Regulatory Volume Decrease in Cultured Kidney Cells (A6): Role of Amino Acids

PATRICK DE SMET,* JEANNINE SIMAELS,* PETER E. DECLERCQ,† and WILLY VAN DRIESSCHE*

From the *Laboratorium voor Fysiologie, K. U. Leuven, Campus Gasthuisberg, B-3000 Leuven; and †Laboratorium voor Klinische Chemie, K. U. Leuven, B-3000 Leuven, Belgium

ABSTRACT Volume regulation was studied in A6 epithelia grown on permeable supports by measuring cell thickness (Tc) while simultaneously recording short circuit current (Isc) and transepithelial conductance (Gt). Lowering the tonicity of the basolateral solution (πb) from 250 or 215 to 140 mOsm/kg elicited a rapid rise in Tc followed by a regulation of the cell volume towards control. This decrease in Tc displays the characteristics of the regulatory volume decrease (RVD). Upon restoring the isoosmotic conditions, Tc decreased rapidly below its control value. A post RVD regulatory volume increase (RVI) as described for other cell types was not observed. The subsequent reduction of the basolateral osmolality increased Tc to the level recorded at the end of the first hypotonic pulse. Because cell content was not altered during the isoosmotic period the second hypotonic challenge was isotonic with the cell and did therefore not evoke an RVD. However, the cell did not lose its ability to volume regulate since an RVD could be elicited by further reduction of πb from 140 to 100 mOsm/kg. The possibility of an involvement of amino acids in the RVD was tested. The amount of amino acids in the cell as well as excreted in the bath was determined by amino acid analysis. Millimolar concentrations of threonine, serine, alanine, glutamate, glycine and aspartate were found in the cell extract. The cellular amino acid concentration was 28.8 ± 0.4 mM. The amounts of glycine, aspartate and glutamate excreted from the cell during the hypotonic treatment were significantly larger than in control conditions. The excretion of these amino acids during hypotonicity decreased the cellular amino acid concentration by 8.4 ± 0.2 mM. This quantity cannot completely account for the RVD during the first hypotonic challenge. The addition of glycine, aspartate and glutamate to the bathing solutions, although used at concentrations higher than intracellularly, did not reduce RVD. On the contrary, this maneuver increased the amplitude of the RVD following both hypotonic pulses. This result suggests a stimulatory role of the amino acids on the processes responsible for the RVD.

INTRODUCTION

The distal parts of the nephron of the kidney are lined with tight epithelia which have a low paracellular conductance. The basolateral membranes are highly per-
meable to water whereas the water permeability of the apical membrane is regulated by insertion and retrieval of aggregates of water channels during and after antidiuretic hormonal action (Chevalier, Bourguet, and Parisi, 1979). Therefore, water flow across the cellular compartment will depend on the osmolality of the tubular and peritubular fluid and the permeability of the apical membrane to water. Transcellular water flow could alter cell volume during time periods where an imbalance between inflow and outflow is present. Alterations in the activity of ion transport processes at the opposing barriers of the epithelium will change cellular solute content and cell volume (Strange, 1990). The volume of epithelial cells could thus be affected by changes in solute as well as water transport across the cells and by alterations of the permeability of the cellular barriers. On the other hand, like other cell types, epithelial cells swell after reducing the basolateral osmolality. However, only minor effects of apical hypotonicity on cell volume can be expected because of the negligible water permeability.

Cultured epithelial cells derived from the distal segments of the kidney of *Xenopus laevis* (A6) form flat monolayers. When grown on permeable supports Na⁺ channels are expressed in the apical membranes (Perkins and Handler, 1981) which in concert with the basolateral Na⁺-K⁺-ATPase enable transepithelial Na⁺ transport. As other distal epithelia of the kidney, the apical membrane has a negligible water permeability in the absence of antidiuretic hormone. We used this preparation to investigate effects of basolateral hypotonicity on cell volume. After reducing the tonicity of the incubation media most cells swell rapidly followed by a gradual decline towards their original volume. This decline phenomenon has been termed regulatory volume decrease (RVD). In many epithelial (Kirk, Schafer, and DiBona, 1987; Lopes and Guggino, 1987; McCarty and O'Neil, 1991) and nonepithelial cells (Hoffman, Lambert, and Simonsen, 1986) the readjustment of cell volume is achieved by the loss of KCl either through ion conductive channels or by means of a coupled KCl cotransport system (Hoffmann, Lambert, and Simonsen, 1988). However, the mechanisms which trigger the activation of these pathways are still poorly understood. The involvement of protein phosphorylation/dephosphorylation has been demonstrated and a link with the mechanisms involved in regulatory volume increase (RVI) has been suggested (Tilly, van den Berghe, Tertoolen, Edixhoven, and de Jonghe, 1993; Jennings and Schulz, 1991).

The present study is concerned with the characteristics of RVD during two successive challenges with hypoosmotic solutions. We found that the first hypoosmotic, hypotonic challenge gave rise to the RVD behavior as described in other cell types. However, no post-RVD regulatory volume increase (RVI) was observed when isoosmotic conditions were restored. Cells just revealed a pure osmometric behavior. Consequently, during a second treatment with hypoosmotic solution cell volume returned to the size reached at the end of the first hypotonic pulse and no RVD was observed. We tested the contribution of amino acids to the osmolyte loss during the RVD. However, we found that the cellular concentration as well as the amount of amino acids lost during hypotonic stress could not account for sufficient osmolyte excretion to explain the RVD. Moreover, volume measurements showed a more pronounced RVD in the presence of amino acids, which is the opposite effect expected for compounds excreted during the RVD.
MATERIALS AND METHODS

Cell Culture

A clone of a distal renal cell line of Xenopus laevis (A6) obtained from Dr. J. P. Johnson (WRAIR, Washington, DC) were cultured as described previously (Van Driessche, De Smet, and De Smedt, 1994). Serial passages 100-110 were used for our experiments. For the volume measurements confluent monolayers were grown on permeable Anopore tissue culture inserts (Nunc Intermed, Roskilde, Denmark). The transparent Anopore inorganic membrane was coated with a gelatin solution containing fluorescent microbeads. Cells were seeded at a density of 10^5 cells/cm^2 and used for our experiments after 6-12 days of growth. The cells used for amino acid analysis were grown in 175 cm^2 plastic bottles (Nunc Intermed, Roskilde, Denmark) until confluence (5 days of growth).

Cell Volume Measurements

Cell height (Tc) was used as an index for cell volume of confluent monolayers. The method has been previously described in detail (Van Driessche, De Smet, and Raskin, 1993). The apical side was labeled with 2 µl of fluorescent biotin-coated microbeads (model L-5251; Molecular Probes, Eugene, OR) in 1 ml of Ringer solution. Focusing of the microbeads was automatically performed under computer control with a piezoelectric focusing device (PIFOC, Physik Instrumente, Waldbronn, Germany). The algorithm for determining the bead's position was based on the determination of the maximum in the fluorescent light intensity. Cell thickness is defined as the vertical distance between the basolateral and apical beads. To take into account the diameter of the microspheres (1 µm), we corrected the Tc values by subtracting 1 µm from the measured cell thickness. The tissues were short-circuited during the entire experiment and the computer program recorded Tc, short-circuit current (Isc) and transepithelial conductance (Gt). Gt was measured as the change in current evoked by a transepithelial voltage of 5 mV. The records of Isc, Gt, and Tc were plotted as percentage of control recorded before imposing the first hypoosmotic challenge. We illustrate the time course of Isc, Gt, and Tc as averaged traces of different experiments. To avoid offsets in the time scale the experiments were precisely timed. Only records where Isc, Gt, and Tc could be recorded over the entire duration of the experiment were used to calculate averaged curves. For all experiments the mean values were depicted as solid lines and the mean ± SEM are shown as dotted lines.

Solutions

Isoosmotic solutions had either an osmolality of 250 or 215 mOsm/kg. 250 mOsm/kg is the osmolality of the growth medium of our cells whereas 215 mOsm/kg equals the osmolality of the standard Cl^- amphibian Ringer solutions. Hypoosmotic solutions had an osmolality of 140 mOsm/kg, which equals the osmolality of the amphibian 57.5 mM Na_2SO_4 Ringer solution. The composition of the solutions used in this study are given in Table I. The osmolality of all solutions was verified with a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany). All experiments were performed at room temperature (22°C).

Determination of Amino Acid Content and Excretion

Cellular amino acid content as well as the amount excreted in control and during a hypotonic challenge were determined with an amino acid analyzer. A6 cells were cultured in plastic bottles until confluence as previously described (Van Driessche et al., 1994). The culture medium was removed and the cells were rinsed three times with isotonic (250-ISO) Ringer solution. Thereafter, cells were incubated for 30 min with 250-ISO solution. The control solution was removed and replaced either with fresh 250-ISO or 140-HYPO solution. After incubation for 30 min, the solutions
TABLE I
Composition of Isoosmotic and Hypoosmotic Solutions in Millimolar

| Isoosmotic solutions | 215-ISO | 250-ISO | AA-ISO | CI-ISO | 100-ISO |
|----------------------|---------|---------|--------|--------|---------|
| Na⁺                  | 70      | 70      | 70     | 70     | 45      |
| K⁺                   | 2.5     | 2.5     | 2.5    | 2.5    | 2.5     |
| Cl⁻                  | 72      | 72      | 37     | 37     | 47      |
| Gluconate            | 0       | 0       | 0      | 0      | 0       |
| Sucrose              | 70      | 107     | 107    | 107    | 157     |
| Glutamate            | 0       | 0       | 10     | 0      | 0       |
| Aspartate            | 0       | 0       | 10     | 0      | 0       |
| Glycine              | 0       | 0       | 15     | 0      | 0       |
| Osmolality (mOsm/kg) | 215     | 250     | 250    | 250    | 250     |

| Hypoosmotic solutions | 140-HYPO | 140-HYPO | AA-HYPO | CI-HYPO | 100-HYPO |
|-----------------------|----------|----------|---------|---------|----------|
| Osmolality (mOsm/kg)  | 140      | 140      | 140     | 140     | 100      |

All solutions listed in this table also contained 1 mM Ca²⁺, 2.5 mM K⁺, and 2.5 mM HCO₃⁻, at pH = 8. The hypoosmotic solutions (140-HYPO, AA-HYPO, CI-HYPO, 100-HYPO) were prepared by removing sucrose.

were removed from the bottles and retained as the control and hypoosmotic effluent samples. The cell contents were extracted with 14% perchloric acid for 20 min after which the extract was adjusted of and of the extract were lyophilized and redissolved in 0.5 ml water. To this solution 0.5 ml of a 10% (wt/vol) sulphosalicylic acid with norleucin (250 μM) as internal standard was added. After centrifugation, 400 μl of the supernatant was brought in a spin X filter (0.22 μm, Costar) and centrifuged in an Eppendorf centrifuge. This filtered solution was mixed with 100 μl of 0.775 N LiOH in “loading buffer” (0.2 M Li-citrate, pH 2.2). Of this mixture 50 μl was applied to the column of the amino acid analyzer (4151 Alpha plus amino acid analyzer, LKB Biochrom Ltd., Cambridge, England). All amino acids as well as taurine, citrulline, α-aminobutyric acid, ornithine, 1-methylhistidine, and 3-methylhistidine were detected as ninhydrin conjugates at 570 or 440 nm. The amounts of amino acids excreted in the incubation medium are expressed as decrease of the cellular concentration, Δ[AA], (see Table IV). To determine Δ[AA], we assumed cell height of 5.0 μm to calculate the total cell volume and calculated Δ[AA], as the amount excreted by cell volume. Similarly we calculated the intracellular concentration ([AA]ᵢ) from the amount found in the cell extract.

Protocols for Tₑ Measurements

(a) Experiments with A6 epithelia cultured on Anopore filters: initially, the tissues were perfused for 50 min with isotonic solutions on both the apical and basolateral surface. After this equilibration period, the osmolality of the apical bath (πₑ) was reduced to 140 mOsm/kg. The subsequent protocol for recording Lₑ, Gₑ, and Tₑ consisted of five periods. During periods I (15 min), III (20 min or 2 h) and V (15 min) the basolateral solution was isoosmotic. The basolateral osmolality (πₑ) was reduced to 140 mOsm/kg during periods II and IV which had a duration of 25 min. Changes in Lₑ, Gₑ, and Tₑ are expressed as percentage of control. The experiments were precisely timed and averaged curves were calculated from different experiments (see Figs. 2, 3, 5, 6, and 8). To characterize the rate of the volume changes elicited by lowering πₑ we fitted an exponential function to the Tₑ data recorded after reaching the peak value in Tₑ (see for instance Fig. 4):

ΔTₑ = Aₑ₀ - Aₑ₀ [1 − exp(−(t − t₀)/τ)]

(1)
where $t_0$ is the time of solution change, $\tau$ the time constant of the exponential decay, $A_{RVD}$ the amplitude of the $T_c$ decline, $A_{INI}$ the initial, instantaneous $T_c$ increase expected at $t_0$ for a perfect osmometric behavior. Although, the RVD might have a more complicated time course, we used this curve fitting procedure to characterize the rate of the volume regulatory processes (Arrazola, Rota, Hannaert, Soler, and Garay, 1993). $T_c^{CTRL}$ is cell height in isoosmotic conditions before the reduction of $\pi_b$. These symbols are explained in Fig. 1. $A_{RVD}$ and $A_{INI}$ were determined by nonlinear regression of Eq. 1 to the averaged curves of $T_c$. Mean values of the peak ($T_c^{peak}$) and plateau ($T_c^{plateau}$) values of $T_c$ are calculated from data collected in individual records.

(b) The measurements of $T_c$ in A6 cells cultured on glass cover slips consisted of two periods. During the first period (30 min) the cells were bathed with 250-ISO solution which was replaced by 140-HYPO during the subsequent period of 30 min. The same incubation media and timing were used to study the excretion of amino acids during an hypotonic challenge.

**Statistics**

Averaged values are represented as means ± SEM. $t$-test was used to verify statistical significance.

**RESULTS**

The protocol for cell height measurements used in this paper consisted of five periods in which $\pi_b$ was changed while maintaining $\pi_a$ at 140 mOsm/kg. Changes in $\pi_a$ did not affect cell volume which demonstrates a negligible permeability of this barrier to water. We kept the $\pi_a$ constant to avoid changes in the apical sucrose concentration. Removing sucrose in this compartment gives rise to changes in the optical refractory index during the solution change which might cause focusing problems and a short interruption of the experiment. We used hypoosmotic solutions at the apical side to avoid, during periods II and IV, an osmotic gradient directed from the apical to basolateral side which opens the paracellular pathway. Initially we performed a series of experiments in which the isoosmotic conditions were defined as 215 mOsm/kg, which is the osmolality of our 115 mM containing NaCl Ringer solution. An example of such an experiment is depicted in Fig. 2. During period I, cell height ($T_c$) was 4.61 μm. When $\pi_b$ was lowered (period II) $T_c$ increased rapidly to a peak value of 6.41 μm and then returned to a value close to control (4.71 μm). After restoring isoosmotic conditions during period III, $T_c$ decreased rapidly to 3.30 μm. Unlike other cell types the A6 epithelia did not regulate their volume back to control, but remained markedly thinner than during the control period before the hypotonic stress. The subsequent reduction of $\pi_b$ in pe-
initially the A6 epithelia were incubated with 140-HYPO and 215-ISO solutions on the apical and basolateral side respectively. An hypoosmotic challenge was applied during periods II and IV during 25 min. \( \pi_b \) was restored to 215 mOsm/kg during period III (20 min). \( T_c \), \( I_c \), and \( G_t \) are in this and following figures expressed as percentage of control values recorded immediately before reducing the basolateral tonicity. The control values were: \( T_c = 4.61 \pm 0.32 \mu m \ (n = 19) \), \( I_c = 11.0 \pm 2.8 \mu A/cm^2 \ (n = 6) \) and \( G_t = 0.20 \pm 0.03 \mu S/cm^2 \ (n = 6) \).

period IV increased \( T_c \) to the value recorded at the end of period II and the cells did not regulate their volume noticeably. After restoring isoosmotic conditions (period V), \( T_c \) decreased to a value close to \( T_c \) recorded in period III.

The experiment shown in Fig. 2 illustrates clearly that A6 cells demonstrate a regulatory volume decrease (RVD) as described for other epithelial cell types (Hoffmann et al., 1988; Welling and Linshaw, 1988; Kirk et al., 1987). Yet, the most striking result obtained from this type of experiment was the failure of A6 cells to restore their volume to control after establishing isoosmotic conditions (period III). The lack of mechanisms which could evoke a post-RVD RVI resulted in a pure osmometric behavior of the cell during period III. At the end of period II the cells had equilibrated their water and solute content with the hypoosmotic incubation media and restored their volume near normal volume. Because of the absence of solute uptake during period III, the second hypoosmotic shock will elevate \( T_c \) to \( T_c^{\text{CTRL}} \) reached during period II, which resembles \( T_c^{\text{CTRL}} \) (Table II). So, due to the absence of a post-RVD RVI, the hypoosmotic media used during period IV are experienced by the cell as isotonic. Similarly we found that the hypoosmotic challenge also caused irreversible effects on short-circuit current (\( I_c \)) and transepithelial conductance (\( G_t \)). Indeed Fig. 2 demonstrates that \( I_c \) and \( G_t \) were markedly lower in period III than in period I. Also the time course of \( I_c \) and \( G_t \) during the two hypoosmotic pulses showed striking differences. In period IV, the increase of both parameters was much slower and the decline after the peak was strongly diminished. In the absence of \( Na^+ \) in the apical bath the changes in \( I_c \) and \( G_t \) were negligible whereas the pattern of the time course of \( T_c \) resembled the one recorded with \( Na^+ \) on both sides (not shown). This finding demonstrates that the \( I_c \) trace in Fig. 2 reflects closely the changes in \( Na^+ \) current. Mean values of \( T_c \), \( I_c \), and \( G_t \) obtained from 13 experiments are summarized in Table II. In six experiments the protocol
was accurately timed as described in Materials and Methods. In seven other experiments, executed in the initial phase of this project, the length of the isoosmotic and hypoosmotic periods was more variable and the data were not used to calculate the averaged curve in Fig. 2.

During the transfer of the epithelium from the culture medium into the Ussing chamber, the effects of hypotonicity discussed so far, could also have been introduced to a certain extent. Indeed during this maneuver the osmolality of the bathing medium was reduced from 250 to 215 mOsm/kg. In the following experiments we wanted to avoid possible alterations of the cells during this phase of the experiment and we therefore redefined our initial isoosmotic conditions by using the osmolality of the growth medium of our cells, which was 250 mOsm/kg. In experiments where the cells were initially perfused with growth medium the solution change to 250-ISO did not alter $T_c$ which demonstrates the isotonicity of this medium. Fig. 3 shows the averaged traces of $T_c$, $I_o$, and $G$ calculated from 8 experiments in which we were able to obtain complete records of $T_c$ with 13 beads. $\pi_0$ was reduced from 250 to 140 mOsm/kg. As in the experiments with 215 mOsm/kg, $T_c$ increased rapidly after applying the hypotonic stress (period II) and declined back towards the value recorded in isotonic conditions. However, $T_c^{\text{Plat}}$ was significantly larger than $T_c^{\text{CTRL}}$ (see Table III). As in Fig. 2 we did not observe the post-RVD RVI after restoring the isoosmotic conditions in period III which resulted in a similar osmotic behavior as described above. $I_o$ and $G$ displayed a similar time course as in the experiment in Fig. 2. Mean values of $T_c$, $I_o$, and $G$ are listed in Table III. It is noteworthy that $T_c^{\text{Plat}}$ obtained during the second osmotic challenge (4.30 $\mu$m) was not significantly different from $T_c^{\text{CTRL}}$ (4.23 $\mu$m) recorded before the first hypoosmo-

| Table 11 | Changes of $T_c$, $I_o$ and $G$ Caused by the Decrease of Bath Osmolality after 215 mOsm/kg Isotonic Conditions
|---|---|---|---|
| | $T_c$ | $I_o$ | $G$
| | $\mu m$ | $\mu A/cm^2$ | $mS/cm^2$
| n | 32 | 15 | 15 |
| First pulse | | | |
| $T_c^{\text{CTRL}}$ | $4.75 \pm 0.25$ | $10.0 \pm 1.43$ | $0.36 \pm 0.07$
| $T_c^{\text{Peak}}$ | $6.58 \pm 0.28^*$ | $16.2 \pm 2.21^*$ | $0.54 \pm 0.07^*$
| $T_c^{\text{Plat}}$ | $4.81 \pm 0.30$ | $11.1 \pm 1.80$ | $0.43 \pm 0.07^*$
| $T_c^{\text{Iso}}$ | $5.52 \pm 0.26^*$ | $5.14 \pm 0.50^*$ | $0.28 \pm 0.07^*$
| Second pulse | | | |
| $T_c^{\text{CTRL}}$ | $3.52 \pm 0.26$ | $3.14 \pm 0.50$ | $0.28 \pm 0.07$
| $T_c^{\text{Peak}}$ | $5.09 \pm 0.28^*$ | $8.82 \pm 1.09^*$ | $0.39 \pm 0.06^*$
| $T_c^{\text{Plat}}$ | $4.55 \pm 0.30^*$ | $9.38 \pm 1.09^*$ | $0.36 \pm 0.06^*$
| $T_c^{\text{Iso}}$ | $5.23 \pm 0.26^*$ | $2.67 \pm 0.43^*$ | $0.26 \pm 0.06^*$

In control conditions, A6 epithelia were incubated in NaCl solutions which had an osmolality of 140 and 215 mOsm/kg on the apical and basolateral side respectively. $T_c$, $I_o$, and $G$ were recorded just before reducing bath osmolality (CTRL), when the maximal value was reached (Peak), after reaching a plateau (Plat) and after the isoosmotic conditions (Iso) were restored on the basolateral side. Numbers are given as means $\pm$ SEM. Values which are significantly different from control are marked with $^*$ for $0.001 < P < 0.05$ or with $^{**}$ for $P \leq 0.001$. 


FIGURE 3. Effects of reducing $\pi_b$ from 250 to 140 mOsm/kg on $T_c$, $I_c$, and $G_i$. The solid lines are means of eight experiments (eight records for $I_c$ and $G_i$) and 13 traces of $T_c$ recorded with beads which remained attached to the apical surface during the whole experiment. As in Fig. 2, the dotted lines are means ± SEM. Initially the A6 epithelia were incubated with 140-HYPO and 250-ISO solutions on the apical and basolateral side respectively. $\pi_b$ was 140 mOsm/kg during periods II and IV (25 min) and 250 mOsm/kg during periods I, III, and V. The control values recorded at the end of period I were: $T_c = 4.23 ± 0.41$ μm ($n = 13$), $I_c = 5.01 ± 0.70$ μA/cm$^2$ ($n = 8$) and $G_i = 0.25 ± 0.06$ mS/cm$^2$ ($n = 8$).

 isotonic pulse. So cell volume completely returned to control during this second hypo-osmotic challenge whereas $T_c^{\text{Plat}}$ remained significantly above $T_c^{\text{CTRL}}$ during period II. Fig. 4 compares the time course of $T_c$ recorded during periods II and IV. The time constant of the decline obtained by nonlinear regression of Eq. 1 to the data was 3.50 min during the first pulse. The magnitude of the decline which we defined as the amplitude of the exponential function ($A_{\text{RVD}}$) was 68% and $A_{\text{RVD}}$ was 83%. This figure clearly shows the absence of a second RVD.

So far our results demonstrate that A6 cells are not able to develop a post-RVD

| Table III |
|---|
| Changes of $T_c$, $I_c$ and $G_i$ Caused by the Decrease of Bath Osmolality after 250 mOsm/kg Isotonic Conditions |

|   | $T_c$ | $I_c$ | $G_i$ |
|---|---|---|---|
|   | μm | μA/cm$^2$ | mS/cm$^2$ |
| n | 13 | 8 | 8 |
| First pulse | | | |
| $T_c^{\text{CTRL}}$ | 4.23 ± 0.41 | 5.18 ± 0.71 | 0.24 ± 0.06 |
| $T_c^{\text{Peak}}$ | 6.38 ± 0.50$^f$ | 13.6 ± 1.60$^f$ | 0.42 ± 0.04$^f$ |
| $T_c^{\text{Plat}}$ | 4.89 ± 0.46$^*$ | 14.2 ± 1.27$^*$ | 0.35 ± 0.04$^*$ |
| $I_c^{\text{s}}$ | 2.82 ± 0.33$^t$ | 1.59 ± 0.26$^t$ | 0.21 ± 0.06$^t$ |
| Second pulse | | | |
| $T_c^{\text{CTRL}}$ | 2.85 ± 0.32 | 1.59 ± 0.26 | 0.21 ± 0.06 |
| $T_c^{\text{Peak}}$ | 4.71 ± 0.39$^f$ | 5.58 ± 0.49$^f$ | 0.27 ± 0.05$^f$ |
| $T_c^{\text{Plat}}$ | 4.30 ± 0.40$^t$ | 10.6 ± 0.72$^t$ | 0.30 ± 0.04$^f$ |
| $I_c^{\text{s}}$ | 2.37 ± 0.30$^*$ | 1.52 ± 0.23 | 0.20 ± 0.05 |

In control conditions, A6 epithelia were incubated in NaCl solutions which had an osmolality of 140 and 250 mOsm/kg on the apical and basolateral side, respectively. $T_c$, $I_c$, and $G_i$ were recorded just before reducing bath osmolality (CTRL), when the maximal value was reached (Peak), after reaching a plateau (Plat) and after the isosmotic conditions (Iso) were restored on the basolateral side. Numbers are given as means ± SEM. Values which are significantly different from control are marked with * for 0.001 < $P$ < 0.05 or with $^f$ for $P$ < 0.001.
RVI. Indeed, the cells behave as perfect osmometers when exposed to isoosmotic solutions after an hypotonic challenge, which is reflected in the absence of a post-RVD RVI. As a consequence, our isoosmotic solution is experienced as hypertonic for the cell. As the cells are only kept for a short time in hypertonic solutions during period III, we checked whether an increase of the incubation time in these conditions could result in solute uptake and thus reduce the irreversibility. We therefore performed similar experiments as in Fig. 3 but prolonged the duration of period III from 20 min to 2 h. The averaged trace of $T_e$ obtained from seven experiments is depicted in Fig. 5. Also in this series of experiments $T_e$ did not demonstrate a significant increase after exposure to hypertonic medium in Period III and the RVD response was barely present during the second hypoosmotic challenge. So, even during the extended hypertonic period cells did not reaccumulate solute and as a consequence did not display an RVD during the subsequent hypoosmotic challenge.

The data presented so far demonstrate the lack of a post-RVD RVI that impedes volume regulation during the second hypoosmotic challenge. Because of the absence of solute uptake in period III the hypoosmotic incubation media in period IV...
is isotonic with the cell that had regulated its volume by losing solute during period II. Within this concept a further reduction of the osmolality during period IV is experienced as hypotonic and might result in an activation of an RVD. The experiment illustrated in Fig. 6 shows that such an additional reduction of the osmolality to 100 mOsm/kg during period IVb gives rise to an RVD and thus to an additional solute loss. After restoring the osmolality to 140 mOsm/kg (period Va) \( T_c \) clearly declined below the plateau recorded at the end of period IVa. So it seems as if a net solute loss had taken place during the additional hypoosmotic challenge. Restoring isoosmotic conditions during period Vb returns \( T_c \) back to the level below values recorded during period III. This experiment demonstrates that the cell maintains the ability to activate the RVD machinery.

Several studies of volume regulation in other cell types have demonstrated the involvement of amino acids such as taurine, glycine, alanine, phenylalanine, tryptophan, aspartate, glutamate, lysine, and arginine, in the RVD (Schousboe, Sánchez Olea, Morán and Pasantes-Morales, 1991; Lambert and Hoffmann, 1993; Rasmusson et al., 1993; Roy and Malo, 1992; Roy and Sauvé, 1987). We therefore determined the amino acid release from the cells in the incubation medium as well as the cellular content in control and after the cells had been exposed to hypotonic solutions. To obtain sufficient material we cultured our cells in plastic bottles. To verify whether cells grown on such impermeable supports also display the RVD after a hypotonic challenge, we measured cell height of monolayers cultured on glass cover slips (Fig. 7). The changes in \( T_c \) recorded with these tissues also demonstrated an RVD which justifies the use of this preparation for the amino acid analysis. In paired experiments we compared amino acid release from cells incubated in isotonic and hypotonic solutions. Before starting the experimental phase, both samples were exposed to isotonic Ringer solution for 30 min. The incubation medium was then removed and replaced by either a hypotonic or isotonic solution. After a 30-min exposure time, the medium was collected and analyzed as described in Materials and Methods. The amino acid analysis of cell content of both samples revealed the presence of six amino acids: serine, alanine, threonine, glycine, glutamate, and aspartate. The total concentration of the other amino acids (see list

Figure 6. Effect of an additional reduction of \( \pi_o \) during the second hypoosmotic challenge. Initially the tissue was incubated with 100-HYPO and 100-ISO solution on the apical and basolateral side respectively. During period II and IVa we reduced \( \pi_o \) to 140 mOsm/kg by partial removal of sucrose. The additional decrease of \( \pi_o \) to 100 mOsm/kg was established during period IVb. The osmolality was restored to 140 and 250 mOsm/kg during period Va and Vb respectively.
in Materials and Methods) in the cell and excreted from the cells under hypotonic conditions was less than 1.11 and 0.18 mM respectively, as determined by analysis. The results are shown in Table IV. Although a significant amount of alanine was measured in the cells, the amount released in the hypotonic conditions was not significantly different from control. However, the amount of threonine, serine, glutamate, glycine, and aspartate excreted by the hypotonic shock was significantly larger than in control. As far as glutamate and aspartate are concerned, this is also reflected in the decrease of the cellular content elicited by hypotonicity. For glycine, threonine and serine, however, we did not measure a significant decrease.

The measurements of amino acid content demonstrate that the total amount accounts for an intracellular concentration of 28.8 ± 0.4 mM (see Table IV). During the 30-min period of hypotonicity the amino acid concentration in the cell was diminished by 8.4 ± 0.2 mM. Considering the large osmotic perturbation imposed to the cells (110 mOsm/kg), it seems unlikely that the loss of this amount of osmolytes plays a major role in the RVD. To test this hypothesis further we performed cell thickness measurements with apical and basolateral solutions which contained 10 mM glutamate, 10 mM aspartate and 15 mM glycine. These concentrations are larger than those found in the cell with amino acid analysis so that the release of

**Table IV**

|       | [AA]c | Δ[AA]c |       | [AA]c | Δ[AA]c |
|-------|-------|--------|-------|-------|--------|
|       | CTRL  | HYPO   | CTRL  | HYPO   |        |
| THR   | 0.36 ± 0.07 | 0.59 ± 0.08* | 3.36 ± 0.32 | 3.28 ± 0.03 |
| SER   | 0.17 ± 0.02 | 0.29 ± 0.03* | 1.13 ± 0.12 | 1.23 ± 0.09* |
| ALA   | 0.43 ± 0.07 | 0.61 ± 0.07 | 2.29 ± 0.10 | 1.89 ± 0.12* |
| ASP   | 0.18 ± 0.01 | 2.39 ± 0.35* | 6.05 ± 0.16 | 3.72 ± 0.58* |
| GLU   | 0.12 ± 0.01 | 1.28 ± 0.20* | 4.40 ± 0.12 | 3.24 ± 0.17* |
| GLY   | 0.47 ± 0.03 | 3.25 ± 0.35* | 11.6 ± 1.3  | 11.4 ± 2.7 |
| Total amount | 1.73 ± 0.04 | 8.41 ± 0.21* | 28.81 ± 0.40 | 24.76 ± 0.81* |

[AA]c was obtained from the amino acid analysis in the cell extract after incubating the cells in 250-ISO (CTRL) or 140-HYPO (HYPO) solution. Δ[AA]c was determined by analyzing the amino acids in the incubation media and expressing the release as decrease in cellular concentration (see Materials and Methods). Numbers are given as means ± SEM (n = 6). * Marks experimental values which are significantly different from control (P < 0.05).
These amino acids should be considerably impeded. Fig. 8 shows the averaged trace of $T_c$ recorded with amino acids containing solutions. The time course of $T_c$ is very similar to the one recorded in absence of amino acids (Fig. 3). Fig. 9 compares the time course of $T_c$ after reducing $\pi_b$ during periods II and IV. The increase of $T_c$ was 49.1 ± 2.8% and 85.5 ± 6.1% during the first and second pulse respectively. To keep the osmolality of the amino acid containing solution identical to the 250-ISO and 140-HYPO solutions, we had to reduce the Cl$^-$ concentration. As Cl$^-$ plays an important role in volume regulatory processes of most cells (Hoffmann et al., 1988) we performed experiments with low Cl$^-$ containing solutions (Cl$^-$ISO and Cl$^-$HYPO). Our result showed that the reduction of Cl$^-$ in the bathing solution impaired volume regulation. This is illustrated in Fig. 10 which compares the time course of $T_c$ in the absence and presence of amino acids (curves I and 3, respectively). It is clear that in the presence of amino acids the RVD during the first as well as second hypotonic challenge is more pronounced. This result excludes the notion that the amino acids provide a significant contribution to the osmolyte loss during RVD. On the contrary, the amino acids clearly stimulate the processes which are involved in the solute losses needed for volume recovery. The reduction of Cl$^-$ in amino acid free bathing solutions markedly changed the time course of $T_c$ during periods II and IV. The RVD was clearly reduced and $T_c^{Peak}$ was larger and approached the increase expected for a perfect osmometer (86%). This experiment.

**FIGURE 8.** Effect of two successive hypotonic challenges in the presence of amino acids in the apical and basolateral solutions. The apical solution was AA-HYPO during the entire experiment. AA-ISO was the basolateral solution during periods I, III, and V. During periods II and IV, $\pi_b$ was reduced by replacing AA-ISO by AA-HYPO. The time in between the two hypotonic pulses was 20 min. Data were obtained from eight experiments in which 23 beads remained attached to the membrane. The solid lines represent mean values. Dotted lines are means ± SEM. The control value was: $T_c = 5.85 ± 0.19 \mu m (n = 23)$.

**FIGURE 9.** Comparison of the time course of $T_c$ during the first (curve 1) and second (curve 2) hypotonic pulse recorded in Fig. 8 in the presence of amino acids. The solid lines are the means recorded during the experiment depicted in Fig. 8. The dashed line was obtained by fitting an exponential function through the $T_c$ data during the first hypotonic pulse. The number at the arrow is $T_c^{CTRL} + A_{inj}$. 
suggests a role of the anion in the volume regulation of A6 epithelia, either through an ion conductive pathway or by a KCl cotransport system.

**DISCUSSION**

The method for volume measurements used in this paper enables the recording of the thickness of individual cells over several hours while the transepithelial voltage and current were controlled. This technique provides an excellent tool to study the processes involved in the regulation of cell volume which are reflected in the changes of cell height. The data enable us to correlate the activation of the transepithelial currents with changes in cell volume. This is of special interest for the investigation of the involvement of ion transport processes in the regulation of cell volume. Moreover, this method uses the same cell to perform the measurements during control and experimental conditions. The microbeads can be observed in transmitted light and the localization can be verified. In heterogeneous tissues the measurements can focus on a specific cell type. Cell thickness was used by others (MacRobbie and Ussing, 1961; Roy and Sauvé, 1987) as an index for cell volume. A discrepancy between cell volume and cell thickness could be introduced by changes in cell shape which are occurring during volume expansion. This could result from alterations in the cytoskeletal structure and by unfolding of the cellular barrier. These processes might be modulated by intracellular Ca^{2+} which is known to increase during a hypotonic treatment. By comparing cell volume measurements of isolated MDCK cells with changes in thickness of monolayers Roy and Sauvé (1987) calculated that the cross-section of the cells was increased by a factor of 1.29 whereas cell height was increased by a factor of 1.55 for cells which doubled their volume upon reducing the bath tonicity. The relatively small increase in cross section is expected because of the limited intercellular space in confluent monolayers. Although cell thickness will underestimate cell volume, it is a good index to describe the time course of the regulatory processes. Moreover, our data demonstrate

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**Figure 10.** Comparison of the time course of T_c in the absence and presence of amino acids in the bathing solutions. The curves 2 and 3 are the means recorded during the experiments depicted in Figs. 3 and 8 respectively. Curve 1 is the average of 40 beads recorded with low Cl^{−}-containing solutions. During the entire experiment the apical side was perfused with Cl-HYPO. The basolateral solution was Cl-ISO during periods I, III, and V which was replaced by Cl-HYPO during periods II and IV.
that $T_{c}^{\text{Peak}}$ approaches in some conditions the theoretical increase in volume expected for a perfect osmometer, as seen during period IV in experiments with 250-ISO and CI-ISO solutions (Figs. 3 and 10, Table V). Therefore, it seems that cell thickness gives a rather accurate estimate of changes in cell volume in A6 epithelia.

Our results demonstrate that A6 epithelia increase their volume upon reducing basolateral tonicity and that these cells are able to restore their volume to its original value. The RVD as recorded in A6 cells during the first hypotonic challenge has been found in many nonepithelial (Hoffmann, Simonsen, and Lambert, 1984; Brugnara, Van Ha, and Tosteson, 1989; Colclasure and Parker, 1992) as well as epithelial cells (Onuchic, Arenstein, and Lopes, 1992; Kirk et al., 1987). The volume regulation by these cells has mostly been attributed to the loss of KCl either through ion conductive channels or through an electroneutral K$^{+}$-Cl$^{-}$ cotransporter. This study did not aim to investigate the involvement of ion transport systems in the loss of cellular osmolytes during volume recovery. We confined our experiments to the comparison of volume regulation during two successive exposures to hypoosmotic solutions and the involvement of amino acids in volume regulation. Our data demonstrate that under the experimental conditions used in this paper, the A6 epithelia do not develop a post-RVD RVI. As a consequence, intracellular osmolyte content is not altered during period III, which results in a perfect osmometric behaviour of the cell. This is reflected in the identical values of $T_{c}$ recorded at the beginning and end of period III (see Tables II and III). During the first hypotonic challenge, cells restored their volume to control (set-point volume) by losing solutes (Parker, 1993). As the cells do not alter their solute content during the isoosmotic period III, the subsequent reduction of bath osmolality will bring cell volume immediately back to the set point. Within the set-point concept, volume regulatory processes (RVD) are only activated when the set-point volume is exceeded. Because during period IV, cell volume equals the volume at the end of period II, which is just the set-point volume, RVD machinery will not be activated and cells will not noticeably decrease their volume. As an alternative for the set-

| TABLE V |
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| Comparison of the Parameters of Peak and Plateau Values of $T_{c}$ while Reducing the Basolateral Osmolality |

|          | 215-ISO | 250-ISO | CI-ISO | AA-ISO |
|----------|---------|---------|--------|--------|
| $n$      | 19      | 13      | 40     | 23     |
| First pulse |         |         |        |        |
| $T_{c}^{\text{Peak}}$ | 189 ± 4 | 148 ± 4 | 164 ± 5 | 149 ± 4 |
| $T_{c}^{\text{Plat}}$ | 102 ± 3 | 117 ± 7 | 134 ± 6 | 109 ± 5 |
| $T_{ch}^{\text{Plat}}$ | 72 ± 4  | 68 ± 6  | 81 ± 5  | 69 ± 5  |
| Second pulse |         |         |        |        |
| $T_{c}^{\text{Peak}}$ | 142 ± 4 | 171 ± 8 | 175 ± 6 | 159 ± 7 |
| $T_{c}^{\text{Plat}}$ | 185 ± 5 | 156 ± 7 | 159 ± 6 | 133 ± 5 |
| $T_{ch}^{\text{Plat}}$ | 96 ± 6  | 82 ± 5  | 89 ± 4  | 84 ± 3  |

Data are expressed as percentage of control. $T_{c}^{\text{Peak}}$ is the maximum in $T_{c}$ reached after reducing bath osmolality, $T_{c}^{\text{Plat}}$ is measured at the end of period II and $T_{ch}^{\text{Plat}}$ values are the cell heights recorded after restoring the isoosmotic conditions in the basolateral bath. Control values for the second hypoosmotic pulse were recorded at the end of period III where the basolateral perfusate was isoosmotic.
point hypothesis, one could consider substrate depletion as cause for the absence of volume regulation during the second hypotonic challenge. This implies also that, the substrate is not reaccumulated during period III which is indeed observed by the absence of the post-RVD RVI. However, substrate depletion would markedly diminish the ability of the cells to regulate their volume upon a further reduction of bath osmolality. On the contrary, we observed in Fig. 6 that the decrease in osmolality from 140 to 100 mOsm/kg elicited a well pronounced RVD, which renders the hypothesis of substrate depletion unlikely. In conclusion, the major cause of the absence of volume regulation during the second hypoosmotic challenge remains the lack of a post-RVD RVI. Several mechanisms could contribute to the absence of the post-RVD RVI. We will discuss below two major mechanisms which could be involved: (a) the loss of amino acids; (b) the lack of the ability to restore the concentration of K\(^+\) and Cl\(^-\) to their resting state.

Amino acids play an important role in cell volume regulation in MDCK cells (Roy and Malo, 1992), cultured chick heart cells (Rasmusson et al., 1993), glial cells (Pasantes-Morales, Maar, and Morán, 1993), and Ehrlich ascites tumor cells (Lambert and Hoffmann, 1993). Most studies focused on the contribution of these solutes to cell osmolality and to the decrease of the osmolyte concentration during hypotonic treatment. In these cell types taurine plays an important role and its excretion contributes largely to the back regulation of cell volume. Our analysis did not demonstrate the presence of taurine. Mainly aspartate, glutamate and glycine and to a smaller extent threonine and serine are excreted during the hypotonic challenge. These amounts do not account for a loss of cell osmolytes which could markedly influence the RVD. This is confirmed by comparing the time course of the RVD during the first hypotonic challenge in experiments with and without amino acids in the bathing solutions (Fig. 10) and by inspecting the mean values of peak and plateau (Table V). Comparison of the plateaus reached during the first pulse with low Cl\(^-\) shows that RVD was even more pronounced in the presence of the amino acids (Table V). If the amino acids merely had a role as osmotic substances, the opposite result would have been obtained. Cell osmolality will nevertheless slightly decrease due to the excretion of the amino acids. The data thus suggest that besides the relatively small contribution to the osmotic loss of solutes, the amino acids seem to favor the RVD. This stimulation of RVD could be caused by the alteration of the set-point volume by the amino acids. If indeed the set point is lowered, RVD after a hypotonic shock will be more pronounced. These indirect effects of the amino acids on volume regulation are also observed during the second hypoosmotic challenge. Indeed, during period IV the RVD is more pronounced in the presence of amino acids and the volume is regulated back below control. This observation favors the suggestion that the amino acids affect the set-point volume of the cell. This again is the opposite result from what would be expected if the amino acids had merely a role as osmolytes which were lost during volume recovery.

The regulation of cell volume of most cell types after lowering extracellular tonicity occurs through the loss of KCl (Samman, Ohtsuyama, Sato, and Sato, 1993). Most cells, restore their volume to normal by the concerted action of the Na\(^+\)-H\(^+\) and Cl\(^-\)-HCO\(_3\) \(-\) exchangers in collaboration with the electrogenic Na\(^+\)-K\(^+\) pump or by the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport. Under our experimental conditions with A6 cells,
these mechanisms seem to be in an inactive state. We also tried to evoke an RVI by increasing the osmolality of the basolateral solution from 250 to 380 mOsm/kg. Such a hypertonic treatment also did not result in an RVI. Glial cells (C6) at room temperature and primary cultured proximal epithelia from rabbit kidney at 37°C also failed to demonstrate volume regulation after a hypertonic challenge.

Our amino acid analysis was performed with cells cultured on glass coverslips, whereas we used cells on permeable supports for most volume measurements. There exists the possibility that the cellular composition of amino acids in polarized and nonpolarized cells is different. This could lead to the failure of external amino acids to oppose the efflux during hypotonic treatment. Unfortunately we are unable to perform the amino acid analysis on cells grown on permeable supports because the amount of material is insufficient for the analysis.

It is noteworthy that the initial relative increase in $T_c$ observed upon reducing $\pi_b$ was markedly larger during the second hyposmotic perturbation (see Figs. 4 and 9 and $T_c^{\text{peak}}$ values in Table V). This discrepancy can be understood in the light of the pure osmometric behavior of the cell after the first hypotonic challenge. With 250-ISO as well as Cl-ISO solutions we found during the second osmotic pulse an initial $T_c$ increase of 71 and 75%. Both values approach the theoretical increase of 78% expected for the perfect osmometer and are smaller during the first hyposmotic challenge (48 and 64% respectively). The deviation of the perfect osmometric behavior during the first hyposmotic challenge might be due to the rapid onset of the regulatory mechanism for cell volume which would cause solute loss during the phase where $T_c$ rapidly increases. Indeed $A_{\text{ini}}$ (183%) obtained from exponential curve fitting approached the value expected for ideal osmometric behavior.

Under the conditions used in this paper $I_{sc}$ reflects transepithelial Na$^+$ transport. Indeed replacing Na$^+$ by N-methyl-D-glucamine (NMDG) abolished $I_{sc}$ without an effect on $T_c$. Moreover, hypotonicity did not activate $I_{sc}$ in experiments in which apical Na$^+$ was replaced by NMDG. The data in this paper confirm previous observations that hypotonicity markedly stimulates transepithelial Na$^+$ transport (Costa, Fernandes, Ferreira, Ferreira, and Giraldez, 1987; Wills, Millinoff, and Crowe, 1991).

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