmed to displace the focal plane at 3-μm intervals and record each frame on the video disk. This procedure took about 1.5 sec. After termination of the experiment, the images were displayed and the boundary of the selected cell was traced for calculation of its cross-sectional area. The volume was calculated from the estimated areas and the distance between the focal planes [Eqs. (1) and (2) of Marsh, Jensen & Spring, 1985].

Determination of the volume of a single cell in a steady state could be carried out with very good reproducibility. The volume of eight unperturbed cells was repeatedly determined by the above procedure. The coefficient of variation [(SD/mean volume) × 100%] of estimated volumes ranged from 1.17% ($n = 12$) to 2.92% ($n = 11$). It turned out to be somewhat more difficult to reproduce the volume determination of a cell undergoing a transient volume change. This was explored by repeating the whole scan procedure twice, within a time interval (10 sec) which was short as compared to the halftime of the cell volume change (minutes). The error (e) of the estimate of the volume is calculated from

$$e = [2|V_n - V_{n+1}|/(V_n + V_{n+1})] \times 100\%$$

where (V_n, V_{n+1}) is the pair of observations. For $n = 69$, we found $e = 4.4 \pm 3.4\%$ (mean ± SD).

Experimental Solutions

The standard Ringer's solution contained (mM): 115 Na, 2.4 K, 1 Ca, 112 Cl, 2.4 HCO_3, and 5 acetate; pH was kept at 8.2 by bubbling the solution with air. In gluconate Ringer's Cl was replaced by gluconate, and Na-free Ringer's was made by replacing Na with either K or N-methyl-D-glucosamine.

Hypertonic Ringer's was made from the standard Ringer's by the addition of 47.6 mmol sucrose per liter. Hypotonic Ringer's was made by reducing the Na and Cl concentrations to half of their concentrations in the standard Ringer's, and its control solution was made by raising the osmolarity by the addition of sucrose. The osmolarity of the solutions was measured before use.

Amiloride was a gift from Merch Sharp and Dohme (West Point, PA), and ouabain (g-strophanthin) was purchased from Sigma.

The data are presented as mean values with their standard errors (±SE).

Results

M.R. Cell Volume under Short-Circuit Conditions

Exposure of the short-circuited epithelium to bilateral Cl-free solutions resulted in a fast decrease of the cell volume (Fig. 2, upper panel). The new steady state, achieved within the first few minutes of solution shift, was maintained for 10 min, indicating that the intracellular diffusible Cl ion pool was depleted. The response was fully reversible as a return to Cl-Ringer's led to restoration of the initial steady-state volume. Control cell volume for six cells was 471 ± 81 μm^3, comparable to the steady-state volume of 467 ± 65 μm^3 following return to Cl-Ringer's after Cl removal. In these experiments the Cl-free cell volume amounted to 85.8 ± 0.6% ($n = 6$) of the control volume, indicating that most of the cell water is associated with the nondiffusable intracellular anions.

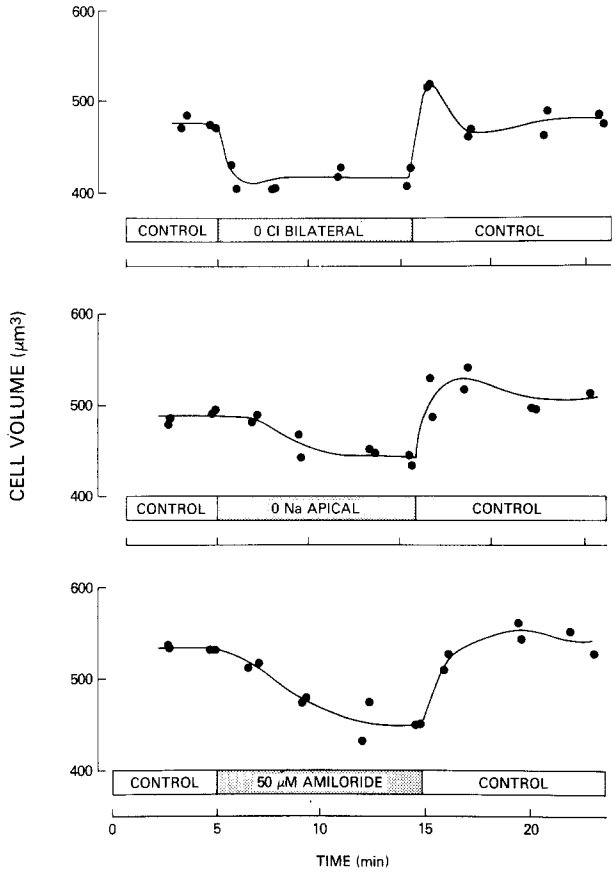

Fig. 2. Volume of single m.r. cells of short-circuited preparations (V = 0 mV). *Upper panel*: Response to bilateral exposure of a Cl-free Ringer's solution (gluconate substitution). *Mid panel*: Response to perfusing the apical chamber with Na-free Ringer's (K substitution). *Bottom panel:* Effect of exposing the apical side of the epithelium to 50 μM amiloride

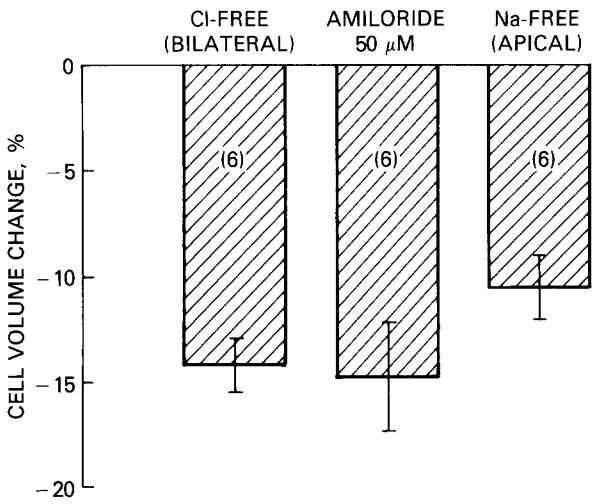

Fig. 3. Comparison of the response of the m.r. cell volume to bilateral Cl-free-, amiloride-, and apical Na-free exposure. The transepithelial potential was clamped at 0 mV. Mean ±SE with the number of cells indicated

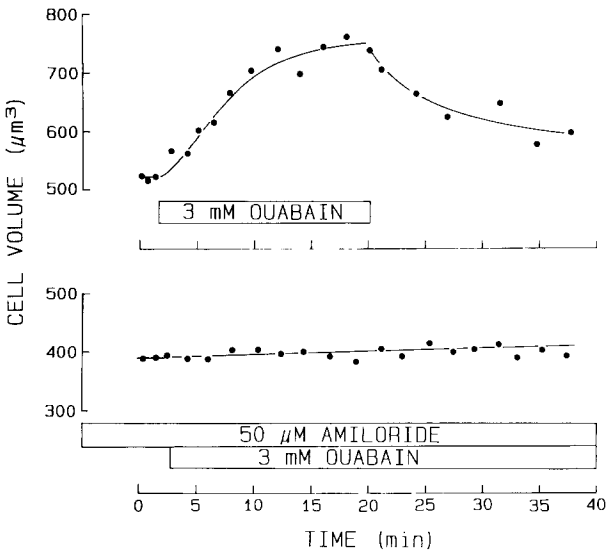

Fig. 4. Effect of the addition of 3 mM ouabain to the serosal bath on the m.r. cell volume under short-circuit conditions (V = 0 mV). *Upper panel:* The preparation was exposed to NaCl-Ringer's on both sides, and ouabain was added during the period indicated below the graph. *Lower panel:* About 15 min prior to ouabain treatment, the preparation was exposed to 50 μM amiloride on its apical side. As indicated below the graph, in these experiments the serosal side of the preparations was not re-exposed to fresh Ringer's solution

The middle panel of Fig. 2 shows that in response to a Na-free apical solution (K substitution) the cell volume also decreased; a similar response was obtained when Na was substituted by N-methyl-D-glucosamine. In the bottom panel of Fig. 2 it is shown that the addition of 50 μM amiloride to the apical solution resulted in a similar reduction in cell volume. In all of these experiments exposure of the epithelium to the control NaCl-Ringer's lead to a full restoration of the initial steady-state volume.

Figure 3 summarizes the results of these experiments. In response to bilateral Cl-free Ringer's, cell volume decreased on the average by 14.2 ± 1.4%. Amiloride treatment resulted in a volume loss of a similar magnitude (14.8 ± 2.8%). The decrease in volume following exposure to a Na-free apical solution was lower, 10.6 ± 1.6%, but the mean value of this series and those of the two other series are not statistically significant ($P > 0.05$).

The addition of 3 mM ouabain to the serosal bath resulted in a significant volume increase. Ouabain inhibition was reversible as shown by the decrease of cell volume toward its initial control value following exposure of the serosal side to fresh Ringer's solution (*see* Fig. 4). It can also be seen that the ouabain-induced volume increase could be prevented if the tissue was pretreated with amiloride.

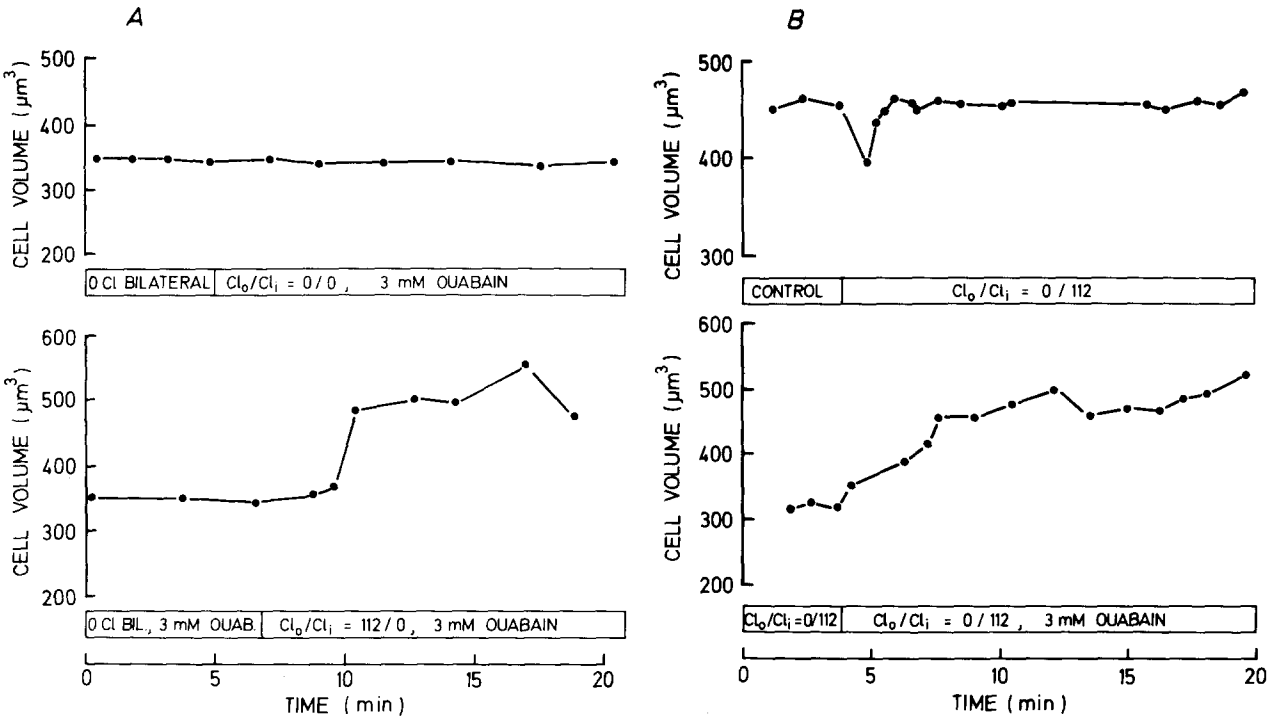

Fig. 5. (*A*) The effect of the external anion composition on the response of m.r. cell volume to serosal ouabain treatment under short-circuit conditions. *Upper panel:* The addition of 3 mM ouabain did not result in volume expansion when the preparation was bathed in Cl-free (gluconate) Ringer's on both sides. *Lower panel:* During the initial period, the preparation was bathed in bilateral Cl-free Ringer's with 3 mM ouabain added to the serosal solution (continued from the experiment shown in the upper panel). At the time indicated below the graph, the apical side of the preparation was perfused with NaCl-Ringer's. The m.r. cell responded with a significant volume increase. (*B*) *Upper panel*: Effect of a sudden removal of Cl from the apical bathing solution on the volume of the m.r. cell ($V = 0$ mV). Notice that initially the volume decreased, but at the new steady state the volume was not different from that recorded with bilateral NaCl-Ringer's. *Bottom panel:* During the initial period, the preparation was bathed in a Cl-free Ringer's on the apical side (gluconate substitution). The serosal solution contained 112 mM Cl. At the time indicated, 3 mM ouabain was added to the serosal side, and the m.r. cell responded with a significant gain in volume

The results of this series of experiments are summarized in Table 1. Ouabain caused a large volume gain characterized by an initial swelling rate of 4% per min. The prior addition of amiloride to the external bath decreased this rate by a factor of 50 to a nonsignificant value. X-ray microanalysis (Rick et al., 1978) also revealed a ouabain-sensitive Na-exit mechanism in the serosal membrane and a Na-entrance pathway in the apical membrane of m.r. cells of frog skin. In their study, however, amiloride did not significantly affect ouabain-induced cation redistributions. The basis of this result, apparently in conflict with ours, is not clear.

Table 1. Ouabain-induced cell volume changes of mitochondria-rich cells of the short-circuited toad skin epithelium: Dependence on the passive Na permeability of the apical membrane (±amiloride)[a]

Protocol	V_o (μm^3)	3 mM ouabain in serosal bath		Cell number
		V_{max}/V_o	Swelling rate (%/min)	
Control	448 ± 36	1.38 ± 0.04[b]	3.98 ± 0.42[d]	6
Amiloride	429 ± 43	1.02 ± 0.01[c]	0.082 ± 0.036[e]	6

[a] Mean ±SE (6 preparations in each group).
[b] V_{max} measured 10-15 min after the addition of ouabain.
[c] V_{max} measured 25-30 min after the addition of ouabain.
[d] $P < 0.001$.
[e] $P < 0.05$.

The Chloride Permeability under Short-Circuit Conditions

From the above results, it follows that the Cl ions exchange between the cell and the bathing solutions, but whether a major pathway is located in both membranes was not revealed. In order to answer this question, we studied the dependence of the ouabain-induced volume increase on the anion composition of the bathing solutions. Figure 5*A* shows that if the tissue was exposed to bilateral Cl-

Table 2. Ouabain-induced cell volume changes of mitochondria-rich cells of short-circuited toad skin epithelium: Dependence on external chloride (gluconate substitution)[a]

Cl concentration in bathing solutions (mM) apical/serosal	V_o (μm^3)	3 mM ouabain in serosal bath		Cell number
		V_{max}/V_o	Swelling rate (%/min)	
0/0	463 ± 56	1.00 ± 0.01	0.03 ± 0.03	7
0/112	408 ± 37	1.46 ± 0.09	22.9 ± 3.7	6
112/0	462 ± 55	1.46 ± 0.06	21.1 ± 2.9	7

[a] Mean ±SE (5 preparations in each group).

Table 3. Cell volume of mitochondria-rich cells of short-circuited toad skin epithelium: Dependence on the major anion of the apical bathing solution[a]

Anion in apical bath. . . .	Steady-state cell volume			Cell number
	Chloride (μm^3)	Gluconate (μm^3)	Diff. (μm^3)	
	441 ± 18	441 ± 18	0.0 ± 1.5	4

[a] Mean ±SE (4 preparations).

free solutions (gluconate substitution), the cell volume was not changed by the addition of ouabain. However, in the presence of Cl in either bathing solution, ouabain addition resulted in significant cell volume increase (Fig. 5*A* and *B*). The results summarized in Table 2 show that the rate of the ouabain-induced volume increase was not significantly dependent on whether the Cl ions were taken up from the apical or the serosal bath. Thus, under short-circuit conditions a pathway specific for Cl ions is present in both membranes. In this series of experiments we also investigated the anion permeability of the cell by following the response of the volume to a sudden removal of Cl from the apical solution. The upper panel of Fig. 5*B* shows that perfusion of the apical chamber with Na-gluconate Ringer's resulted in a modest, transient loss of cell volume. At the new steady state, with gluconate Ringer's on the outside, cell volume was not significantly different from its value with Cl Ringer's on the outside (Table 3).

Response of M.R. Cell Volume to Voltage Clamping

Previous experiments have shown that the passive Cl permeability of the skin of the European toads, *B. bufo* and *B. viridis*, is activated by clamping the preparation in the physiological region of potentials (Larsen & Kristensen, 1978; Larsen & Rasmussen, 1982; Katz & Larsen, 1984). In an initial series of experiments we investigated whether the Cl permeability of the skin of *B. marinus* exhibits a similar potential dependence. In the following experiments the active Na currents were eliminated by amiloride so that the remaining currents are expected to be governed by the Cl conductance. In Fig. 6 is shown a family of voltage-clamp currents obtained by pulsing the transepithelial potential (V) from 30 mV (apical positive) to the series of eight potentials indicated on top, and back. With Cl-Ringer's in the apical bath, current activations were seen when V was pulsed to the negative region, and returning V to 30 mV resulted in a large inward current that slowly decreased to the initial steady-state value. It is also seen that the rate of the current activation increases as the tissue is clamped to more negative potentials. These reversible conductance activations could be eliminated by replacing the Cl ions of the apical solution with gluconate (Fig. 6, right-hand panel). This result shows that the slow, time-dependent outward currents are carried by an inward flow of Cl ions across the apical border of the epithelium. The Cl conductance was further characterized by measuring the steady-state tissue conductance as a function of the transepithelial potential. Examples of conductance-voltage relationships are depicted in Fig. 7. Provided Cl ions were present in the apical solution, the conductance was a steep function of potential over the range, -60 mV $< V <$ 30 mV. The graphs also reveal that the

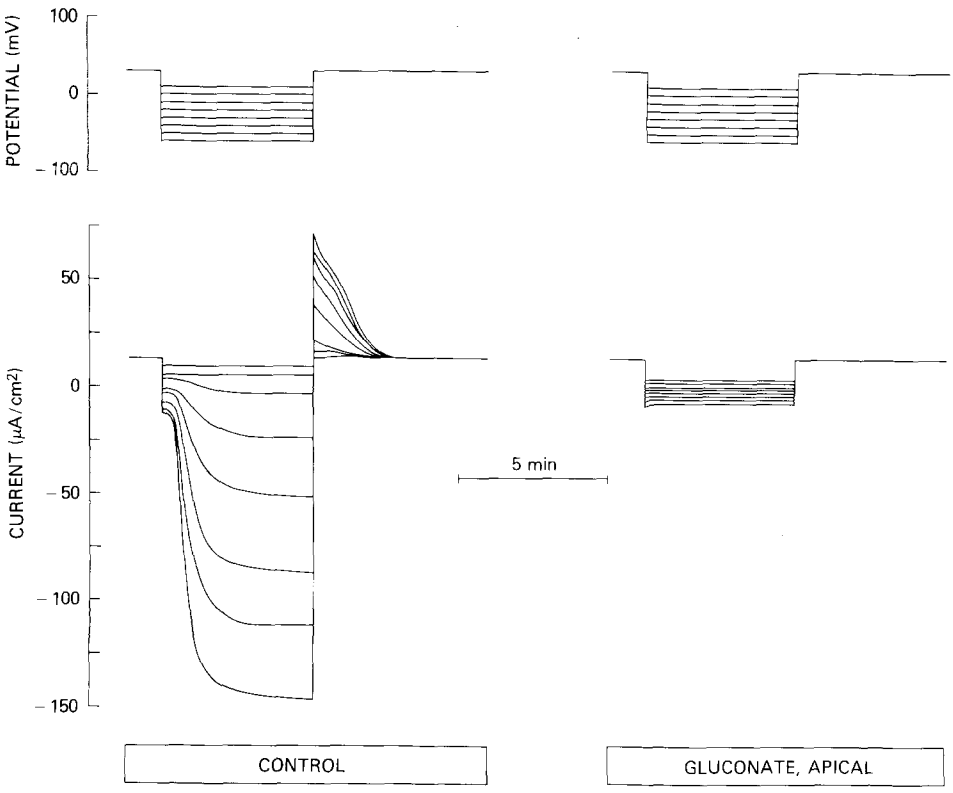

Fig. 6. Superimposed family of voltage-clamp currents obtained by pulsing the transepithelial potential from a holding value of 30 mV to the values indicated on top, and back. *Left-hand panel:* Control, NaCl-Ringer's on both sides with 50 μM amiloride in the apical bathing solution. *Right-hand panel:* Na-gluconate Ringer's on the apical side (with 50 μM amiloride), and NaCl-Ringer's on the serosal side

skin of this species exhibits a significant Cl conductance under short-circuit conditions (V = 0 mV). The associated transepithelial currents are shown in Fig. 8. The additional conclusion to be drawn from these relationships is that the steady-state current under equilibrium conditions (bilateral Cl-Ringer's, V = 0 mV) is zero, which is in agreement with the notion (Larsen & Rasmussen, 1982; Kristensen, 1983) that the potential-dependent Cl conductance controls a purely passive flux.

Associated with activation of the Cl conductance, the m.r. cells swelled (Fig. 9, left-hand panel). It is seen that the rate of swelling following clamping of the tissue to V = −80 mV occurred with a time course similar to that of the current activation and that the volume decreased again as the conductance was deactivated at V = 30 mV. M.r. cell swelling was prevented when the tissue was exposed to a Cl-free apical solution (Fig. 9, right-hand panel). Similar results were obtained when the tissue was hyperpolarized to −100 mV from a holding potential of 0 mV (Fig. 10). As shown in Fig. 7, the tissue exhibited a significant Cl permeability at the 0-mV holding potential. Despite the fact that the Cl conductance was partially activated at 0 mV, the current activation and cell swelling occurred with similar time courses. The volume gained by clamping V to −100 mV was lost again following return of V to 0 mV in parallel with the slow conductance deactivation.

Figure 11 shows that the steady-state m.r. cell volume increased as the tissue was clamped to more negative potentials. Thus, as the inward driving force for the Cl ion flow is increased and the Cl conductance is activated, m.r. cells respond by swelling. Swelling and conductance activation were both eliminated in experiments where the Cl ions of the apical bath were replaced by gluconate. From these observations we conclude that the voltage-induced swelling is associated with uptake of Cl ions from the apical solution through an activated Cl permeability in the apical membrane of the mitochondria-rich cells. The observed swelling must be due to the accumulation of Cl ions and cations in the m.r. cells, presumably because the rate of solute entry exceeds that of solute exit.

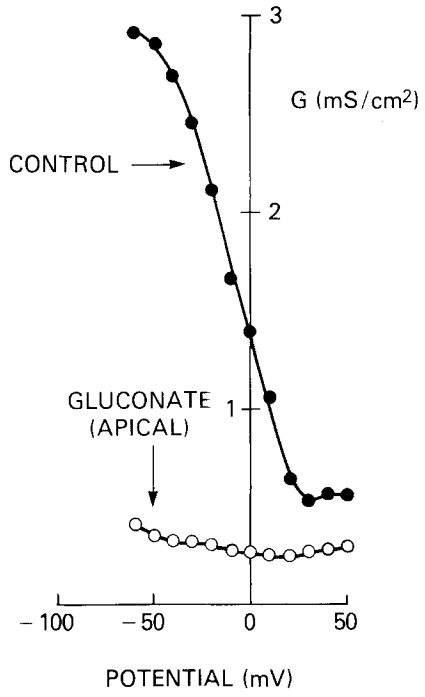

Fig. 7. Relationship between the transepithelial potential and the steady-state conductance (G) of the toad skin exposed to NaCl-Ringer's on both sides (●, *CONTROL*), or a Cl-free Ringer's in the apical chamber (○, *GLUCONATE (APICAL)*). The apical membrane's Na conductance was eliminated by adding 50 μM amiloride to the apical bathing solution

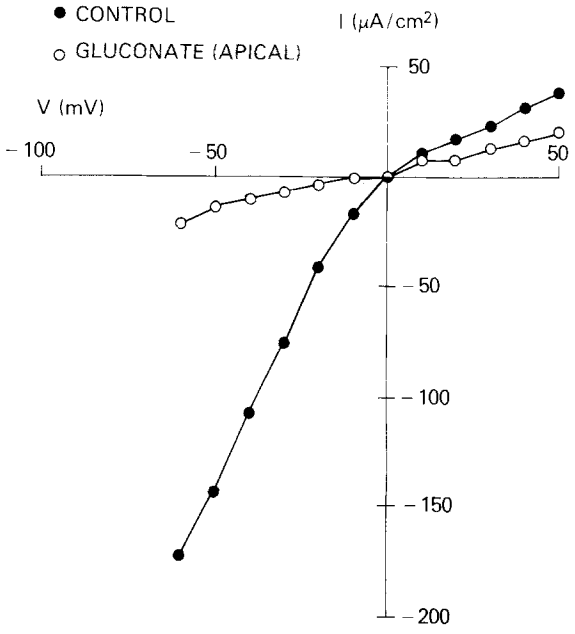

Fig. 8. Steady-state current-voltage (I-V) relationships of the amiloride (50 μM) treated toad skin. ●: *CONTROL*, NaCl-Ringer's bathing both sides of the preparations. ○: *GLUCONATE (APICAL)*, the apical solution was made Cl free by replacing the Cl ions with gluconate

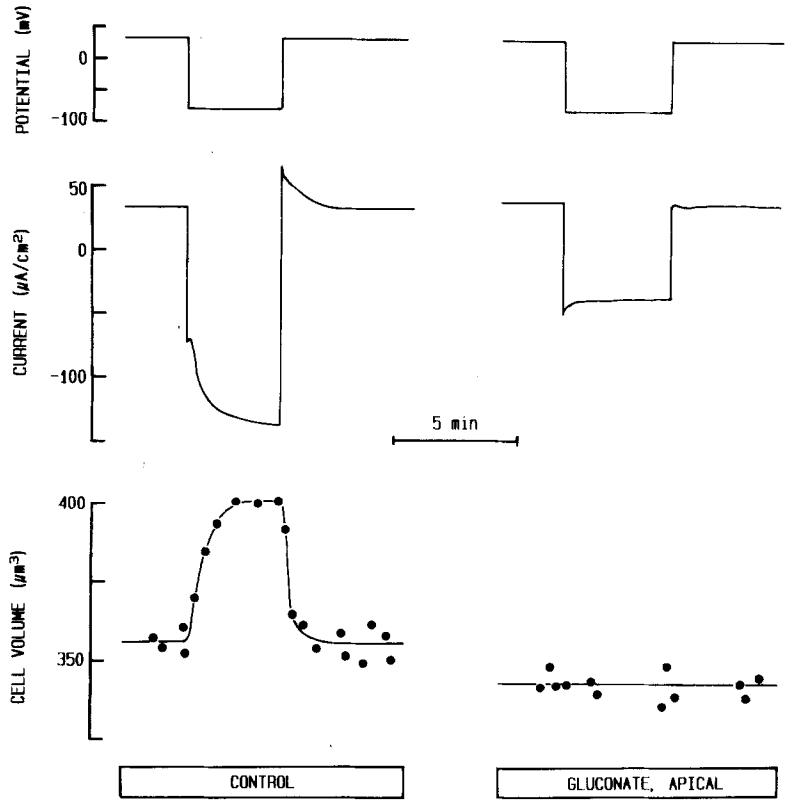

Fig. 9. Response of transepithelial current and m.r. cell volume to a change of the transepithelial potential from 30 to −80 mV, and back. *Left-hand panels:* Control, NaCl-Ringer's bathing both sides of the preparations. 50 μM amiloride in the apical bathing solution. *Right-hand panels:* Cl ions of the apical bathing solution was replaced with gluconate, and 50 μM amiloride was present in the apical bath

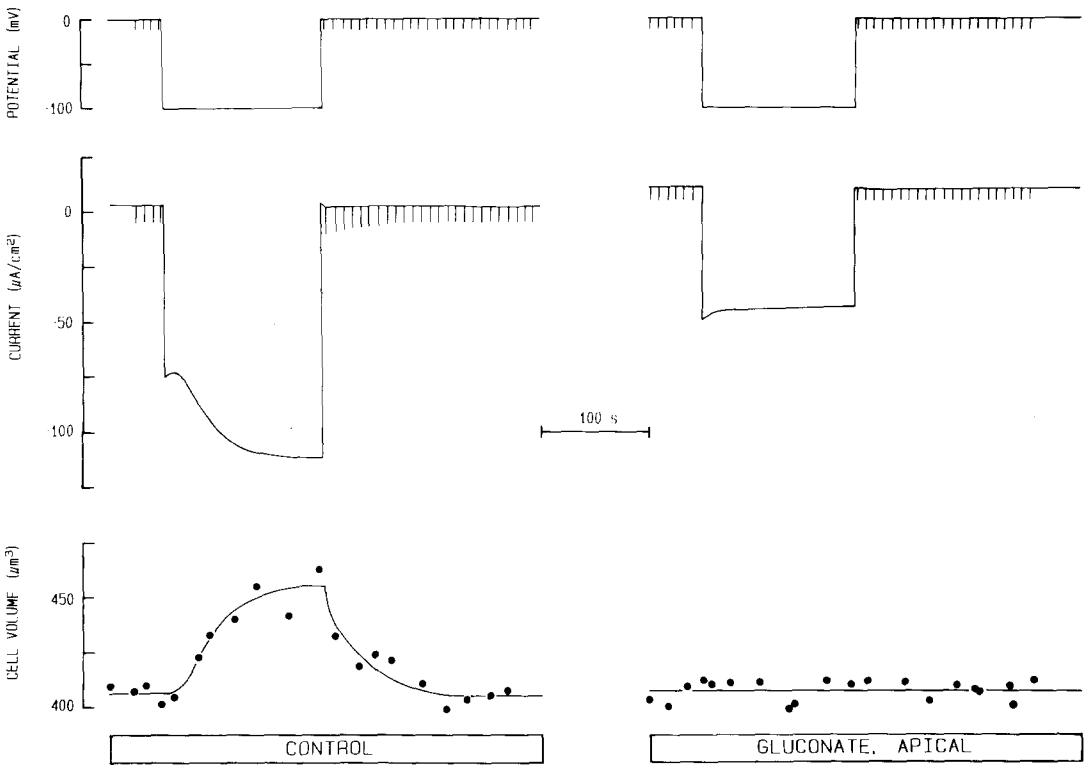

Fig. 10. Response of transepithelial current and m.r. cell volume to a change of the transepithelial potential from 0 to −100 mV, and back. The active sodium current was inhibited with 50 μM amiloride in the apical bathing solution. At approximately 10-sec intervals a 10-mV voltage pulse of 1 sec duration was superimposed on the 0-mV clamping voltage for monitoring the change of the tissue conductance. *Left-hand panels:* NaCl-Ringer's bathing both sides of the preparation. Notice that the (passive) conductance is deactivated following a return of the potential to 0 mV and that the volume of the m.r. cell, which was increased when the tissue was clamped to −100 mV, decreased with a time course similar to the conductance deactivation. *Right-hand panels:* Cl ions of the apical bathing solution replaced with gluconate

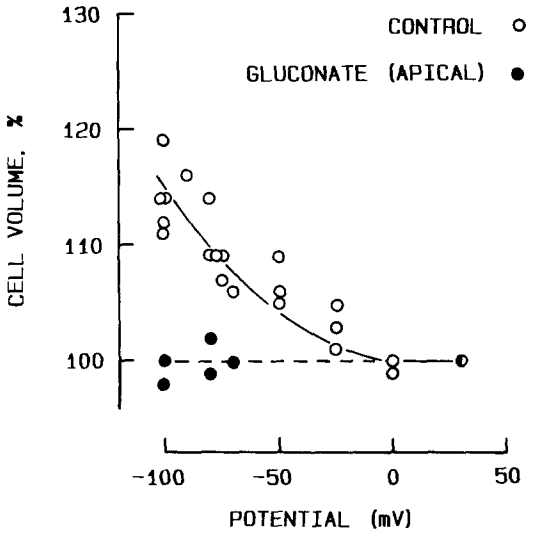

Fig. 11. Relationship between transepithelial potential and the steady-state volume of m.r. cells. The volume is expressed relative to the volume measured at 30 mV (at which potential the voltage-dependent Cl conductance is deactivated, *see* Fig. 7). All of the preparations were exposed to 50 μM amiloride on the apical side. ○, *CONTROL*; NaCl-Ringer's in the apical chamber. ●, *GLUCONATE (APICAL)*; Cl ions of the apical bathing solution substituted with gluconate

The Dependence of the Time Course of Volume Changes on the Serosal Potassium Concentration

The cations accompanying the Cl ions during the voltage-induced volume changes are unknown from the experiments presented above. However, as the apical membrane's Na conductance was eliminated in these experiments, and as the Na permeability of the inner membrane was shown to be vanishingly small (Fig. 4, Table 1), it is most likely that K ions from the serosal bath are taken up together with the Cl ions. To investigate this possibility we repeated the above protocols but with varying serosal K concentrations, K_i. The results of individual experiments are collected in Fig. 12. It can be seen that, following a hyperpolarizing voltage step, current activation and cell volume increase always occurred with a similar time course, and that lowering K_i to 0.5 mM significantly slowed down the rate at which the new steady states of both processes were achieved. In Fig. 13 the results are summarized by depicting, as a function of K_i, the relative rate of volume changes during volume gain and loss, re-

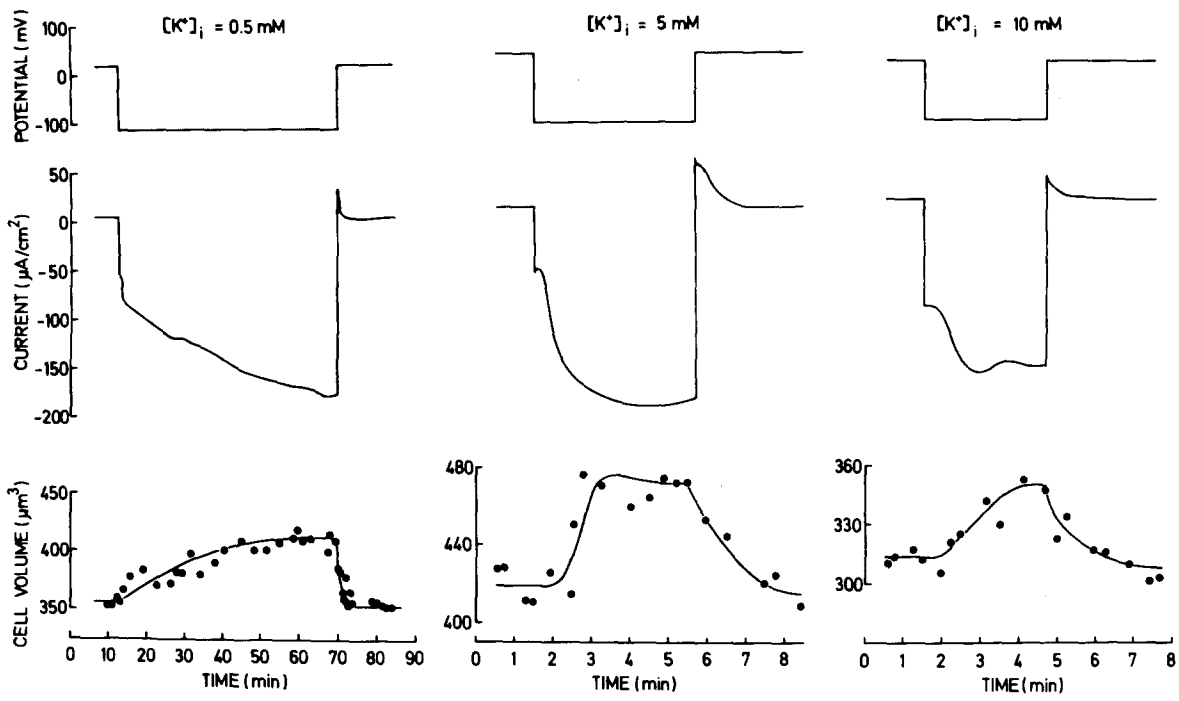

Fig. 12. Effect of varying the serosal K concentration on the time course of the clamping current- and the m.r. cell volume response to voltage activation/deactivation of the Cl conductance. The serosal K concentrations are indicated above each set of graphs. In all of the experiments, 50 μM amiloride was added to the apical bathing solution

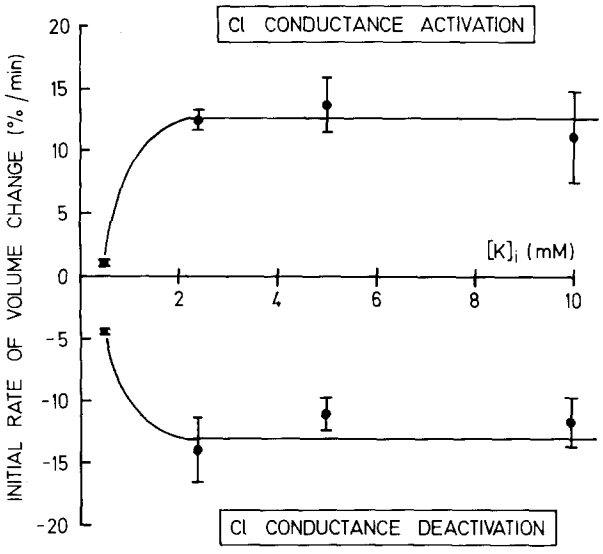

Fig 13. Initial rate of m.r. cell volume change (mean ± SE) as a function of serosal K concentration during activation of the Cl conductance ($V = -100$ mV) and deactivation of the Cl conductance ($V = 30$ or 0 mV). 23 m.r. cells of 13 preparations

spectively. The rate of volume change was independent of the serosal K concentration for $K_i \geq 2.4$ mM. However, for $K_i = 0.5$ mM, the time it took the cell to reach its new steady state was significantly increased. At this K concentration, the rate of voltage-induced volume gain was 0.97 ± 0.24% ($n = 9$) while the rate of volume loss significantly ($P < 0.001$) greater (−4.48 ± 0.76%, $n = 11$). The above results show that the serosal membrane K conductance can become rate limiting for volume changes at low, serosal K concentration. These observations are consistent with the conclusion that K ions derived from the serosal bath are taken up with the Cl ions derived from the apical bath during the voltage-induced swelling.

Response of M.R. Cell Volume to Osmotic Pertubations

The steady-state volume of the m.r. cell increased with increasing Cl conductance (Figs. 7, 11), and the time courses for the development of the current activation and of the volume gain were identical (Figs. 9, 10, 12). Thus, from this type of experiments it appears that cell volume increase and Cl-current activation might be causally related. In a series of experiments we investigated whether these two processes could be dissociated from one another. In one group of experiments the tissue was exposed to standard Ringer's on both sides and clamped at 30 mV (Fig. 14*A*). When the steady state was achieved, V was stepped to −100 mV, which

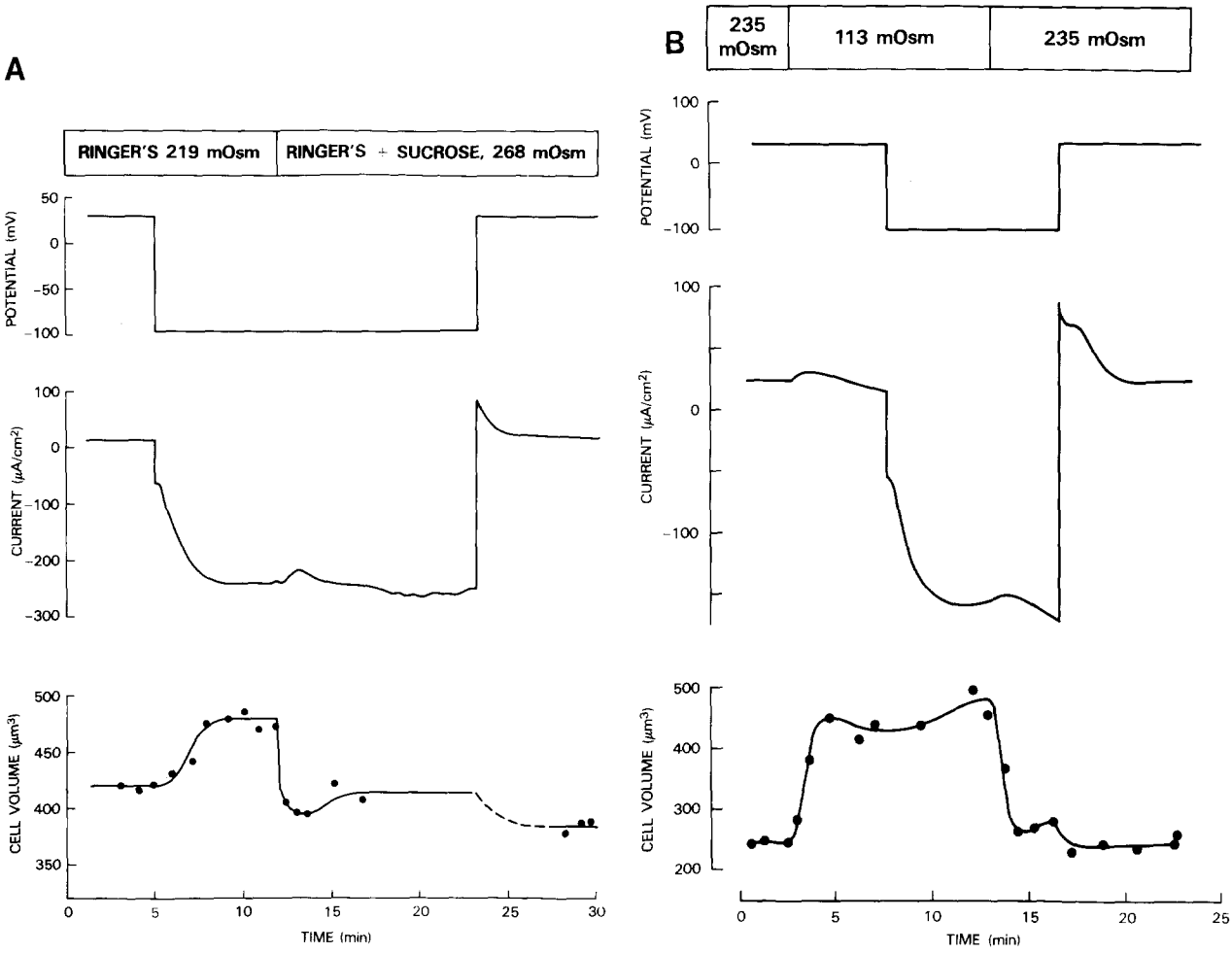

Fig. 14. (*A*, *B*) Response of transepithelial current (Cl conductance) and m.r. cell volume to transepithelial voltage clamping and to changing bilaterally the osmolarity of the bathing solution with sucrose. (*A*) From the steady state at 30 mV and with the preparation bathed with NaCl-Ringer's on both sides (50 μM amiloride in the apical solution), the protocol started with an activation of the tissue conductance by clamping the preparation to −100 mV. (*B*) The preparation was bathed initially with Ringer's solutions of half strength with respect to NaCl, and with sucrose added to obtain an osmotic pressure of 235 mOsm (50 μM amiloride was also added to the apical bathing solution). The protocol was started by osmotically swelling the m.r. cell at V = 30 mV. The clamping current (the Cl conductance) responds to shifts in clamping potential, but not (or little) to the osmotic pertubations which result in large m.r. cell volume changes

resulted in a Cl-current activation of about 150 μA cm^{-2} and a volume increase from 420 μm^3 to about 475 μm^3. The tissue was then exposed on both sides to a Ringer's made hypertonic by the addition of sucrose, which resulted in a rapid return of the volume to about 400 μm^3. However, the Cl conductance remained activated as revealed by constancy of the current. Following a return of V to 30 mV, the conductance slowly decayed to its initial low value associated with a further, but small, decrease in volume. This small decrease in volume probably reflects the amount of KCl taken up during voltage clamping at −100 mV that was lost again. In the second group of experiments, cell volume was increased under conditions where the Cl conductance was deactivated (Fig. 14*B*, V = 30 mV). In order to maintain the ionic concentrations of the bathing solutions constant, the control Ringer's was diluted to about half Ringer's strength and sufficient sucrose was added to obtain an osmolarity equal to that of standard Ringer's. It can be seen (Fig. 14*B*) that, following removal of sucrose, the m.r. cell responded with a fast volume increase of more than 250 μm^3, but that the tissue conductance remained low, as revealed by a time-invariant small current. The Cl conductance was then activated by clamping the tissue to −100 mV, which resulted in a small further increase in volume. Cell volume could be returned to near its initial value by increasing the osmolarity of the bathing solutions without affect-

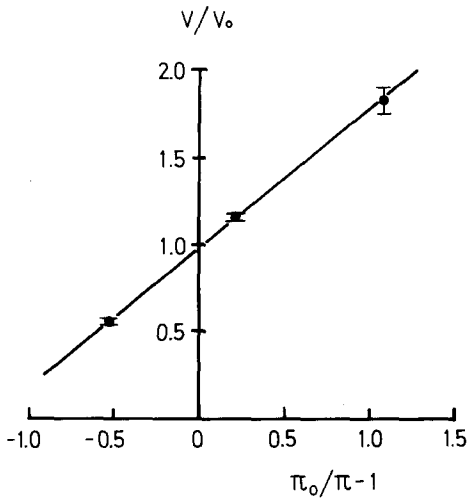

Fig. 15. Relationship between the relative volume of m.r. cells and the relative osmotic pressure of the external bathing solutions. V_o is the cell volume when the tissue is bathed in isotonic solutions of the osmotic pressure, π_o. The apical bathing solution contained 50 μM amiloride. Linear regression analysis gave the following relationship, $V/V_o = 0.79(\pi_o/\pi - 1) + 0.98$ ($r^2 = 0.979$). The line of regression is drawn through the experimental points (12 cells of 5 preparations)

ing the Cl conductance significantly. Not until V was returned to 30 mV was the conductance deactivated.

The osmotic behavior of the m.r. cell was further analyzed by calculating the relative change of the steady-state volume as a function of the imposed change of the external osmotic concentration (Fig. 15). The graph shows that the relationship is well described by the following mathematical expression for an osmometer (Lucke & McCutcheon, 1932):

$$V/V_o = [(V_o - x)/V_o](\pi_o/\pi - 1) + 1 \quad (1)$$

where V_o and π_o are the initial cell volume and osmotic pressure, respectively, V and π are their new values following osmotic pertubation, and x is the osmotically inactive volume of the cell. From the slope of the regression equation, we have:

$$(V_o - x)/V_o = 0.79$$

from which we obtain, $x/V_o = 0.21$. Therefore, under isosmotic conditions and with the apical membrane's Na permeability blocked by amiloride, the osmotically inactive volume amounts to about 21% of the total m.r. cell volume.

The results of these experiments do not imply that the m.r. cells are incapable of volume regulation. Cells exposed to amiloride on the outside and clamped at 0 or 30 mV have almost emptied their Cl pool (Figs. 3 and 11). Osmotical swelling of the m.r. cells under these conditions, therefore, is not expected to induce fast regulatory volume decrease which in the principal cells is associated with loss of Cl (Ussing, 1982*a*). In the principal cells of frog skin, regulatory cell volume increase in response to an increase in tonicity of the bathing solutions is associated with activation of a Na-dependent, Cl cotransport system in the serosal membrane (Ussing, 1982*a*; 1985). If a mechanism of volume regulatory volume increase is present also in the m.r. cells, the time course of its activation is long as compared with the time scale of the present experiments.

Discussion

It is a prerequisite for a method that uses volume changes of a minority cell type for exploring its membrane transport pathways that the cell does not communicate with the principal cells through intercellular junctions. There is good evidence that this condition is fulfilled for the amphibian skin. (i) The steady-state Cl concentration of principal cells is two to three times that of m.r. cells (Rick et al., 1980; 1984). (ii) The addition of antidiuretic hormone results in a significant increase in the Na concentration of the principal cells with no detectable changes in the Na concentration of m.r. cells (Rick et al., 1984). (iii) Under open circuit conditions, the volume of principal cells of frog skin does not respond to removal of external Cl (Ussing, 1982*a*), however, the m.r. cell volume decreases (Spring & Ussing, 1986). (iv) The principal cell volume remains constant (MacRobbie & Ussing, 1961), and the m.r. cells swell (Spring & Ussing, 1986) following exposure of the apical side of frog skin to a hypotonic solution. The above studies demonstrate that, at steady state, the two cell types maintain large differences in intracellular ion concentrations and that a change in composition and volume of one cell type does not lead to a similar change in composition and volume of the other. It is strongly indicated, therefore, that the m.r. cells and principal cells of amphibian skin are not coupled to one another.

The Cellular Chloride Concentration under Short-Circuit Conditions

Following bilateral exposure of the short-circuited epithelium to a Cl-free solution, the m.r. cell volume decreased (Fig. 3). As these experiments were performed under isosmotic conditions and assum-

ing that the Cl ions leave the cell together with monovalent ions (Na and K), only, the initial cellular Cl concentration, Cl_c, can be estimated by:

$$Cl_c = C_o(V_1 - V_2)/[2(V_1 - x'V_2)] \quad (2)$$

where V_1 and V_2 are the initial and final steady-state volume, respectively, C_o is the concentration of an isosmotic NaCl solution (C_o = 115 mM), and x' is the fraction of the osmotically inactive volume of the Cl-depleted cell (x' = 0.21, Fig. 15). Applying Eq. (2) to the individual experiments, we calculate, Cl_c = 19.8 ± 1.7 mM (n = 6). This value is comparable with the Cl concentration of m.r. cells of frog skin, Cl_c = 14 mM (Rick et al., 1984), and toad skin, Cl_c = 25 mM (Rick et al., 1980), measured with X-ray microanalysis.

Exposure of the tissue to 50 μM amiloride resulted in a significant loss of cell water (Fig. 2). This concentration of amiloride completely eliminated the apical Na conductance of the principal cells (Materials and Methods). An amiloride-sensitive Na/H exchanger has been found in the plasma membrane of both cellular and epithelial preparations. Inhibition of this electroneutral transport system, however, requires an amiloride concentration of one to two orders of magnitude higher than the apical Na conductance of high resistance epithelia (Benos, 1982). Our results indicate, therefore, that the amiloride-inhibitable apical pathway of the m.r. cells is a passive conductance. Since exposure of the tissue to a Na-free apical solution also resulted in a significant loss of cell water (Fig. 2) we conclude that the effect of amiloride is not caused by a direct effect on the Cl permeability, but associated with elimination of the apical Na conductance. At the new steady state the cell volume was not significantly different from the Cl-free volume of the cell (Fig. 3). Thus, following elimination of the apical membrane's Na conductance, the intracellular diffusible Cl ion pool becomes virtually zero. Such a response is to be expected if the cellular Cl concentration is in thermodynamic equilibrium with the Cl concentration of the external bathing solutions. Hyperpolarization of the cell caused by the elimination of its passive Na conductance drives the Cl ions out of the cell, and at the new steady state the cellular Cl concentration is in Donnan equilibrium with the K concentration of the serosal bath, i.e., Cl_c is about 2 to 3 mM (the associated volume is too small to be detected with the present method). Our results indicate, therefore, that in the short-circuited skin the steady-state intracellular Cl concentration is set by the intracellular potential with minor, or no, contribution from an active transport mechanism.

The experiments where the cell volume was increased with ouabain showed that the Cl ions can be taken up from either side of the cell (Fig. 5, Table 2), indicating that under short-circuit conditions passive pathways are available in the apical as well as in the serosal membrane. This conclusion is supported by the observation of a significant Cl ion-specific transepithelial conductance at V = 0 mV (Fig. 7). Nevertheless, m.r. cell volume did not decrease following removal of Cl from the apical bath, i.e., at the new steady state the cell volume was not significantly different from the volume before removal of Cl_o (Table 3). Taken together with the observation that Cl ions were taken up from the apical solution following ouabain treatment (Fig. 5), this result indicates that apical membrane Cl permeability becomes virtually eliminated by removal of external Cl. A dependence of the Cl permeability of amphibian skin on Cl_o was suggested from permselectivity studies (Kirschner, 1970; Boulan et al., 1978) and analysis of the Cl_o dependence of unidirectional Cl fluxes (Mandel & Curran, 1972; Kristensen, 1978; 1982; Ques-von Petery et al., 1978; Harck & Larsen, 1986). At the new steady state, m.r. cell volume was not changed (Fig. 5; Table 3), indicating that it is the apical, rather than the cellular, Cl concentration which controls the apical membrane's Cl permeability.

It was previously shown that exposure of the frog skin to a Cl-free apical solution under open-circuit conditions resulted in a significant m.r. cell volume loss (Spring & Ussing, 1986). Under open-circuit conditions there is a steady-state net flux of Cl through the cell, and elimination of the Cl flux from the apical bath to the cell results in a net loss of Cl from the cell through the serosal membrane. In these studies the transepithelial potential was not measured, but it is known that replacement of the external Cl by a nonpermeant anion leads to a hyperpolarization of the epithelium (Koefoed-Johnsen & Ussing, 1958). The associated depolarization of the apical membrane should also decrease the passive Na flux from the apical bath to the cell. In this manner both the cellular Na and Cl pools would be depleted and consequently cell water lost.

The Active Na Flux through a Mitochondria-Rich Cell

With an amiloride-blockable Na-entry pathway in the apical membrane and an ouabain-sensitive Na-exit pathway in the serosal membrane, it follows that the m.r. cells are furnished with Na pathways typical for a Na-absorbing epithelial cell. Accordingly, the m.r. cells contribute to the short-circuit current of the skin. Since the ouabain-induced cell

swelling was prevented by pretreatment with amiloride (Fig. 4, Table 1) Na entry across the serosal membrane is probably insignificant. Thus, the initial rate of volume gain is a measure of the rate at which Na ions derived from the apical bath accumulate in the cell while K ions passively leave the cell through the serosal membrane. Due to the large conductance of the surface membrane of the m.r. cells, we may assume that the contribution of the electrogenic pump to the membrane potential is so small that it can be disregarded. Furthermore, if the passive K permeability of the serosal membrane is not affected directly by ouabain, initially following the ouabain inhibition of the pumps, the passive serosal K flux should not be significantly disturbed. This means that 2 K passively leave the cell into the serosal bath for each 3 Na entering the cell from the apical bath. Accordingly, the steady-state active Na flux J_{Na}^{active}, prevailing before ouabain inhibition of the pumps can be estimated by:

$$J_{Na}^{active} = 3\ C_o(\Delta V/\Delta t) \quad (3)$$

where $(\Delta V/\Delta t)$ is the initial rate of volume expansion, and C_o, as above, is the Na concentration of an isosmotic NaCl solution. The relative rate of volume gain of 3.98 ± 0.42% per min and the mean volume of 448 ± 36 μm^3 (Table 1) correspond to an absolute rate of volume gain, $(\Delta V/\Delta t) = 0.30 \pm 0.04$ $\mu m^3\ sec^{-1}$ from which we obtain [Eq. (3), $C_o = 115$ mM],

$J_{Na}^{active} = 0.10 \pm 0.01$ fmol sec^{-1} per m.r. cell.

The associated short-circuit current, I_{Na} (= FxJ_{Na}^{active}) is equal to 9.9 (±1.3) pA per m.r. cell ($n = 6$). With an average apical membrane area of 12 μm^2 this estimate corresponds to a current density of 83 $\mu A\ cm^{-2}$, which is three times larger than the current density of 23 $\mu A\ cm^{-2}$ for the whole epithelium (*see* Materials and Methods). However, with an average density of about 60,000 m.r. cells per cm^2 (Willumsen & Larsen, 1985) they contribute with a few percent, only, to the total short-circuit current of the skin.

The Na Dependence of Cl Fluxes in the Short-Circuited Skin

It is well established that elimination of the apical Na conductance of the short-circuited amphibian skin, either by amiloride (Candia, 1978; Kristensen, 1978) or by removal of the Na ions of the apical bathing solution (Macey & Meyers, 1963; Kristensen, 1978; Ques-von Petery et al., 1978), results in a significant reduction of the transepithelial unidirectional Cl fluxes. It was also found that the Na channel activator, BIG (benzimidazolylguanidin (Fuchs, Larsen & Lindemann, 1977)), when added to the apical solution, stimulates the Cl flux through the short-circuited frog skin (Kristensen, 1981). Based on these results it was suggested that Cl and Na ions pass through a common cellular compartment (Candia, 1978; Kristensen, 1981), interactions predicted by a three-compartment computer model with electrodiffusive Cl pathways in the apical and the serosal membrane. Such effects were predicted whether the model contained fixed Cl permeabilities in both membranes (Kristensen, 1982), or incorporated a voltage-dependent Cl permeability in the apical membrane (Larsen & Rasmussen, 1985).

The present study has shown that the m.r. cell, under short-circuit conditions, possesses Cl ion-specific permeabilities in the apical as well as in the serosal membrane. We have also shown that the m.r. cells transport Na actively and that the sodium permeability of the m.r. cell apical membrane is amiloride sensitive. Thus, the hypothesis that Cl equilibrium fluxes flow through a cellular compartment which also transports Na is verified by the present study.

Previous experiments produced the puzzling result that although the short-circuit current was eliminated almost immediately following amiloride exposure, the inhibition of the Cl conductance developed slowly over a period of several minutes (Kristensen, 1983; Nagel, Garcia-Diaz & Essig, 1983). Such a difference in the time courses for the inhibition of the Na current and the Cl conductance was also predicted by the computer model (Larsen & Rasmussen, 1985). The effects of amiloride application on steady-state Cl fluxes as well as on the time course for the inhibition of the Cl ('shunt') conductance may now be explained as resulting from amiloride block of the m.r. cell apical membrane's Na channels. Delayed inhibition of the Cl conductance by amiloride does not require direct interaction of amiloride with the Cl channels.

The Voltage-Activated Cl Conductance

In a previous study it was found that clamping the frog skin to −150 mV (mucosa negative) results in a significant increase in m.r. cell volume (Foskett & Ussing, 1986). In the present study it was shown that this response is also typical of m.r. cells of the toad skin submitted to voltage clamping in the physiological range of transepithelial potentials (Figs. 10, 11, 12). Furthermore, it was shown that the time course for the development of the voltage-activated

Cl current was similar to the time course of cell volume increase, and that both responses were absent in preparations exposed to a Cl-free apical solution. These results lead to the conclusion that a Cl conductance is localized to the apical membrane of the m.r. cells. This was also the conclusion from studies using the vibrating probe technique for the localization of the Cl_o-dependent clamping current (Foskett & Ussing, 1986; Katz & Scheffey, 1986), and from a microelectrode study of identified cells of toad skin epithelium (Willumsen & Larsen, 1986). The principal cells were found to possess virtually no apical Cl conductance. However, the Cl currents (at $V = -100$ mV) and the m.r. cell densities of the preparations were found to be proportionally related. The low epithelial sulfate permeability of $1.6(\pm 0.3)\ 10^{-9}$ cm sec^{-1} (Harck & Larsen, 1986) and a similar low Cl permeability of $9.7(\pm 1.5)\ 10^{-9}$ cm sec^{-1} of toad skin exposed to a Cl-free apical solution (Bruus et al., 1976) indicate a very low junctional membrane anion conductance. Thus, the m.r. cells constitute the only significant physiological pathway for the influx of Cl ions driven by the transepithelial electrical potential difference.

The activated Cl conductance was virtually unaffected by osmotically imposed cell volume changes. These results show that the Cl conductance activation does not result from the cell volume increase but from the potential change, probably depolarization of the apical membrane of the m.r. cell. Voltage-dependent Cl channels which are activated by membrane depolarization have been found in the serosal membrane of the rabbit urinary bladder (Hanrahan, Alles & Lewis, 1985), and in the apical membrane of A6 cells, derived from amphibian kidney (Nelson, Tang & Palmer, 1984)). A similar voltage-dependent Cl permeability is also present in the serosal membrane of the principal cells of frog skin (Ussing, 1986). This type of Cl pathway exhibits a weak anion selectivity (Hanrahan et al., 1985; Ussing, 1986); which also characterizes the voltage-dependent transepithelial Cl conductance of the present study (Kristensen, 1982; Harck & Larsen, 1986).

In the present experiments, since the apical membrane Na conductance was blocked with amiloride and the passive Na permeability of the serosal membrane was shown to be vanishingly small, we hypothesized that the cation accompanying the Cl uptake was potassium derived from the serosal bath. Support for this hypothesis came from the finding that m.r. cell volume increase was slowed down significantly when the serosal bath Ringer's contained 0.5 mM K (Figs. 12, 13). The rate of volume change was not affected by raising the serosal K concentration above the concentration of 2.4 mM of the standard Ringer's solution (Fig. 13), suggesting that, at a physiological serosal K concentration, the rate-limiting process for the development of the new steady state is the uptake of Cl from the apical bath. As previously discussed (Larsen & Rasmussen, 1985), in the absence of Na conductance of the apical membrane, the steady-state volume of the hyperpolarized cell is expected to be determined by the ratio of the apical and the serosal membrane's Cl conductances. Thus, a measurable cell volume gain in response to hyperpolarization, e.g., 0 to -100 mV, leads to the conclusion that, at the new steady state, the apical membrane's Cl conductance exceeds that of the serosal membrane. The slow increase of the m.r. cell volume following hyperpolarizing voltage clamp probably reflects the slowly increasing Cl conductance of the apical membrane.

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