Loss of cell volume regulation during metabolic inhibition in renal epithelial cells (A6): role of intracellular pH

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Received 3 August 2001; accepted in final form 26 March 2002

Smets, Ilse, Marcel Ameloot, Paul Steels, and Willy Van Driessche. Loss of cell volume regulation during metabolic inhibition in renal epithelial cells (A6): role of intracellular pH. Am J Physiol Cell Physiol 283: C535–C544, 2002.—In renal ischemia, tubular obstruction induced by swelling of epithelial cells might be an important mechanism for reduction of the glomerular filtration rate. We investigated isometric cell swelling by examining volume regulation of A6 cells during metabolic inhibition (MI) induced by cyanide and 2-deoxyglucose. Changes in cell volume were monitored by recording cell thickness (Tc). Intracellular pH (pHc) measurements were performed with the pH-sensitive probe 5-chloromethyl-fluorescein diacetate. Tc measurements showed that MI increases cell volume. Cell swelling during MI is proportional to the rate of Na+ transport and is not followed by a volume regulatory response. Furthermore, MI prevents the regulatory volume decrease (RVD) elicited by a hyposmotic shock. MI induces an intracellular acidification that is conserved during a subsequent hypotonic shock. A transient acidification induced by a NH4Cl prepulse causes a marked delay of the RVD in response to a hypotonic shock. On the other hand, acute lowering of external pH to 5, simultaneously with the hypotonic shock, allowed the onset of RVD. However, this RVD was completely arrested 10 min after the initiation of the hypotonic challenge. The inhibition of RVD appears to be related to the pronounced acidification that occurred within this time period. In contrast, when external pH was lowered 20 min before the hypotonic shock, RVD was absent. These data suggest that internal acidification inhibits cellular volume regulation in A6 cells. Therefore, the intracellular acidification associated with MI might at least partly account for the failure of volume regulation in swollen epithelial cells.

distal tubular epithelial cell line; chemical ischemia; cyanide; 2-deoxyglucose; pH-sensitive fluorescent probe 5-chloromethyl-fluorescein diacetate; hyposmotic shock; regulatory volume decrease

A FUNDAMENTAL PROPERTY of animal cells is the ability to maintain cell volume constant. Cellular volume is determined ultimately by two factors: the total cell content of osmotically active particles and the osmolality of the bathing medium. The principal threat to cell volume in vivo is a change in the amount of osmotically active molecules in cells bathing in isosmotic solutions. A change in total solute content of cells can occur if the balance is altered between solute entry and extrusion from the cells. A dramatic example of such isosmotic swelling in vivo occurs during ischemia. In ischemic renal injury, cellular ATP depletion inhibits the basolateral Na+ pump that normally maintains constant cell volume by offsetting the tendency of cells to swell because of impermeant cellular solutes. The resulting swelling of tubular cells is an early, important step in the development of tubular necrosis. It has also been proposed that cell swelling contributes to renal dysfunction by leading to obstruction of the tubular lumen and, thus, to a decrease of glomerular filtration rate (GFR) (2). Furthermore, cell swelling occurs at the expense of vascular space, and the compression of vessels may impede reflow through injured tissue (18). The understanding of epithelial cell volume regulatory mechanisms and their modulation by ischemia could be extremely valuable for designing novel therapeutic strategies to improve organ function, e.g., in transplants.

In this study, polarized A6 epithelia derived from the distal part of the nephron of the kidney of Xenopus laevis were used as model cells. The A6 cell line is a well-established cell line with transport properties specific for mammalian collecting duct cells (30). The inhibition of cellular energy metabolism was used as an experimental model to simulate ischemic cell injury and was realized by inhibiting both cellular glycolysis (with 2-deoxyglucose) and oxidative phosphorylation (with cyanide). The aim of the present study was to examine whether metabolic inhibition (MI) causes swelling of these distal epithelial cells and, if so, whether the cells can readjust their volume by a mechanism known as regulatory volume decrease (RVD). Furthermore, we investigated whether and how MI interferes with cell volume control mechanisms in anisotonic solutions. In most cell types, including A6 cells (8), exposure to a hypotonic solution elicits a RVD that is accomplished mainly by KCl efflux through
specific volume-activated K⁺ and Cl⁻ channels, different from the native K⁺ and Cl⁻ channels. A fall in intracellular pH (pHᵢ) occurs in most cells exposed to ischemic conditions (2). Recently, it was reported that internal protons are able to inactivate apical epithelial Na⁺ channels (ENaCs) (14, 41) and the native basolateral K⁺ channels (14) in A6 cells. Although activation and regulation of volume-activated channels have been studied in detail, data on proton modulation are rather scarce. In freshly isolated S2 segments of renal proximal tubules of rabbit kidney (34), it was shown that a rise in PCO₂ of the perfusate reduces the rate of volume regulation in response to a hypotonic shock without affecting the extent of the RVD response. Moreover, it was shown that lowering of the external perfusate pH exhibits an RVD-inhibitory effect in A6 cells (21). Neither study distinguished between the effects of extracellular acidification and the intracellular acidosis associated with the acidifying maneuver on the observed RVD retardation. Some very recent reports indicate pHₗ-sensitive volume-activated K⁺ channels in primary cultures of seawater fish gill cells (11), in villus epithelial cells (22), and in Ehrlich ascites tumor cells (15). Therefore, we verified whether intracellular protons might inhibit RVD, possibly via inhibition of volume-activated ion channels.

This report shows that cell swelling during MI depends on the salt transport rate of the epithelial cell. We demonstrate that MI inhibits the RVD in response to isosmotic cell swelling (due to the MI itself) as well as to anisosmotic cell swelling (elicited via a reduction of the extracellular osmotic pressure). Furthermore, our findings suggest that the intracellular acidification that accompanies MI might play a role in RVD inhibition.

**MATERIALS AND METHODS**

**Cell Culture**

A6 cells (passes 105–111) obtained from Dr. J. Johnson (University of Pittsburgh, PA) were cultured at 28°C in a humidified incubator in the presence of 1% CO₂ in the air (for details, see Ref. 36). A6 cells were seeded on a permeable support (25 mm in diameter; Nunc Anopore; details, see Ref. 36). A6 cells were seeded on a permeable support (25 mm in diameter; Nunc Anopore; details, see Ref. 36).

**Cell Volume Measurements**

This method has been described previously in detail (37). Briefly, tissues were mounted in an Ussing-type chamber enabling solution exchange on both sides. Cell thickness (Tₑ) was used as an index for cell volume of confluent monolayers. The apical (upper) side of the monolayer was labeled with fluorescent biotin-coated microbeads. Focusing of the microbeads was automatically performed with a piezoelectric focusing device (PIFOC; Physik Instrumente, Waldbronn, Germany). Tₑ is defined as the vertical distance between the basolateral and apical beads. Measured Tₑ values were corrected for the diameter of the fluorescent microbeads by subtracting 1 μm. Changes in cell height are expressed as a percentage of the value recorded just before the hypoosmotic challenge or metabolic inhibition was imposed. Average values of Tₑ were calculated from the recordings from a number of beads (nB) that remained attached to the monolayer during the entire experiment.

The tissues were short-circuited during the entire course of the experiment by using Ag-AgCl voltage and current electrodes that were connected to the bath solutions with agar bridges containing 3% agar in 1 M KCl medium. Transepithelial conductance (Gₑ) and short-circuit current (Iₑ) were recorded. Iₑ mainly reflects transepithelial Na⁺ absorption in A6 cells because, in general, Iₑ disappears in the presence of 0.1 mM apical amiloride.

**Fluorescence Imaging Microscopy to Measure pH**

**With CMFDA**

**Use of CMFDA as a pH-sensitive probe.** CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes, Eugene, OR) is an analog of 2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Except for one abstract in which the use of CMFDA as a pH probe is mentioned without details (28), this is, to our knowledge, the first full report illustrating the suitability of CMFDA for pHₑ measurements. CMFDA shows pH-dependent spectral shifts in a somewhat more acidic cytosolic pH range than BCECF because the pKₐ is ~6.4 (determined for the unconjugated hydrolyzed product in buffer (after conjugation to an intracellular thiol or amine, the pKₐ value may be different) compared with a pKₐ of ~7 for BCECF. The advantage of CMFDA is that it accumulates and retains far better into A6 cells than BCECF. Once the membrane-permeant form of the probe enters a cell, esterase-mediated hydrolysis converts nonfluorescent CMFDA to fluorescent 5-chloromethylfluoresceine, which can then react with intracellular thiols to yield well-retained products (16). Cytotoxic effects caused by this intracellular reaction are unlikely, because many cell types loaded with CMFDA remain viable for at least 24 h after loading and often through several cell divisions (1).

At first, we checked whether this fluorescein derivative was a suitable pH probe for A6 cells. CMFDA calibrations were performed as shown for one typical experiment in Fig. 1A. CMFDA was used in the dual-excitation (495 and 440 nm) mode (for details, see Use of fluorescence imaging microscopy to measure pHₑ). Intracellular pH was forced to equilibrate with the external pH according to the method of Thomas et al. (35). Cells were exposed to solutions containing 137 mM KCl, 1 mM CaCl₂, 10 mM HEPES, and 13 μM nigericin. The K⁺ concentration used approximates the reported cytosolic K⁺ concentration in A6 cells (25). Different pH values of the solution (pHₛ) were obtained with Tris. The CMFDA fluorescence (F) ratio R = F(495)/F(440) vs. the different imposed pHₛ values is depicted in Fig. 1B.

Second, pHₑ values of A6 cells measured with the commonly used pH dye BCECF were compared with those measured with CMFDA. Table 1 reveals that both dyes indicate similar absolute pH values in control conditions as well as after an acidifying treatment. Therefore, CMFDA is a reliable pHₑ indicator. Moreover, the improved cellular retention of the fluorescent probe allowed us to perform long-lasting experiments (5–6 h) and overcome the cell leakage problem encountered when using BCECF.
cells were loaded from the apical side with the probe CM-
fluorescein diacetate (CM-FDA). After the background signal was measured, tissues were mounted in an Ussing-type chamber (chamber for details). The different symbols indicate FDA).

![Graph A](image1)

**Fig. 1.** pH-sensitivity of 5-chloromethylfluorescein diacetate (CM-FDA). A: ratio of CMFDA fluorescence intensities (F) measured at 535 nm due to excitation at 495 and 440 nm, F(495)/F(440), is shown at various pH values for 1 representative example of a CMFDA calibration. Intracellular pH (pHi) was forced to equilibrate with the solution pH (pHios), according to the method of Thomas et al. (35) (see MATERIALS AND METHODS for details). The different symbols indicate the cellular response in 6 different regions in the monolayer. B: the 6 imposed pH values in A and their corresponding CMFDA ratios for 1 representative region are plotted and fitted (solid line) in B.

**Use of fluorescence imaging microscopy to measure pHi.** A6 tissues were mounted in an Ussing-type chamber (chamber opening 0.7 cm²). After the background signal was measured, cells were loaded from the apical side with the probe CM-FDA. Cells were then exposed to a final concentration of 10 μM of the acetoxymethyl ester form of the dye (stock solution 5 mM in DMSO). Loading was performed for 60 min at room temperature in apical control Ringer solution (Table 2). After loading, excessive dye was removed by replacing the apical bath solution several times. During the experiment, monolayers were continuously superfused (0.75 ml/min) on both sides. The fluorescence was measured with an inverted epifluorescence microscope Zeiss Axiovert 100 (Jena, Germany). Excitation light of a 75-W xenon lamp (Osram, Berlin-München, Germany) was filtered at 440 and 495 nm with excitation filters (bandwidth of 10 nm; Chroma Technology, Brattleboro, VT), which were inserted in a computer-controlled filter wheel (Lambda 10–2; Sutter Instruments, Novato, CA). The fluorescence collected by the objective (Zeiss LD Achromplan ×20/0.4 corr.) was transmitted through a >500-nm long-pass dichroic mirror and a 535/50-nm band-pass emission filter (Chroma Technology) to a Quantix charge-coupled device (CCD) camera (Photometrics, Tucson, AZ). The camera was equipped with a Kodak KAF 1400 CCD (grade 2, MPP) with 1317 × 1035 pixels and cooled to −25°C by a thermoelectric cooler. The acquisition of pairs of images for this dual-excitation radiometric dye, CMFDA, was controlled by a homemade program that uses V for Windows software (Digital Optics, Auckland, New Zealand). Camera exposure time for 1 image was 1 s. Signals were obtained by spatially integrating pixels over the field of view. The background image, because of reading noise and the dark current of the CCD camera and autofluorescence of tissue and tissue support, was automatically subtracted pixel by pixel from the image of the loaded cells. At the end of each experiment, a calibration was performed by using the nigericin-high-K+ technique (35) as described earlier. Heterogeneity in the cellular response of different regions in the monolayer was checked after each experiment with a homemade program that uses V for Windows software. This analysis revealed that no significant differences existed in the cellular response of different regions in one monolayer to pH-changing manipulations (Fig. 1A).

**Statistics**

Values from n experiments (different monolayers) are given as means ± SE.

**Solutions and Chemicals**

The compositions of the solutions used in this study are given in Table 2. MI was induced by bilateral sodium cyanide (CN, 2.5 mmol/l) and 2-deoxy-d-glucose (2-DG, 20 mmol/l). The mitochondrial substrate pyruvate was substituted for glucose in the recovery solution after MI because it has been reported that 2-DG has an irreversible action on cellular glycolysis (20).

Isosmotic solutions had an osmolality of 260 ± 4 mosmol/kg H₂O, which is the osmolality of the growth medium for the cells. Hyposmotic solutions had an osmolality of 140 ± 4 mosmol/kgH₂O. All apical solutions in Figs. 3–8 were hyposmotic to avoid an osmotic gradient from apical to basolateral side during the basolateral hypoxic challenge. Reduction of apical osmolality does not alter the volume of A6 cells (9). The osmolality of the solutions was verified with a cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany).

Ethylisopropylamiloride (EIPA) was purchased from Research Biochemicals (Natick, MA). CN was from UCB (Brussels, Belgium). 2-DG, nigericin, omeprazole, magnesium ni-

![Graph B](image2)

**Table 1. Comparison of pHc values obtained with CMFDA and BCECF**

<table>
<thead>
<tr>
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<th>CMFDA</th>
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<td></td>
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<tr>
<td>Control</td>
<td>7.00 ± 0.03</td>
<td>14</td>
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<tr>
<td>After 45 min</td>
<td>6.44 ± 0.04</td>
<td>6</td>
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<td>CN ± 2-DG</td>
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Intracellular pH (pHi) values indicated are means ± SE for n different monolayers. The compositions of the control Ringer and the Ringer with 2.5 mM NaCN (CN) in combination with 20 mM 2-deoxy-d-glucose (2-DG) are given in Table 2. THE pH of both solutions was 7.4; and osmolalities were 200 mosmol/kgH₂O. CMFDA, 5-chloromethyl fluorescein diacetate; BCECF, 2’,7’-bis(2-carboxyethyl)-5(6)-carboxy fluorescein.
between high- and low-rate Na\(^{+}\)-transporting epithelia. We found a striking difference in cell volume regulation after metabolic inhibition in these epithelia resulted in a progressive increase of cell volume, reaching a plateau value of 120% within 25 min (Fig. 2B). Perfusion with recovery Ringer for 30 min allowed the cells to partially recover to 107%.

Figure 2 illustrates the behavior of low-rate Na\(^{+}\)-transporting epithelia (1.7 \(\pm\) 0.2 \(\mu\)A/cm\(^2\), \(n = 5\)) during a basolateral hypotonic shock from 260 to 140 mosmol/kgH\(_2\)O and subsequent perfusion with isotonic Ringer. When basolateral osmolality was lowered, A6 cells swelled quickly due to basolateral water influx. Subsequently, this volume increase was rapidly counteracted by a RVD, accomplished by the loss of K\(^{+}\) and Cl\(^{-}\) (8) and, to a lesser extent, of amino acids (7). The behavior of metabolically inhibited cells with a low rate of Na\(^{+}\) transport (2.7 \(\pm\) 0.2 \(\mu\)A/cm\(^2\), \(n = 5\)) during a basolateral hypotonic shock is indicated with a solid line. A 45-min preincubation period with CN and 2-DG did not alter the cell volume significantly (see also Fig. 2B), but a subsequent hypotonic shock revealed the complete disappearance of RVD. Ischemic cells, thus,
METABOLIC INHIBITION AND CELL VOLUME REGULATION

A

Fig. 2. Effect of metabolic inhibition (MI) on cell thickness ($T_c$) of A6 epithelia. A: during MI, $T_c$ increases up to 120% in tissues with high rates of Na$^+$ transport (22.3 ± 0.8 μA/cm$^2$) (solid line). Tonicity of the Ringer solution was 200 mosmol/kgH$2$O at both sides. Initial absolute $T_c$ value was 9.32 ± 0.33 μm ($n = 6$, no. of beads $n_B = 92$). Dotted lines represent SE. B: $T_c$ response to MI for low-rate salt-transporting (2.8 ± 0.3 μA/cm$^2$) epithelia. Initial absolute $T_c$ value was 9.15 ± 0.27 μm ($n = 4$, $n_B = 54$). Tonicity of the Ringer solution was 260 mosmol/kgH$2$O. CN, cyanide; 2-DG, 2-deoxy-d-glucose.

Effect of Metabolic Inhibition With Minimized Na$^+$ Influx on RVD

One possible explanation for the absence of RVD seen in ischemic cells might be that the inhibition of active Na$^+$ excretion from the cell leads to an accumulation of Na$^+$ in exchange for K$^+$. Because the swelling-activated cation channels in A6 cells are impermeable to Na$^+$ (21), cellular K$^+$ depletion will impede cell volume regulation. To test the role of K$^+$ depletion in the inhibition of RVD after MI, we designed a protocol to minimize Na$^+$ influx into the cells. First, apical Na$^+$ was replaced with N-methyl-d-glucamine (NMDG$^+$). This apical Na$^+$ uptake-abolishing manipulation did not affect cell volume in isotonic conditions (9). Second, the basolateral Na$^+$ concentration was lowered to 10 mM to reduce the chemical driving force for Na$^+$ influx. In this protocol, metabolic inhibition was realized with 2-DG (20 mM) at both sides of the monolayer and CN (2.5 mM) exclusively at the basolateral side to maintain Na$^+$-free conditions at the apical side. The solid line in Fig. 4 illustrates the behavior of cells that were exposed to CN and 2-DG 45 min before and during the hypotonic challenge. Although Na$^+$ accumulation and K$^+$ depletion were prevented, these ischemic cells still failed to downregulate their volume after a hypotonic shock. Because the reduction of the basolateral Na$^+$ concentration had a pronounced volume decreasing effect in isotonic conditions, we checked to see whether this manipulation as such had an influence on RVD evoked by a hypotonic shock. However, RVD was still present in cells that were superfused for 75 min with a basolateral Ringer that contained only 10 mM Na$^+$. These results suggest that another MI-associated phenomenon must play a role in RVD inhibition.

Intracellular Acidification During Metabolic Inhibition in A6 Cells

Recently, we showed that intracellular protons are able to close Na$^+$ channels in A6 cells (41). As a consequence, the hypothesis arose that intracellular pro-
tons might also inhibit volume-activated K⁺ or Cl⁻ channels that are responsible for RVD. To verify this hypothesis, pHc was monitored during a 45-min period of MI. As shown in Fig. 5, the mean pHc for 7 different monolayers displayed a pronounced acidification from pHc 6.93 ± 0.04 to 6.54 ± 0.02 during MI. Because this acidification was maintained during the subsequent basolateral hypotonic shock (n = 4), intracellular protons are indeed candidates for interaction with volume-activated channels that are responsible for the RVD.

Influence of Intracellular Acidification on Volume Recovery After a Hyposmotic Challenge

Because pHc changes affect transepithelial Na⁺ transport (INa) changes in A6 cells (41) and even a small INa has an inhibiting effect on RVD (9), the effects of pHc on RVD were examined in nontransporting conditions in which NaCl was replaced with NMDGCl in the apical saline. As mentioned earlier, this apical Na⁺ uptake-abolishing manipulation does not affect cell volume in isosmotic conditions (9).

Intracellular acidification via the NH₄Cl prepulse method in combination with EIPA. First, the NH₄Cl prepulse technique was used to acidify the cells without changing external pH. The basolateral NH₄Cl pulse was performed under strict and volume-controlled experimental conditions (41): 40 mM sucrose in the basolateral control saline was isotonically replaced with 20 mM NH₄Cl for 25 min. Subsequently, cells were bilaterally superfused for 15 min with control saline containing 50 μM EIPA that arrests the basolateral Na⁺/H⁺ exchanger (3). As shown in Fig. 6A, this method evoked an intracellular acidification from pHc 7.00 ± 0.08 to 6.4 ± 0.1 (n = 4). Although EIPA was used, the cells gradually recovered from this pronounced acidification during the subsequent basolateral hypotonic shock (pHc = 6.8 ± 0.1 after 20 min).

Further attempts, including the use of sodium propionate (30 mM), the Na⁺/H⁺ exchanger blockers HOE-642 (10 μM) and S-3226 (10 μM), and the combination of the inhibitors EIPA (50 μM), omeprazole (0.1 mM), and magnesium nitrate (10 mM) to block, respectively, the Na⁺/H⁺ exchanger and the putative K⁺/H⁺-ATPase and H⁺-ATPase, to keep the cells acidic for a longer period failed (results not shown). In general, cell swelling leads to cytosolic acidification (19). Nevertheless, we investigated in control experiments whether the basolateral hypotonic treatment as such was not a pHc-increasing manipulation in A6 cells. If this were the case, it would neutralize previous acid-inducing maneuvers. Replacement of basolateral isotonic Ringer with a hypotonic Ringer solution for 1 h induced no...
significant $p_{Hc}$ change in 6 different monolayers (Fig. 6A). $T_c$ experiments (Fig. 6B) reveal that a hypotonic challenge still elicits a complete RVD in cells that were previously acidified to $pH_{6.4}$ with the NH$_4$Cl prepulse technique. However, RVD was profoundly slowed down during the first 20 min compared with control experiments. This delay in RVD might be attributed to the presence of intracellular protons that gradually leave the cells during the hypotonic shock.

Intracellular acidification via lowering of $pH_{sol}$. Second, $pH_c$ was modified by lowering $pH_{sol}$ at both sides of the epithelium. As indicated in Fig. 7A, a 30-min exposure period to $pH_{6.0}$ reduced the intracellular $pH$ from 7.06 ± 0.02 to 6.59 ± 0.03 ($n = 6$). A subsequent basolateral hypotonic shock of 60 min acidified the cells further to $pH_c = 6.3 ± 0.2$ ($n = 3$). These acidified cells still elicited an RVD that was, however, partially inhibited as shown in the $T_c$ measurements of Fig. 7B. To obtain a more pronounced $pH_c$ drop (from 6.99 ± 0.06 to 6.15 ± 0.07, $n = 6$), other monolayers were superfused for 20 min with a Ringer of $pH_{sol} 5$ (Fig. 7A). The acidifying process continued during the hypotonic shock. Figure 7B shows that, under these conditions, RVD was completely inhibited. The fact that the shrinking of cells still occurred when isotonic Ringer was applied demonstrates that this very low $pH_{sol}$ did not damage cell membranes. Moreover, the mean transepithelial conductance for these acidic monolayers did not exceed a value of 0.85 mS/cm$^2$ during this experimental protocol.

To verify whether the RVD inhibition was due to the pronounced intracellular acidification or simply caused by the action of external protons, it was necessary to distinguish between chronic and acute treatment with an external acidic solution ($pH_{sol} 5$). Chronic treatment refers to the protocol described earlier in which external $pH$ was lowered 20 min before and during the hypotonic challenge (Fig. 7), whereas acute treatment involves lowering of external $pH_{sol}$ simultaneously with the hypotonic shock. If protons were to exert their inhibitory action on RVD from the extracellular side, acute administration of protons would allow a rapid blockage of RVD. On the contrary, it took at least 10 min to arrest the RVD as depicted in Fig. 8A. The $pH_c$ experiments in Fig. 8B reveal that, in the first 10 min of acute treatment with a $pH_{sol}$ of 5, $pH_c$ drops from 6.99 ± 0.05 to 6.39 ± 0.06 ($n = 5$). The clear delay in RVD blockage in acutely treated cells might indicate the necessity for a pronounced intracellular acidification for RVD inhibition.

**DISCUSSION**

In the present study, MI with CN and 2-DG was used as an experimental model to simulate ischemic cell injury. We investigated the effects of MI on cell volume and cell volume regulation of a tight epithelium, A6.

Metabolic Inhibition Induces Cell Swelling in High-Rate Na$^+$-Transporting A6 Epithelia

Under steady-state conditions, epithelial cell volume is maintained by balancing the rates of apical and basolateral ion transport in such a way that intracellular solute content remains constant. When MI is applied, cellular ATP depletion will hinder the active basolateral Na$^+$ extrusion from the cells. In A6 epithelia with high rates of Na$^+$ transport (Fig. 2A), the reduced pump rates during MI were insufficient to match massive apical Na$^+$ influx. During the first 25 min of ischemia, cell volume gradually increased up to a plateau value of 120%. A further rise of cell volume was probably prevented by a reduction of apical Na$^+$ entry by the action of intracellular protons (41) elicited during MI (Fig. 5). Hence, it seems that the plateau phase in Fig. 2A is comparable with the situation in low-rate salt-transporting epithelia (Fig. 2B). There, the hindered Na$^+$ pump was still able to offset apical Na$^+$ influx, and,
Absence of Anisosmotic Volume Regulation in Metabolically Inhibited Cells

This study further explored the capacity of distal epithelial cells (A6) to regulate cellular volume during MI by challenging them with a basolateral hypotonic shock. After swelling, nonischemic A6 cells exhibit a RVD by K⁺ and Cl⁻ efflux through volume-activated channels (8) and, to a lesser extent, by extruding amino acids (7). Our results demonstrate that CN⁻ and 2-DG-treated A6 cells completely lost the ability to regulate their volume after a basolateral hypotonic shock. This result is consistent with previous reports in which RVD was completely inhibited in hypotonically suspended glial cells in anoxic conditions in the presence of iodoacetate (17) or partially inhibited by inhibitors of mitochondrial electron transport in cerebral astrocytes (27). Because the activation of volume-regulated anion channels in different cell types is known to be ATP dependent, either via direct interaction of ATP with the channel (26, 29) or via ATP-dependent phosphorylation steps (5, 23, 31), the lack of ATP might underlie RVD inhibition by inactivation of volume-activated Cl⁻ channels in A6 cells.

In addition, cellular ATP depletion during MI leads to reduced activity of the Na⁺ pump. Experiments that were designed to minimize Na⁺ influx and thus to avoid K⁺ depletion during MI revealed that RVD was still impaired (Fig. 4). Hence, RVD inhibition in metabolically inhibited A6 cells cannot be ascribed solely to intracellular K⁺ depletion.

Role of Intracellular pH on RVD Inhibition

This study indicates that MI is associated with a pronounced intracellular acidification in A6 cells (Fig. 5). A fall in intracellular pH occurs in most cells exposed to ischemic conditions (for review, see Ref. 2). Because we used 2-DG to block cellular glycolysis, the fall in pHc was not due to increased lactate production. Acidosis resulted probably from the imbalance between proton accumulation due to ATP hydrolysis (13, 32, 38, 39) and the constant passive influx of protons on one hand, and the suppression of H⁺ extruding transport processes in ATP depleted cells on the other hand (10).

Li et al. (21) have shown that lowering the external pHsol exhibits an RVD-inhibitory effect in A6 cells. Our data suggest that this inhibitory action is exerted by internal rather than external protons. As shown in Fig. 6B, RVD was clearly delayed in A6 cells, acidified by means of the NH₄Cl prepulse technique, with normal external pHsol. Similarly, Sullivan et al. (34) found that the rate of volume regulation in response to hypotonic media was reduced in rabbit proximal tubules that were acidified by increasing the PCO₂ in the perfusate from 5 to 15%. Because the high PCO₂ treatment in those experiments lowered both the external pHsol (from 7.44 to 6.97) as well as pHc (from 7.39 to 7.08 in isotonic conditions), no distinction was possible between modulation of RVD by external and internal protons. Moreover, pHc was not measured during hypotonic conditions. Thus it is unknown whether pHc therefore, accumulation of intracellular osmolytes and cell swelling could be avoided during MI.

The cell swelling during MI in high-rate salt-transporting epithelia could also be a consequence of basolateral Na⁺ influx rather than apical Na⁺ influx as described above. A candidate for basolateral Na⁺ influx is the basolateral Na⁺/H⁺ exchanger (3) that is probably activated in these acidic cells (Table 1). However, this explanation is less plausible because low-rate salt-transporting epithelia (Fig. 2A) displayed no change in cell volume during MI, although these epithelia acidified to the same extent (Fig. 5), and, therefore, at least a similar activation of the basolateral Na⁺/H⁺ exchanger could be expected.

Absence of Isosmotic Volume Regulation in Metabolically Inhibited A6 Cells

High-rate salt-transporting A6 cells displayed no volume regulatory event after cell swelling due to MI. The similar behavior of Madin-Darby canine kidney cells during MI (preliminary experiments) indicates that the absence of cell volume regulation in metabolically inhibited cells, as described in this study, is not limited to amphibian epithelial cells.
remained acidic in hypotonic media with high PCO₂ pressure. In our study, the extra internal protons resulting from the NH₄Cl prepulse were extruded out of the cells in the first 20 min of the hypotonic shock, so their inhibitory action on RVD was limited in time. Further experiments are needed to elucidate the identity of the H⁺-extruding mechanism(s). In the work of Sullivan et al. (34), RVD was not complete except to the same extent as in control experiments. In contrast, we found in the present study that the RVD in acidified A6 cells was complete in both control and experimental conditions. It has been shown in A6 cells that the extent of RVD is dependent on the Na⁺ transport rate (9). Hence, the apparent discrepancy concerning the completeness of RVD in A6 cells and proximal tubular cells might be explained by the difference in their transport capacity.

Our results indicate that a complete and immediate inhibition of RVD after a hypotonic challenge is achieved only when the following conditions are fulfilled. First, the extra amount of internal protons needs to be trapped inside the cell. This was the case in ATP-depleted cells (Fig. 5), and in cells not ATP depleted, protons remained inside by lowering the external pHsol. Second, a profound acidification is necessary. This was evidenced by the fact that RVD was only retarded (Fig. 7B) in cells that were acidified to some extent (pHc 6.59). In contrast, an immediate and complete blockage of RVD was seen in cells that were profoundly acidified to pHc values as low as 6.15. Acute addition of external protons (pHsol 5), simultaneously with the hypotonic shock, could not inhibit RVD immediately (Fig. 8A). The clear delay in RVD blockage seen in acutely treated cells is accompanied with a rapid intracellular acidification (Fig. 8B). Although it is conceivable that external protons could also interact with the volume-regulated ion channels either directly or indirectly at a site unrelated to the pore, the slow time course of the effect of lowering external pHsol acutely on RVD gives evidence against this explanation. Recently, it has been shown that inactivation of cloned amphibian (derived from A6 cells) and mammalian (human) renal chloride channels (ClC-5) after exposure to external acidic solutions occurred very rapidly (within 1 min) (24). Therefore, the involvement of external protons in RVD inhibition is less likely.

In the present study, the mechanism of RVD inhibition by internal protons in A6 cells was not identified. Further experiments are needed to resolve this issue. Native basolateral K⁺ channels are pHc sensitive in A6 cells (14). The closure of these channels in acidified cells does not imply RVD inhibition because, in many cell types including A6 cells, Cl⁻ and K⁺ efflux occurs through a separate set of volume-activated ion channels (6, 8). Although the underlying molecular mechanism of the inhibitory effect of protons on some types of K⁺ channels is well described, e.g., on ROMK1 (4, 12, 33), the inhibition of volume-activated K⁺ channels by protons is still unclear. However, some recent studies reported that volume-activated channels might indeed be pHc sensitive. In primary cultures of seawater fish gill cells, the swelling-activated K⁺ channel is impaired in acidic cells (11). In addition, a role for intracellular acidification in the inactivation of volume-regulated K⁺ channels is described recently for Ehrlich ascites tumor cells (15).

We acknowledge E. Lariviere for excellent technical assistance with volume measurements. We also thank J. Simeaels, A. Boesen, J. Janssen, R. Van Werde, G. Raskin, P. Pirotte, and W. Leyssens for technical help. We are grateful to Dr. W. Zeiske for critical comments and helpful suggestions.

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