Cell volume control in vestibular dark cells during and after a hyposmotic challenge

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Wangemann, Philine, and Nobuyuki Shiga. Cell volume control in vestibular dark cells during and after a hyposmotic challenge. *Am. J. Physiol.* 266 (*Cell Physiol.* 35): C1046–C1060, 1994.—Cell height was measured as an index of volume in a preparation of vestibular dark cells in which the perfusate had access to both sides of the epithelium. In response to a hyposmotic challenge induced by removal of 75 mM NaCl, cell height increased to 107%; however, cell width did not increase. Significantly larger increases in cell height were observed in the absence of Cl^- or K^+ or in the presence of ouabain, lidocaine, barium, or quinidine, at 7°C, or after fixation with glutaraldehyde. However, no significantly different swelling was observed during a hyposmotic challenge in the absence of Na^+ or in the presence of bumetanide or ethoxyzolamide. Subsequent return to control osmolarity caused a regulatory volume increase that was dependent on Na^+, Cl^-, and K^+, inhibited by bumetanide, ouabain, or 7°C, however not inhibited by ethoxyzolamide, barium, quinidine, or lidocaine. The data suggest that cell volume control during the hyposmotic challenge involved a mechanism dependent on cytosolic KCl and the Na^+-K^+-ATPase and that the Na^+-Cl^--K^+ cotransporter was involved in regulatory volume increase.

regulatory volume decrease; regulatory volume increase; potassium channel blocker; bumetanide; ethoxyzolamide; ouabain; inner ear

VESTIBULAR DARK CELLS in the ampulla of the semicircular canal are known to secrete K^+ from perilymph to endolymph (14). Uptake of K^+ across the basolateral membrane (perilymphatic side) is thought to involve the Na^+-K^+-ATPase and the Na^+-Cl^--K^+ cotransporter (16, 20, 34). Na^+ and Cl^- are taken up by the cotransporter and likely recycle across the basolateral membrane via the Na^+-K^+-ATPase and Cl^- channels, respectively (18, 35). The mechanism of K^+ secretion across the apical membrane is still unclear.

Recently, it has been shown that dark cells swell and regulate their volume when the extracellular K^+ concentration was isosmotically increased (34). Isosmotic K^+-induced cell swelling depended on the piretanide- and bumetanide-sensitive Na^+-Cl^--K^+ cotransporter (34), whereas cell shrinking after K^+-induced cell swelling was abolished in the presence of quinidine or lidocaine and partially inhibited in the presence of barium, suggesting the involvement of a K^+ channel (36).

The aim of the present study was to investigate whether dark cells regulate their volume in the presence of a hyposmotic challenge and upon return to control osmolarity and to characterize the mechanisms involved regarding their ionic requirements and drug sensitivities. In particular, it was of interest to investigate whether volume control mechanisms in the presence of a hyposmotic challenge involve the quinidine-, lidocaine-, and barium-sensitive K^+ channel like cell shrinking after K^+-induced cell swelling and whether volume regulation after the hyposmotic challenge involved the Na^+-Cl^--K^+ cotransporter like K^+-induced cell swelling. Some of the results have been presented at recent meetings (24, 37).

METHODS

Preparation

Gerbils were anesthetized with pentobarbital sodium (50 mg/kg ip) and decapitated. The dissection of dark cell epithelium has been described previously (33). Briefly, dark cell epithelium was dissected at 4°C from an ampulla of the vestibular labyrinth. For measurements of cell height, the tissue was folded into a loop and transferred to the bath chamber on an inverted microscope (IM, Zeiss, Oberleochen, Germany; or Diaphot, Nikon, Tokyo, Japan) where it was clamped to the bottom. The folded tissue was brought into focus such that the apical and basolateral borders of the single-layered epithelium were clearly visible in an optical section (34). If not stated otherwise, the bath chamber, which contained ~0.3 ml, was continuously perfused at a rate of 50–60 ml/min, and experiments were conducted at 37°C. The perfusate had access to both the apical and the basolateral membrane of the epithelium except in the experiment shown in Fig. 3.

Solutions

The compositions of solutions are listed in Table 1. All solutions were adjusted to pH 7.4. The osmolarity of the solutions was measured by freezing point depression (Osmette A; Precision Systems, Natick, MA) to be either 289 ± 2 mosM (n = 23) or 151 ± 1 mosM (n = 16). Bumetanide, ethoxyzolamide, quinidine, lidocaine, and ouabain (Sigma, St. Louis, MO) were predissolved in dimethyl sulfoxide (DMSO) to a final DMSO concentration of 0.1%. At this concentration, DMSO did not have a significant effect on cell height or on the initial rate of K^+-induced cell swelling (unpublished observations). Glutaraldehyde was obtained as a 50% stock solution (EM Science, Gibbstown, NJ).

Data Acquisition

Cell height measurements. For measurements of cell height, the inverted microscope was equipped with a black and white video camera (model WV-1550; Panasonic, Secausus, NJ). The microscope image was mixed with a time signal (Time Code Generator, Fast Forward Video, Irvine, CA) and was displayed on a monitor (model PVM-122; Sony, Park Ridge, NJ) as well as recorded on videotape (model AG-1950 or AG-1960; Panasonic). Two methods (*A* and *B*) were used that yielded nearly identical results when both were applied to the same experiment. For *method A*, a computer-generated image of two vertical cursors was mixed on-line with the microscope image (NTSC Recordable Videocard, USVideo, Stamford, CT). The two cursors could be adjusted independently to overlay the apical and basal borders of the epithelium as shown in Fig. 1. A computer program ("CH") wrote the time and the calibrated

Table 1. *Solutions*

	Solution												
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
NaCl	150.0	75.0	75.0	128.6	75.0			150.0	75.0			138.9	63.9
Na-G						150.0	74.0						
NMG-Cl										150.0	75.0		
KCl				21.4	21.4							3.6	3.6
K_2HPO_4	1.6	1.6	1.6	1.6	1.6	1.6	1.6			1.6	1.6		
KH_2PO_4	0.4	0.4	0.4	0.4	0.4	0.4	0.4			0.4	0.4		
Na_2HPO_4								1.6	1.6				
NaH_2PO_4								0.4	0.4				
HEPES												5.0	5.0
$MgCl_2$	1.0	1.0	1.0	1.0	1.0			1.0	1.0	1.0	1.0	1.0	1.0
$MgSO_4$						1.0	1.0						
$CaCl_2$	0.7	0.7	0.7	0.7	0.7			0.7	0.7	0.7	0.7	0.7	0.7
$Ca\text{-}G_2$						4.0	4.0						
$BaCl_2$												5.0	5.0
Glucose	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Mannitol		150.0			107.2								
Osmolarity, mosM	289	289	151	289	289	289	151	289	151	289	151	289	151

G, gluconate; NMG, *N*-methyl-D-glucamine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid. Solution values are in mM.

distance between the two cursors (cell height) into an ASCII file at a chosen sample rate (0.5 Hz). For analysis of initial rates of cell swelling, another computer program ("SLOPES") read the ASCII files and obtained linear regressions of the data points within specified time intervals. For the initial rate of K^+-induced cell swelling and subsequent shrinking, the chosen time intervals were the initial 10 s during and after the K^+ step, respectively.

Data shown in Figs. 2, 3, and 5–15 were smoothed by a computer program ("SMOOTH") that applied a moving window average. The size of the window was chosen to be five data points. The computer programs CH, SLOPES, and SMOOTH for obtaining data with *method A* are available from the author (P. Wangemann).

Method B has been described previously (34, 36). Briefly, the microscope image was mixed with a time signal and recorded on videotape. The time signal was used for identification of single frames. For analysis of cell height, single frames were selected, and six cell height measurements were averaged from each frame (Java, Jandel Scientific, San Rafael, CA). The locations of the individual measurements on each frame remained constant for all frames in a given experiment from one tissue.

Method A has two advantages over *method B*. *1*) Data were obtained on line; no separate time for analysis was necessary. *2*) Equipment costs were low, since no digitization of the video image was required. However, *method A* has to be employed carefully, since the surface of the apical membrane was not flat when cells were swollen. During swelling, the apical cell perimeter protruded into the bath like hills and valleys (Fig. 1*B*). On the one hand, cell height would have been overestimated if measured between the basal perimeter and the top of these hills; on the other hand, cell height would have been underestimated if measured between the basal perimeter and the valleys. A correct measurement was approximated by placing the cursor at the average between the top of the hills and the bottom of the valleys as shown in Fig. 1*B*. Regarding this latter point, *method B* has an advantage, since six cell height measurements from one video frame were numerically averaged so that hills and valleys in the perimeter of the apical membrane were averaged.

Cell width measurement. In one series of experiments cell width was measured by marking the surface of the tissue with fine carbon particles and monitoring the distance between two particles with *method A* (see above).

Refractive index. The effect of varying refractive indexes on the cell height measurement was tested by measuring the diameter of a glass bead with *method A* (see above) during solution changes. No changes in measured diameter were observed during changes between *solutions 1, 2, 3,* and *4* (data not shown).

Time Course of Solution Changes

The time course of changes between *solutions 1* and *3* near the apical membrane of the tissue and within the fold of the tissue was monitored by measuring the change in the liquid junction potential. This liquid junction potential of ~3 mV was measured as a voltage with a large microelectrode (15 μm OD) filled with 150 mM NaCl. The microelectrode was connected via a Ag/AgCl half-cell to a grounded electrometer (WPI, New Haven, CT). The bath chamber was grounded via a flowing 1 M KCl/calomel reference electrode (Fisher Scientific, Pittsburgh, PA). The signal was amplified 10-fold and recorded on a chart recorder (Fisher Scientific).

Time Course and Application of Solely Apical K^+ Steps

In one experiment, cell height was measured during K^+ steps that were applied solely to the apical membrane (Fig. 3). This experiment was conducted at room temperature (25°C). Immediately before the experiment, perfusion of the bath with *solution 1* was stopped, and flow of trypan blue-colored *solution 4* out of a pipette (20 μm ID) was activated. The pipette was positioned near the apical membrane of the tissue. The time course of the solution change was monitored by measuring the change in the liquid junction potential. According to Ohm's Law ($I = U/R$), the liquid junction potential of ~0.5 mV was measured as a 250-pA current with a large microelectrode (2 MΩ) that was filled with 3 M KCl in 1% agar and was positioned near the apical membrane. The microelectrode was connected via a Ag/AgCl half-cell to the headstage of a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA). The bath chamber was grounded via a flowing 1 M KCl Ag/AgCl half-cell. The signal was recorded on a chart recorder (Fisher Scientific).

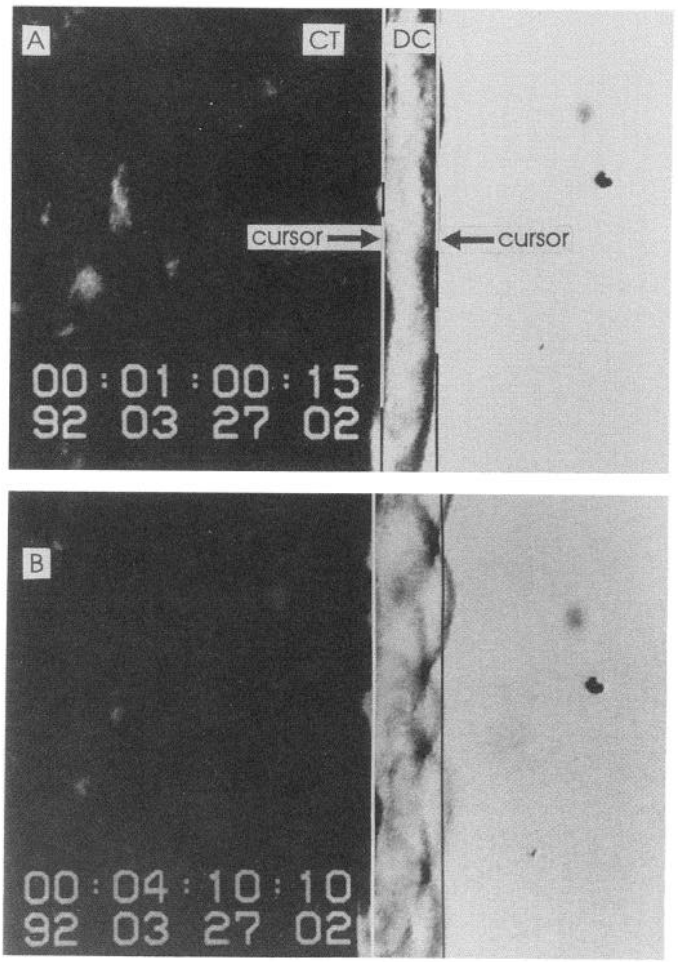

Fig. 1. Data acquisition. Microscope was focused to view an optical cross section of preparation which consists of vestibular dark cell epithelium (DC) and connective tissue (CT). Representative images of epithelium are shown under unswollen (*A*) and swollen conditions (*B*). Microscope image was mixed with a time and day code and with an image of 2 vertical cursors that could be moved pixel by pixel along horizontal axis. Left and right cursors are shown to overlay basal and apical membrane of epithelium. Cell height is distance between left and right cursor. Note that for correct measurement of cell height, "hills" and "valleys" of apical outline of epithelium have to be visually averaged, as shown in *B*.

Data Presentation and Statistics

Data are given as means ± SE of percent cell height or width of the initial rates. The number of observations (n) is equal to the number of epithelial samples. For statistical analysis, averages of original data were compared using Student's t test for paired samples. Averages of percent values were compared using Student's t test after a logarithmic transformation. A logarithmic transformation has been suggested to restore normal distribution, which is required for Student's t test (25). Differences were assumed to be significant when $P < 0.05$.

RESULTS

Hyposmotic Challenges Induced by Removal of 75 mM NaCl or 150 mM Mannitol

Reduction of the osmolarity by removal of 75 mM NaCl (*solution 1* to *solution 3*) caused a slow increase in cell height, reaching 107 ± 1% (n = 55) at the end of 2 min. The average of 16 experiments is shown in Fig. 2*A*. Repeated hyposmotic challenges (12–18 times) did not cause significantly different responses. During the first and last hyposmotic challenge, cell height increased to 102 ± 2 and 104 ± 2%, respectively, at the end of 2 min (n = 4). Upon return to control osmolarity by addition of 75 mM NaCl, cell height first decreased then increased (Fig. 2*A*). This increase in cell height is referred to as a regulatory volume increase (RVI; for review see Ref. 4).

In a parallel series of experiments, the osmolarity was reduced by removal of 150 mM mannitol from a solution containing 75 mM NaCl (*solution 2* to *solution 3*). Cell height increased to 119 ± 2% (n = 6) at the end of 2 min (Fig. 2*B*). Upon return to control osmolarity by addition of 150 mM mannitol, no RVI was observed (Fig. 2*B*).

Isosmotic K^+ Steps as Control Maneuvers

Isosmotic K^+ steps were conducted as control maneuvers. Rapid K^+-induced cell swelling and subsequent shrinking suggested that the preparation was vital, that the perfusion rate was adequate, and that dark cells were water permeable (34, 36).

K^+ was isosmotically elevated for 40 s from 3.6 to 25 mM by replacement for Na^+ (*solution 1* to *solution 4*) before and after a hyposmotic challenge (Fig. 2*A*). During the K^+ step before the hyposmotic challenge, cell height reached a maximum value of 132 ± 1% at 31 ± 1 s (n = 54), after which cell height decreased significantly to 126 ± 2% at the end of the 40 s (isosmotic volume regulation). The initial rate of K^+-induced cell swelling was not significantly different before and after the hyposmotic challenge (0.069 ± 0.006 vs. 0.070 ± 0.006 μm/s; n = 54); however, the initial rate of cell shrinking after the second K^+ step was significantly decreased (−0.088 ± 0.007 vs. −0.048 ± 0.009 μm/s; n = 54). The latter observation may indicate that the ratio of intracellular K^+ to Na^+ had decreased due to volume regulation during and after the hyposmotic challenge, since cell shrinking after isosmotic K^+-induced cell swelling is thought to depend on the ratio of intracellular K^+ to Na^+ (34, 36). Consistent with this view are the findings that the initial rates of cell shrinking after the third K^+ step were slowed more after a long K^+-free period (Fig. 7) or ouabain exposure (Fig. 9) than after a Cl^--free period (Fig. 6) or bumetanide exposure (Fig. 10). Similar observations have been reported earlier (34).

In a parallel series of experiments, K^+ was isosmotically elevated for 40 s from 3.6 to 25 mM by replacement of mannitol (*solution 2* to *solution 5*) before and after a hyposmotic challenge (Fig. 2*B*). Before the hyposmotic challenge, cell height reached a maximum of 124 ± 3% at 38 ± 1 s (n = 6). Isosmotic volume regulation in the presence of elevated K^+ was not observed. After the hyposmotic challenge, the initial rates of K^+-induced cell swelling and subsequent cell shrinking were both significantly decreased (0.060 ± 0.014 vs. 0.036 ± 0.008 μm/s and −0.112 ± 0.016 vs. −0.060 ± 0.019 μm/s, respectively; n = 6), suggesting that the hyposmotic challenge in the presence of only 75 mM NaCl was not fully reversible.

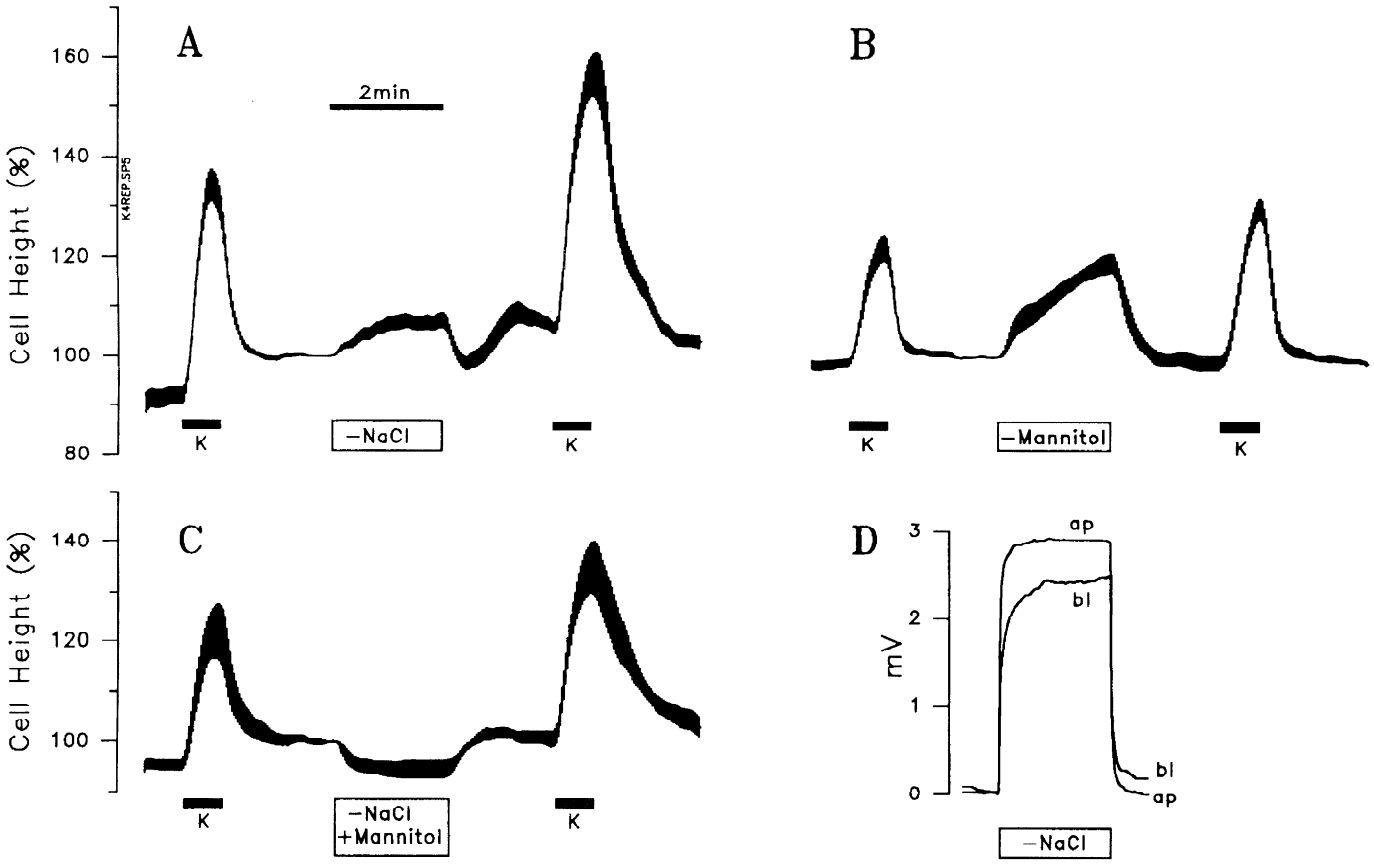

Fig. 2. Hyposmotic challenges, isosmotic NaCl removal, and verification of solution changes. *A–C*: means ± SE of cell height measurements in 2-s intervals are plotted vs. time. *A*: osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl). Cell height at beginning of hyposmotic challenge was 100%, 7.5 ± 0.2 μm (n = 16). *B*: osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 150 mM mannitol (−mannitol). Cell height at beginning of hyposmotic challenge was 100%, 8.2 ± 0.6 μm (n = 6). *C*: isosmotic removal of 75 mM NaCl replaced by 150 mM mannitol (−NaCl +mannitol). Cell height at beginning of hyposmotic challenge was 100%, 7.9 ± 0.4 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM were conducted as control experiments (solid bars) and were obtained in *A* and *C* by K^+ replacing Na^+ and in *B* by K^+ replacing mannitol. *D*: as a verification of solution change, liquid junction potential intrinsic to removal of 75 mM NaCl was obtained near apical membrane (ap) and near connective tissue within fold of preparation (bl). Time base given in *A* applies to whole figure.

Verification of the finding that K^+-induced cell swelling occurred via the Na^+-Cl^--K^+ cotransporter located in the basolateral membrane (34, 36) and not via some mechanism in the apical membrane was obtained with a separate experimental protocol (see METHODS). In this experiment, unlike all other experiments, K^+ steps from 3.6 to 25 mM (*solution 1* to *solution 4*) were applied solely to the apical membrane during measurements of cell height (Fig. 3). The time course of the solution change was monitored by measuring the current driven by the liquid junction potential across the resistance of the large microelectrode (Fig. 3). During this solely apical K^+ step, cell height did not change significantly in contrast to experiments at room temperature (unpublished observations) or at 37°C (see above), where the perfusate had access to both the apical and the basolateral membrane.

Isosmotic Removal of NaCl

Isosmotic removal of 75 mM NaCl by replacement with mannitol (*solution 1* to *solution 2*) caused significant cell shrinkage to 95 ± 2% (n = 6) at the end of 2 min (Fig. 2*C*).

Time Course of Solution Changes

The time course of the arrival of the hyposmotic challenge was monitored near the apical membrane of the epithelium and within the fold of the preparation by measuring the change in the liquid junction potential (Fig. 2*D*). As expected, arrival of hyposmotic challenge was slower within the fold of the tissue than near the apical membrane of the epithelium.

Maximal Increase of Cell Height During a Hyposmotic Challenge

Two series of experiments were conducted to determine the maximal increase in cell height during a hyposmotic challenge induced by removal of 75 mM NaCl.

Cooling of the control solution (*solution 1*) from 37 to 7°C caused significant cell shrinking. Cell height de-

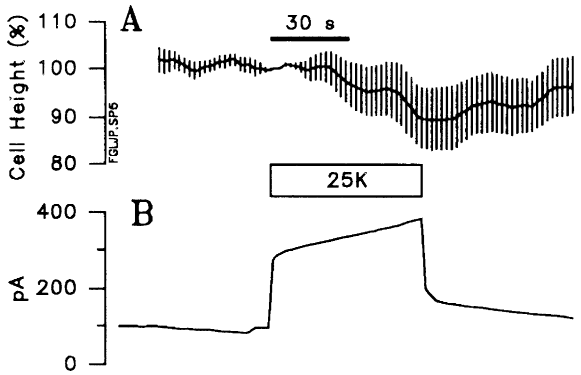

Fig. 3. Effect of solely apical isosmotic K^+ steps from 3.6 to 25 mM. *A*: means ± SE of cell height measurements in 2-s intervals are plotted vs. time. Cell height at beginning of K^+ step was 100%, 5.6 ± 0.8 μm (n = 5). *B*: as a verification of solution change, liquid junction potential intrinsic to K^+ step was measured as current near apical membrane.

creased to 83 ± 2% (n = 6) at the end of 140 s (Fig. 4*A*). Reduction of the osmolarity by removal of 75 mM NaCl under control conditions (37°C) and at 7°C (*solution 1* to *solution 3*) caused cell height to increase to 103 ± 3 and 119 ± 3%, respectively, at the end of 2 min (n = 6; Fig. 4*B*). Hyposmotically induced cell swelling at 7°C was significantly larger than under control conditions at 37°C. Upon return to control osmolarity, RVI was not observed at 7°C (n = 6; Fig. 4).

Fixation of the tissue with 1 mM glutaraldehyde (in *solution 1*) caused no significant change in cell height (99.7 ± 3%; n = 6) at the end of 60 s (Fig. 5*A*). In response to a hyposmotic challenge under control conditions (*solution 1* to *solution 3*), cell height increased to 109 ± 1% (n = 6) at the end of 2 min. During a hyposmotic challenge after the first and fourth exposure to glutaraldehyde, cell height increased to 115 ± 2 and 128 ± 2%, respectively, at the end of 2 min (n = 6). Hyposmotically induced cell swelling during the fourth challenge was significantly larger than under control conditions (Fig. 5*B*). Upon return to control osmolarity, RVI was not observed after glutaraldehyde fixation (n = 6; Fig. 5).

Ionic Requirements of Volume Control Mechanisms

Cl^- dependence. Isosmotic replacement of Cl^- by gluconate (*solution 1* to *solution 6*) caused a significant decrease of cell height to 88 ± 1% (n = 6) at the end of 40 s (Fig. 6*A*) as reported earlier (34). Upon isosmotic reintroduction of Cl^-, cell height increased to higher values than before removal of Cl^-. This overshooting response remains unexplained.

In response to a hyposmotic challenge under control conditions (*solution 1* to *solution 3*), cell height increased to 108 ± 2% (n = 6) at the end of 2 min. During the first and fourth hyposmotic challenge in the absence of Cl^- (*solution 6* to *solution 7*), cell height increased to 108 ± 1 and 119 ± 3%, respectively, at the end of 2 min (n = 6). Hyposmotically induced cell swelling during the fourth challenge was significantly larger than under control conditions (Fig. 6*C*). Upon return to control

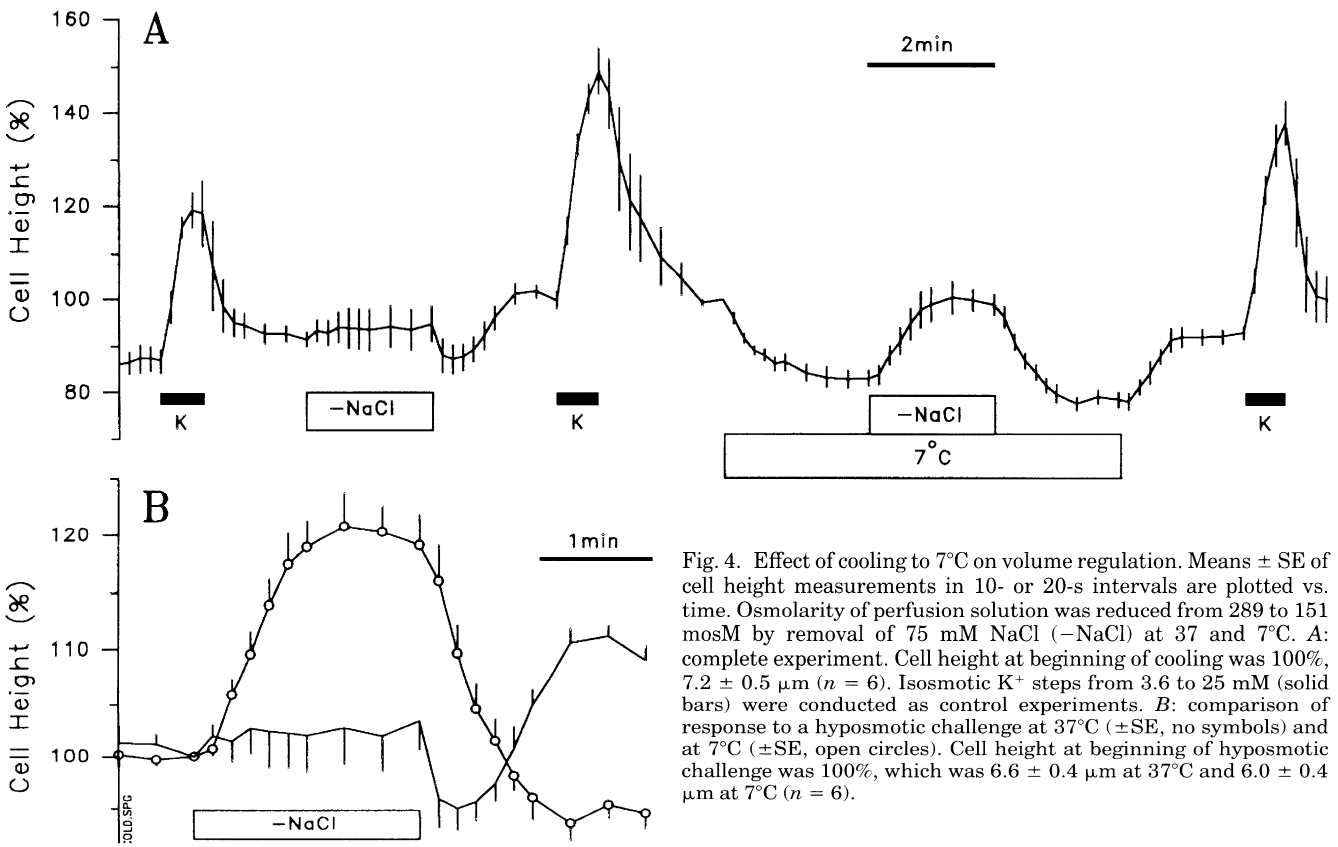

Fig. 4. Effect of cooling to 7°C on volume regulation. Means ± SE of cell height measurements in 10- or 20-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl) at 37 and 7°C. *A*: complete experiment. Cell height at beginning of cooling was 100%, 7.2 ± 0.5 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: comparison of response to a hyposmotic challenge at 37°C (±SE, no symbols) and at 7°C (±SE, open circles). Cell height at beginning of hyposmotic challenge was 100%, which was 6.6 ± 0.4 μm at 37°C and 6.0 ± 0.4 μm at 7°C (n = 6).

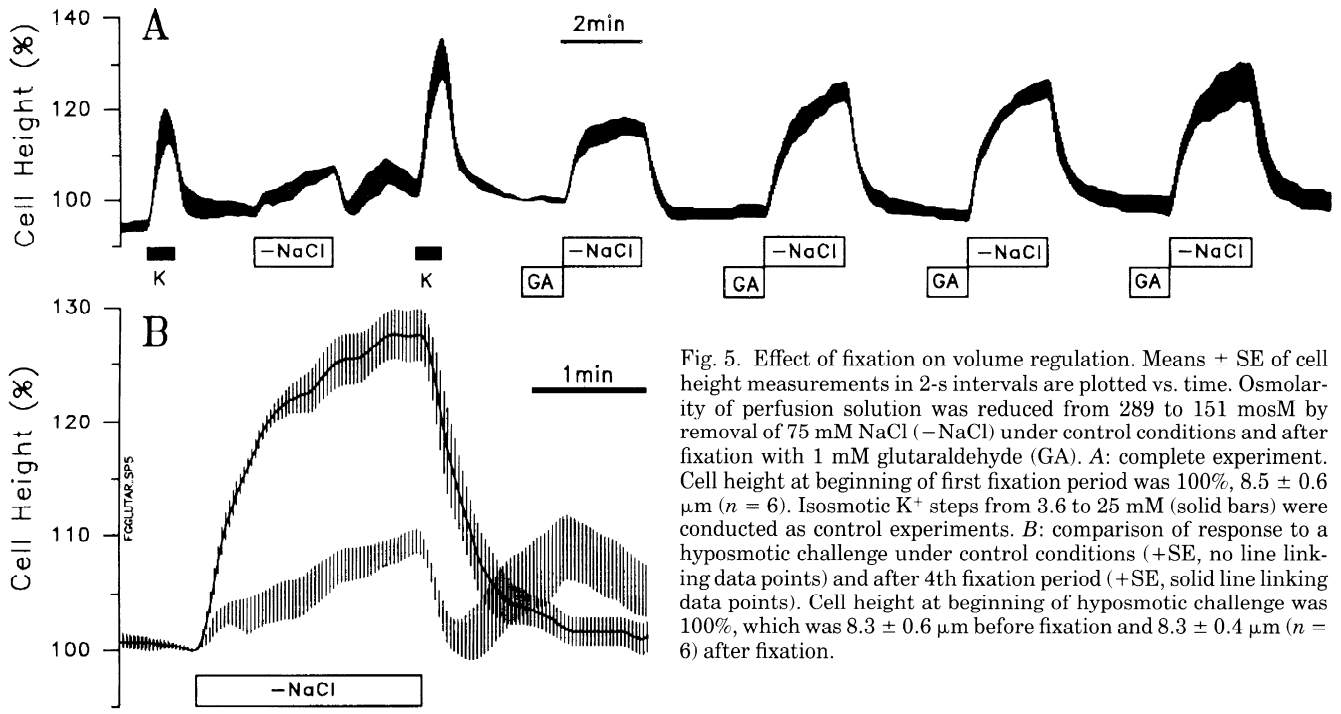

Fig. 5. Effect of fixation on volume regulation. Means + SE of cell height measurements in 2-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl) under control conditions and after fixation with 1 mM glutaraldehyde (GA). *A*: complete experiment. Cell height at beginning of first fixation period was 100%, 8.5 ± 0.6 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: comparison of response to a hyposmotic challenge under control conditions (+SE, no line linking data points) and after 4th fixation period (+SE, solid line linking data points). Cell height at beginning of hyposmotic challenge was 100%, which was 8.3 ± 0.6 μm before fixation and 8.3 ± 0.4 μm (n = 6) after fixation.

osmolarity, RVI was not observed in the absence of Cl^- (n = 6; Fig. 6).

In a similar series of experiments, cell width was measured. No significant changes were detected during isosmotic replacement of Cl^- or during hyposmotic challenges under control or Cl^--free conditions (Fig. 6*B*).

In another series of experiments, hyposmotic challenges were repeated 12 times in the absence of Cl^-. During the 12th hyposmotic challenge, cell height increased to 123 ± 5% (n = 3) at the end of 2 min.

K^+ dependence. Isosmotic replacement of K^+ by Na^+ (*solution 1* to *solution 8*) caused a significant decrease in cell height to 95 ± 1% (n = 6) at the end of 40 s (Fig. 7*A*). Upon isosmotic reintroduction of K^+, cell height decreased again. This response remains unexplained.

In response to a hyposmotic challenge under control conditions (*solution 1* to *solution 3*), cell height increased to 108 ± 2% (n = 6) at the end of 2 min. During the first and fourth hyposmotic challenge in the absence of K^+ (*solution 8* to *solution 9*), cell height increased to 104 ± 2 and 124 ± 4%, respectively, at the end of 2 min (n = 6). Hyposmotically induced cell swelling during the fourth challenge was significantly larger than under control conditions (Fig. 7*B*). Upon return to control osmolarity, RVI was not observed in the absence of K^+ (n = 6; Fig. 7).

Na^+ dependence. Isosmotic replacement of Na^+ by *N*-methyl-D-glucamine (*solution 1* to *solution 10*) caused a significant decrease of cell height to 95 ± 1% (n = 6) at the end of 40 s (Fig. 8*A*), as reported earlier (34). Upon isosmotic reintroduction of Na^+, cell height returned to control values as observed before removal of Na^+. The mechanism for cell shrinking upon removal of Na^+ remains unexplained.

In response to a hyposmotic challenge under control conditions (*solution 1* to *solution 3*), cell height increased to 110 ± 1% (n = 6) at the end of 2 min. During the first and fourth hyposmotic challenge in the absence of Na^+ (*solution 10* to *solution 11*), cell height increased to 104 ± 1 and 111 ± 1% (n = 6) at the end of 2 min. Hyposmotically induced cell swelling during the fourth challenge was not significantly different from under control conditions (Fig. 8*B*). Upon return to control osmolarity, RVI was not observed in the absence of Na^+ (n = 6; Fig. 8).

In a separate series of experiments, hyposmotic challenges were repeated 12 times in the absence of Na^+. During the 12th hyposmotic challenge, cell height increased to 110 ± 5% (n = 3) at the end of 2 min.

Drug Sensitivity of Volume Control Mechanisms

The drug sensitivity of the volume control mechanisms was determined. Drugs included a blocker of the Na^+-K^+-ATPase (ouabain), a blocker of the Na^+-Cl^--K^+ cotransporter (bumetanide; Ref. 36), and the K^+ channel blockers barium, quinidine, and lidocaine (28).

Ouabain. Addition of 10^{-3} M ouabain (in *solution 1*) caused no significant change in cell height (105 ± 6%; n = 6) at the end of 40 s (Fig. 9*A*). Furthermore, no change in cell height was observed upon removal of ouabain. Similar results have been reported earlier (34).

In response to a hyposmotic challenge under control conditions (*solution 1* to *solution 3*), cell height increased to 104 ± 2% (n = 6) at the end of 2 min. During the first and fourth hyposmotic challenge in the presence of ouabain, cell height increased to 110 ± 3 and 114 ± 2%, respectively, at the end of 2 min (n = 6). Hyposmotically induced cell swelling during the fourth

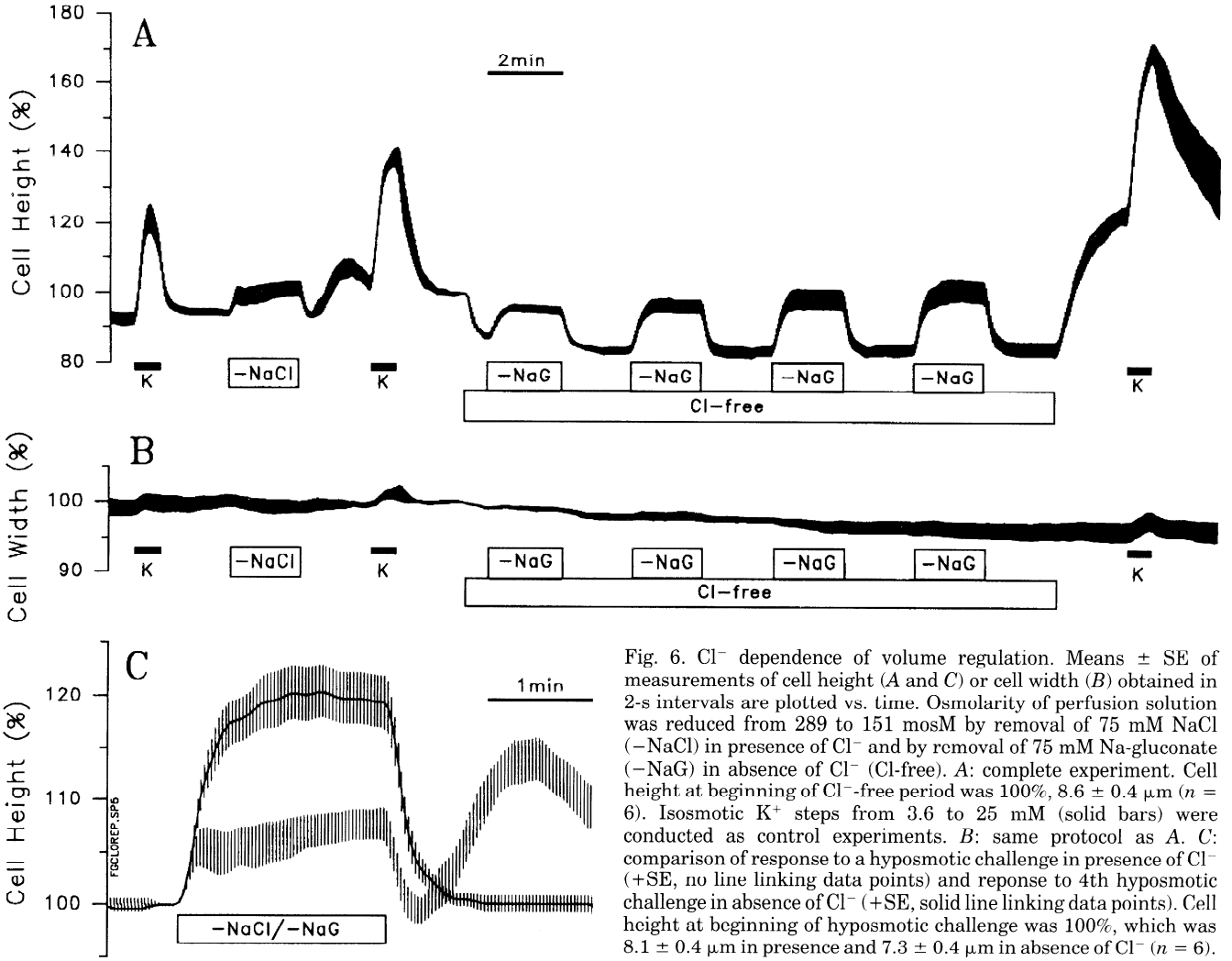

Fig. 6. Cl^- dependence of volume regulation. Means ± SE of measurements of cell height (*A* and *C*) or cell width (*B*) obtained in 2-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl) in presence of Cl^- and by removal of 75 mM Na-gluconate (−NaG) in absence of Cl^- (Cl-free). *A*: complete experiment. Cell height at beginning of Cl^--free period was 100%, 8.6 ± 0.4 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: same protocol as *A*. *C*: comparison of response to a hyposmotic challenge in presence of Cl^- (+SE, no line linking data points) and reponse to 4th hyposmotic challenge in absence of Cl^- (+SE, solid line linking data points). Cell height at beginning of hyposmotic challenge was 100%, which was 8.1 ± 0.4 μm in presence and 7.3 ± 0.4 μm in absence of Cl^- (n = 6).

challenge was significantly larger than under control conditions (Fig. 9*B*). Upon return to control osmolarity, RVI was not observed in the presence of ouabain (n = 6; Fig. 9).

In a separate series of experiments, hyposmotic challenges were repeated 12 times in the presence of ouabain. During the 12th hyposmotic challenge, cell height increased to 112 ± 2% (n = 3) at the end of 2 min.

Bumetanide. Addition of 10^{-5} M bumetanide (in *solution 1*) caused no significant change in cell height (to 99 ± 1%; n = 6) at the end of 40 s (Fig. 10*A*). Furthermore, no change in cell height was observed upon removal of bumetanide. Similar results have been reported earlier (34).

In response to a hyposmotic challenge under control conditions (*solution 1* to *solution 3*), cell height increased within 2 min to 107 ± 1% (n = 6). During the first and fourth hyposmotic challenge in the presence of bumetanide, cell height increased to 108 ± 2 and 108 ± 1%, respectively, at the end of 2 min (n = 6). Hyposmotically induced cell swelling during the fourth challenge was not significantly different from that under control conditions (Fig. 10*B*). Upon return to control osmolarity, RVI was partially inhibited in the presence of bumetanide (n = 6; Fig. 10).

Ethoxyzolamide. Addition of 10^{-4} M ethoxyzolamide (in *solution 1*) caused no significant change in cell height (100.6 ± 0.4%; n = 6) at the end of 40 s (Fig. 11*A*). Furthermore, no change in cell height was observed upon removal of ethoxyzolamide.

In response to a hyposmotic challenge under control conditions (*solution 1* to *solution 3*), cell height increased within 2 min to 109 ± 2% (n = 6). During the first and fourth hyposmotic challenge in the presence of ethoxyzolamide, cell height increased to 107 ± 1 and 105 ± 2%, respectively, at the end of 2 min (n = 6). Hyposmotically induced cell swelling during the fourth challenge was not significantly different from under control conditions (Fig. 11*B*). Upon return to control osmolarity, RVI was observed in the presence of ethoxyzolamide (n = 6; Fig. 11).

Barium. Addition of 5×10^{-3} M barium (*solution 12*) caused a significant increase in cell height to 108 ± 2% (n = 6) at the end of 40 s (Fig. 12*A*). Upon removal of barium, cell height decreased toward control values. Similar observations have been reported earlier (36).

Reduction of the osmolarity under control conditions (*solution 1* to *solution 3*) caused a monophasic increase in cell height to 106 ± 3% (n = 6) at the end of 2 min. In contrast, a hyposmotic challenge in the presence of

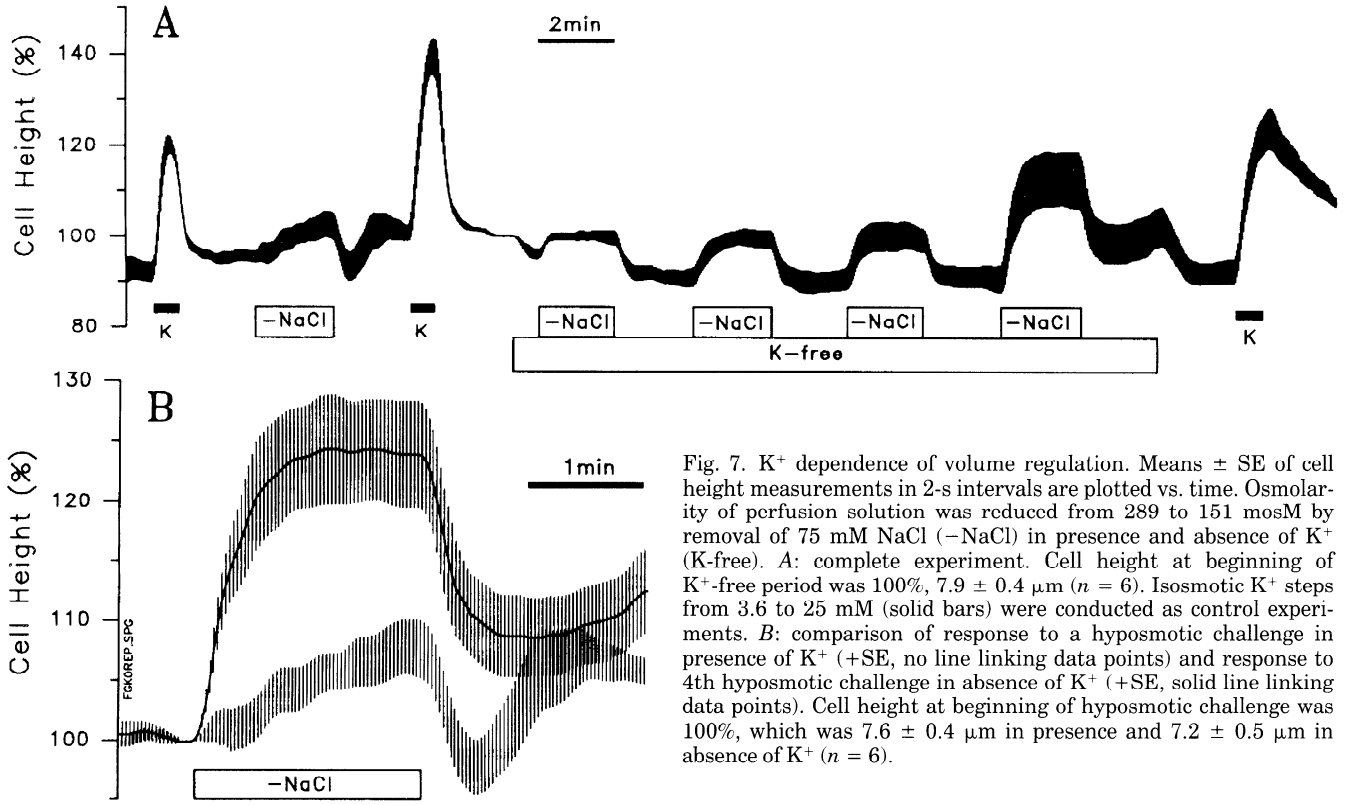

Fig. 7. K^+ dependence of volume regulation. Means ± SE of cell height measurements in 2-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl) in presence and absence of K^+ (K-free). *A*: complete experiment. Cell height at beginning of K^+-free period was 100%, 7.9 ± 0.4 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: comparison of response to a hyposmotic challenge in presence of K^+ (+SE, no line linking data points) and response to 4th hyposmotic challenge in absence of K^+ (+SE, solid line linking data points). Cell height at beginning of hyposmotic challenge was 100%, which was 7.6 ± 0.4 μm in presence and 7.2 ± 0.5 μm in absence of K^+ (n = 6).

barium (*solution 12* to *solution 13*) caused cell height to increase to 113 ± 2% at 22 s followed by a decrease in cell height to 106 ± 2% (n = 6) at the end of 2 min. Upon return to control osmolarity in the presence of barium, cell height first decreased then increased (n = 6; Fig. 12). This increase in cell height could either be due to RVI or to isosmotic barium-induced cell swelling.

Quinidine. Addition of 10^{-3} M quinidine (in *solution 1*) caused no significant change in cell height (to 104 ± 3%; n = 7) at the end of 40 s (Fig. 13*A*), contrary to an earlier report (36). However, upon removal of quinidine, cell height decreased toward control values, suggesting that quinidine caused cell swelling.

Reduction of the osmolarity under control conditions (*solution 1* to *solution 3*) caused a monophasic increase in cell height to 106 ± 3% (n = 7) at the end of 2 min. In contrast, a hyposmotic challenge in the presence of quinidine caused cell height to increase to 109 ± 2% at 10 s followed by a decrease in cell height to 105 ± 5% (n = 7) at the end of 2 min. Upon return to control osmolarity in the presence of quinidine, cell height first decreased then increased (n = 7; Fig. 13). This increase in cell height could either be due to RVI or to isosmotic quinidine-induced cell swelling.

Lidocaine. Addition of 5 × 10^{-3} M lidocaine (in *solution 1*) caused a significant increase in cell height to 104 ± 1% (n = 6) at the end of 40 s (Fig. 14*A*). Upon removal of lidocaine, cell height decreased toward control values. Lidocaine-induced cell swelling has been reported earlier (36).

Reduction of the osmolarity by removal of 75 mM NaCl under control conditions and in the presence of lidocaine (*solution 1* to *solution 3*) caused cell height to increase to 108 ± 1 and 119 ± 2%, respectively, at the end of 2 min (n = 6). Upon return to control osmolarity in the presence of lidocaine, cell height first decreased then increased (n = 6; Fig. 14). This increase in cell height could either be due to RVI or to isosmotic lidocaine-induced cell swelling.

In parallel experiments, the hyposmotic challenge was achieved by removal of 150 mM mannitol from a solution containing 75 mM NaCl. Addition of 5 × 10^{-3} M lidocaine (in *solution 2*) caused no significant change in cell height (to 102 ± 1%; n = 6) at the end of 40 s (Fig. 15*A*). However, upon removal of lidocaine, cell height decreased toward control values.

Reduction of the osmolarity by removal of 150 mM mannitol under control conditions and in the presence of lidocaine (*solution 2* to *solution 3*) caused cell height to increase to 119 ± 2 and 160 ± 4%, respectively, at the end of 2 min (n = 6). Upon return to control osmolarity, RVI was observed neither in the absence nor in the presence of lidocaine (n = 6; Fig. 15).

DISCUSSION

Method

A variety of methods have been developed to monitor changes in volume of epithelial cells (2, 5, 9, 22, 23, 26, 29, 34, 39). Nearly all these methods have been verified as suitable for the epithelia to which they were applied by the simple observation that a change in the osmolarity caused at least transiently a change in cell volume

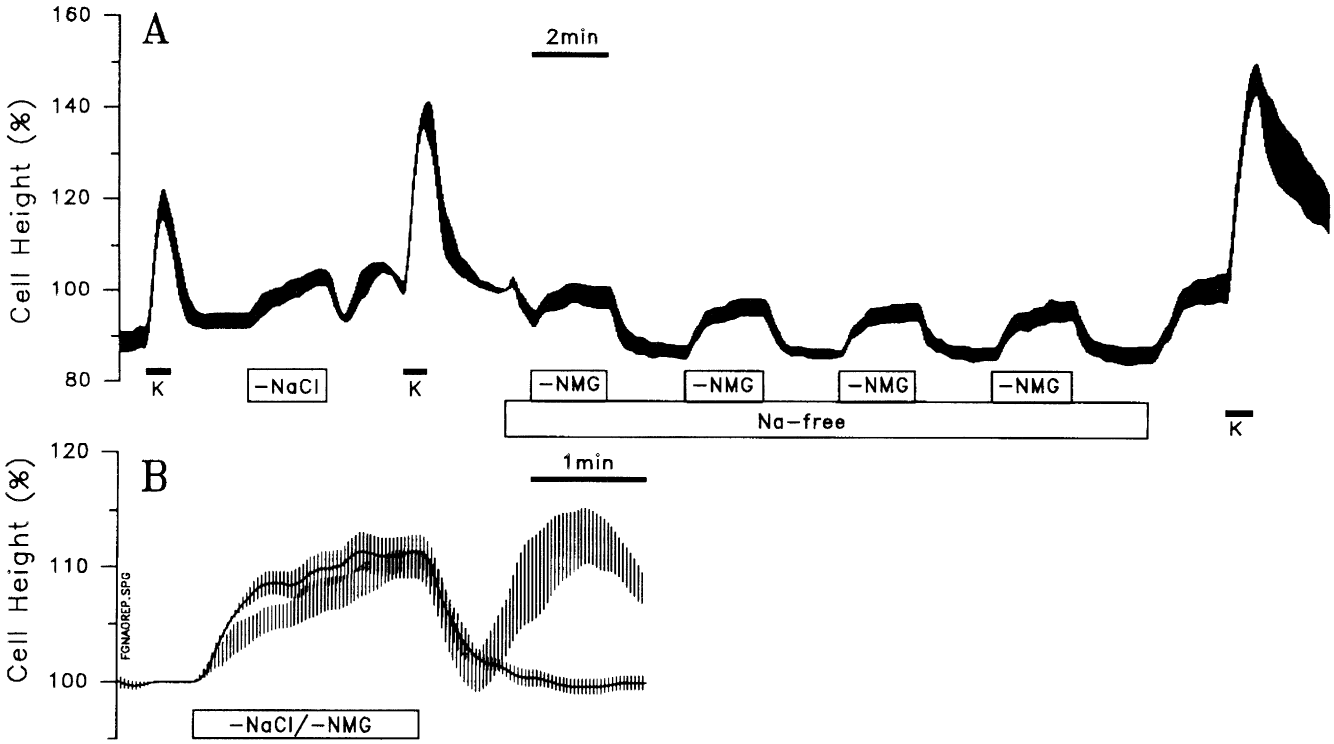

Fig. 8. Na^+ dependence of volume regulation. Means ± SE of cell height measurements in 2-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl) in presence of Na^+ or by removal of 75 mM *N*-methyl-D-glucamine chloride (−NMG) in absence of Na^+ (Na-free). *A*: complete experiment. Cell height at beginning of Na^+-free period was 100%, 7.9 ± 0.2 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: comparison of response to a hyposmotic challenge in presence of Na^+ (+SE, no line linking data points) and response to 4th hyposmotic challenge in absence of Na^+ (+SE, solid line linking data points). Cell height at beginning of hyposmotic challenge was 100%, which was 7.3 ± 0.1 μm in presence and 6.8 ± 0.2 μm in absence of Na^+ (n = 6).

that was within 10–20% of what was expected from an ideal osmometer. Several of these methods monitor cell height or tubular diameter within a homogeneous epithelium and have been successfully applied to epithelia that are morphologically similar to vestibular dark cells with regard to the high degree of basolateral infoldings.

Two experimental methods are commonly applied to achieve a hyposmotic challenge: partial removal of the major salt, mostly NaCl (e.g., Refs. 10, 21, 27, 38), and isosmotic partial replacement of NaCl with the impermeant osmolyte mannitol with subsequent removal of the mannitol (9, 31, 32). The former method has the advantage that the ionic contents of the solutions before the hyposmotic challenge is physiological; however, Na^+ and Cl^- concentration steps are superimposed on the hyposmotic challenge. The latter method has the advantage that no ion concentration steps are superimposed; however, volume regulatory mechanisms might be partially impaired due to the low Na^+ and Cl^- concentrations.

Several observations suggest that volume regulatory mechanisms and ion transport mechanisms in dark cells were impaired in the presence of 75 mM NaCl and 150 mM mannitol. *1*) Isosmotic volume regulation during the K^+ step was absent (Fig. 2*B*). *2*) RVI after the hyposmotic challenge was absent (Fig. 2*B*). *3*) Lidocaine-induced cell swelling was absent (Fig. 15*A*). Therefore, most hyposmotic challenges in the present study were obtained by removal of 75 mM NaCl.

Response to a Hyposmotic Challenge

The volume of an ideal osmometer would be expected to increase to 191% during a reduction of the osmolarity from 289 to 151 mosM. If dark cells would be ideal osmometers and expand only in height (one dimension), cell height would be expected to increase to 191%, or if they would expand in three dimensions, cell height as well as width and depth would be expected to each increase to 124%. However, the maximal increase in cell height during a hyposmotic challenge induced by removal of NaCl was 119–128% as determined under most unspecific inhibitory conditions such as cooling or fixation (Figs. 4 and 5); however, no increase in cell width was detected under Cl^--free conditions (Fig. 6*B*).

This discrepancy cannot be explained by a large unstirred layer, since solution changes were conducted rapidly (Fig. 2*D*) and since cell volume under isosmotic conditions changed rapidly when K^+ was elevated or when K^+, Cl^-, or Na^+ was reduced (Figs. 2 and 6–8). The mechanism for cell shrinking upon isosmotic removal of K^+, Cl^-, or Na^+ might involve K^+, Cl^-, nonselective cation channels, or the Na^+-Cl^--K^+ cotransporter present in dark cells (17, 18, 28, 34, 36). However,

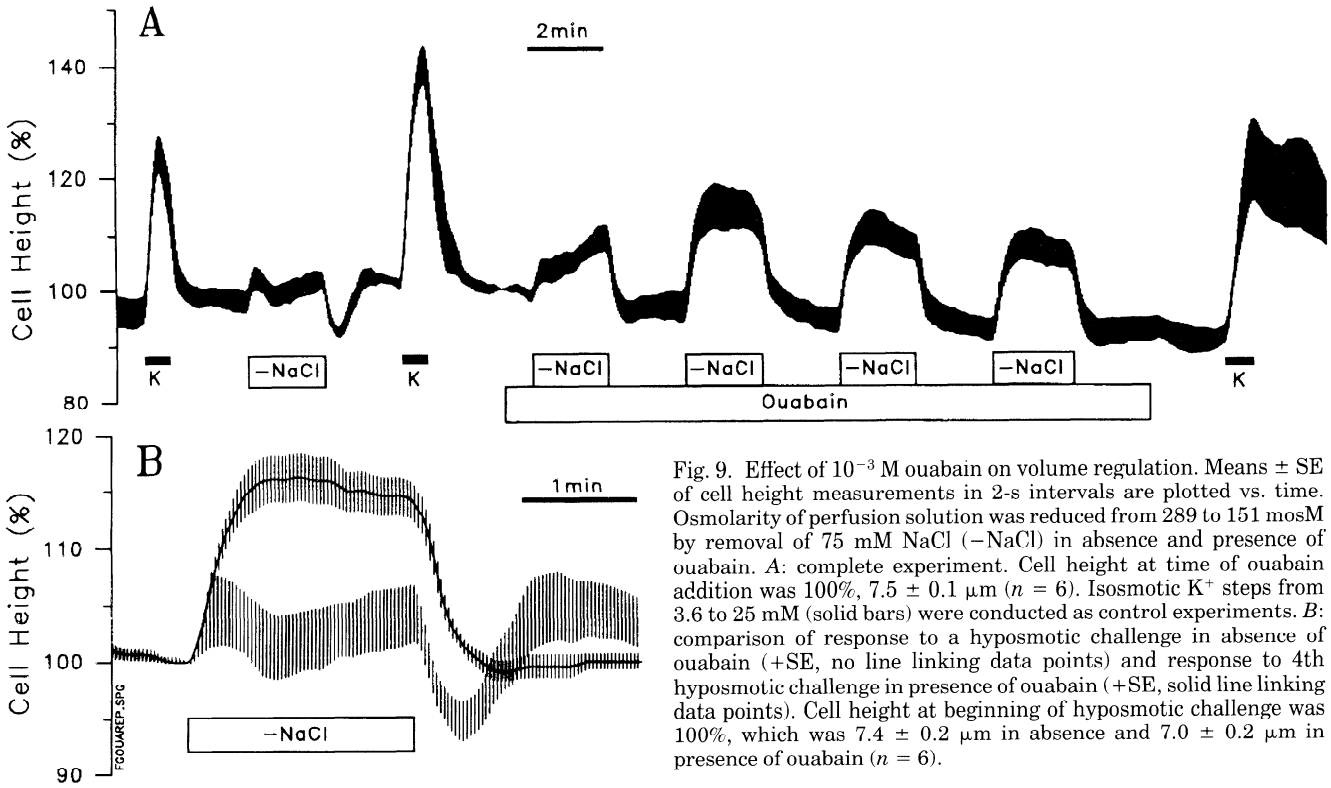

Fig. 9. Effect of 10^{-3} M ouabain on volume regulation. Means ± SE of cell height measurements in 2-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl) in absence and presence of ouabain. *A*: complete experiment. Cell height at time of ouabain addition was 100%, 7.5 ± 0.1 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: comparison of response to a hyposmotic challenge in absence of ouabain (+SE, no line linking data points) and response to 4th hyposmotic challenge in presence of ouabain (+SE, solid line linking data points). Cell height at beginning of hyposmotic challenge was 100%, which was 7.4 ± 0.2 μm in absence and 7.0 ± 0.2 μm in presence of ouabain (n = 6).

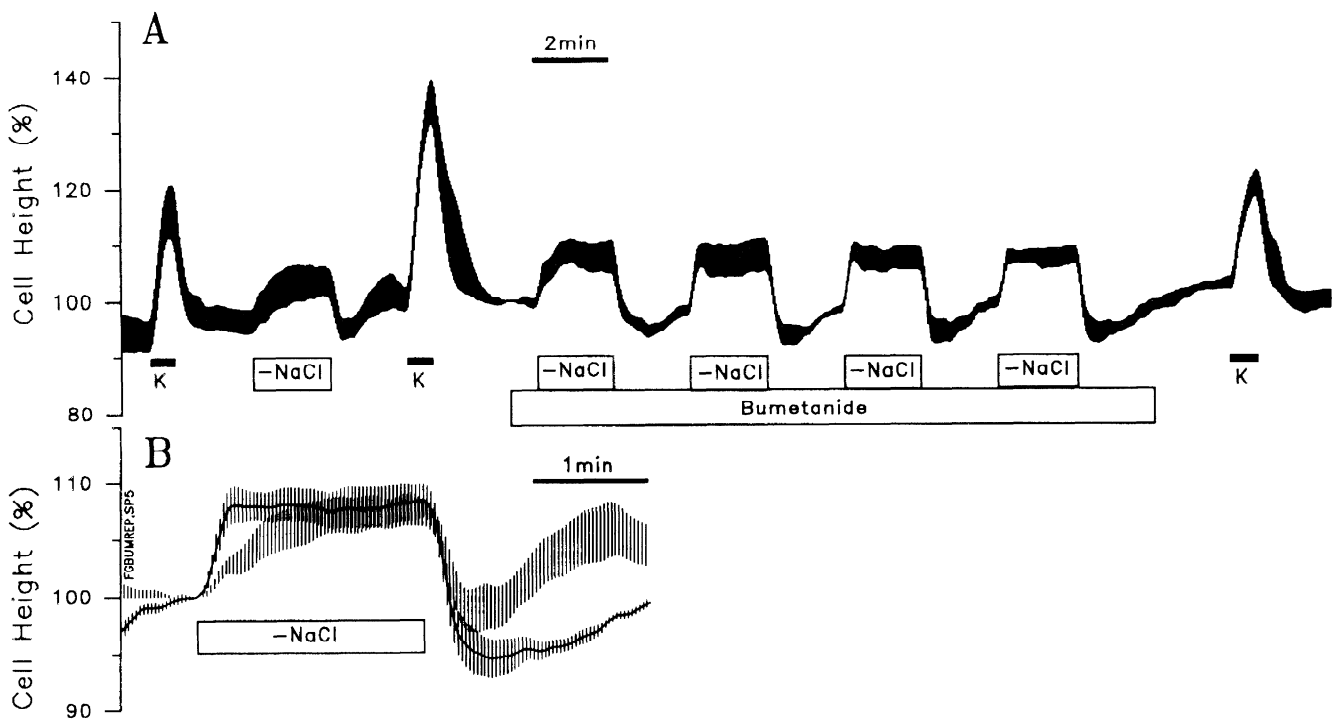

Fig. 10. Effect of 10^{-5} M bumetanide on volume regulation. Means ± SE of cell height measurements in 2-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl) in absence and presence of bumetanide. *A*: complete experiment. Cell height at time of bumetanide addition was 100%, 8.0 ± 0.3 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: comparison of response to a hyposmotic challenge in absence of bumetanide (+SE, no line linking data points) and response to 4th hyposmotic challenge in presence of bumetanide (+SE, solid line linking data points). Cell height at beginning of hyposmotic challenge was 100%, which was 7.7 ± 0.2 μm in absence and 8.0 ± 0.3 μm in presence of bumetanide (n = 6).

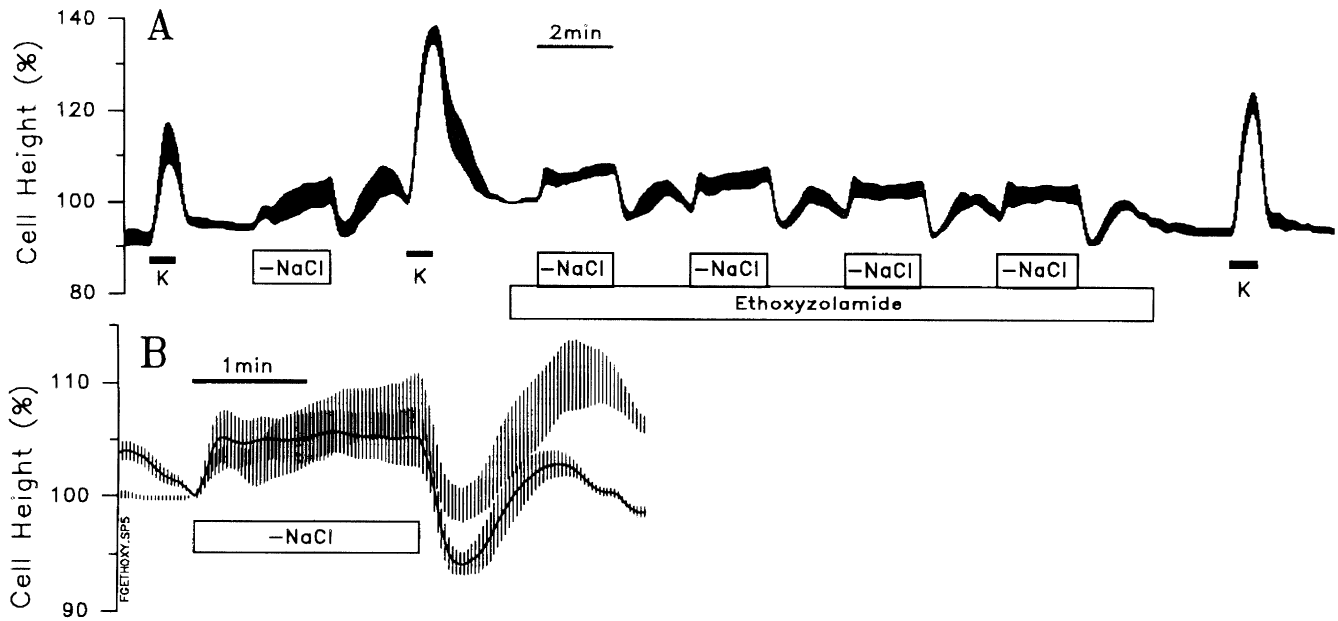

Fig. 11. Effect of 10^{-4} M ethoxyzolamide on volume regulation. Means ± SE of cell height measurements in 2-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl) in absence and presence of ethoxyzolamide. *A*: complete experiment. Cell height at time of ethoxyzolamide addition was 100%, 8.2 ± 0.4 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: comparison of response to a hyposmotic challenge in absence of ethoxyzolamide (+SE, no line linking data points) and response to 4th hyposmotic challenge in presence of ethoxyzolamide (+SE, solid line linking data points). Cell height at beginning of hyposmotic challenge was 100%, which was 7.8 ± 0.4 μm in absence and 8.0 ± 0.4 μm in presence of ethoxyzolamide (n = 6).

the finding that dark cells shrunk rapidly during isosmotic removal of Cl^- supports the assumption that the unstirred layer was not protecting the basolateral membrane from solution changes, including the hyposmotic challenge, since a Cl^- conductance and Cl^- channels constituting this Cl^- conductance have only been found in the basolateral membrane (14, 15, 18).

The observation that cell height and width measurements in dark cells did not yield a response close to what was expected from an ideal osmometer could be ex-

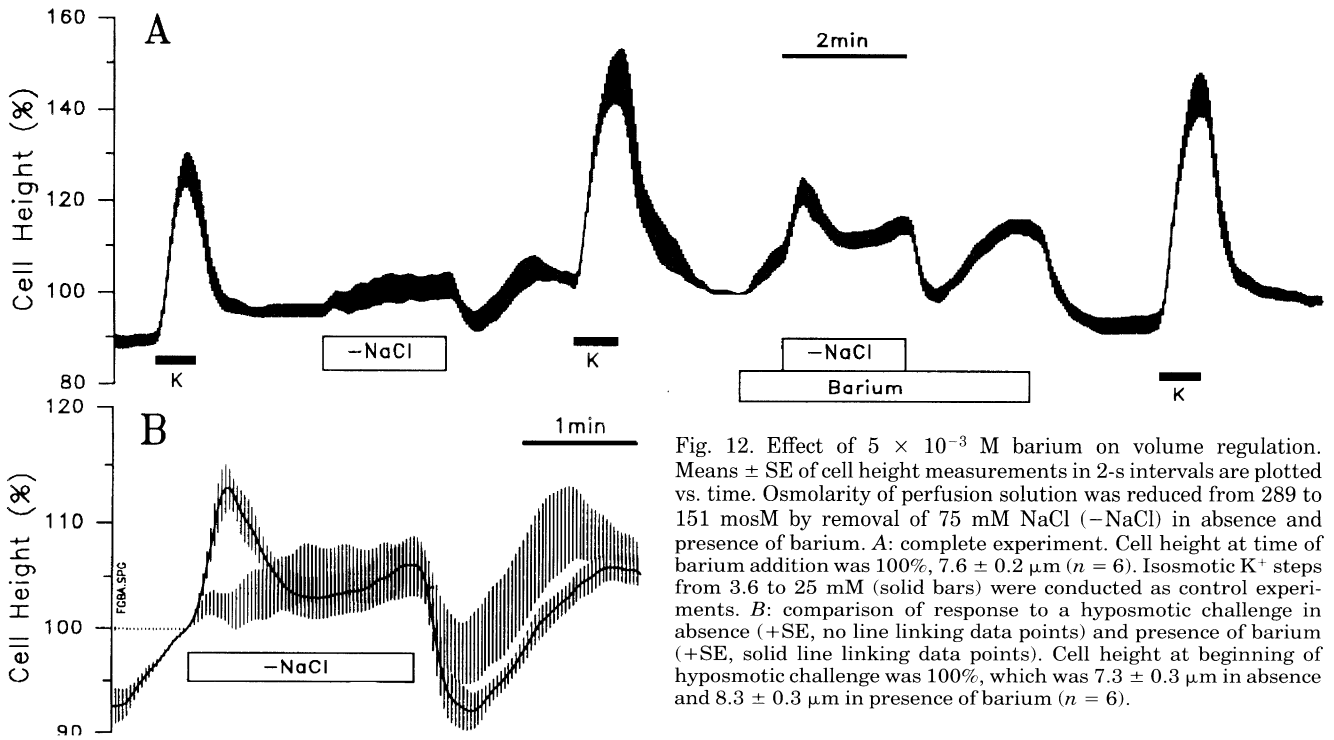

Fig. 12. Effect of 5 × 10^{-3} M barium on volume regulation. Means ± SE of cell height measurements in 2-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl) in absence and presence of barium. *A*: complete experiment. Cell height at time of barium addition was 100%, 7.6 ± 0.2 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: comparison of response to a hyposmotic challenge in absence (+SE, no line linking data points) and presence of barium (+SE, solid line linking data points). Cell height at beginning of hyposmotic challenge was 100%, which was 7.3 ± 0.3 μm in absence and 8.3 ± 0.3 μm in presence of barium (n = 6).

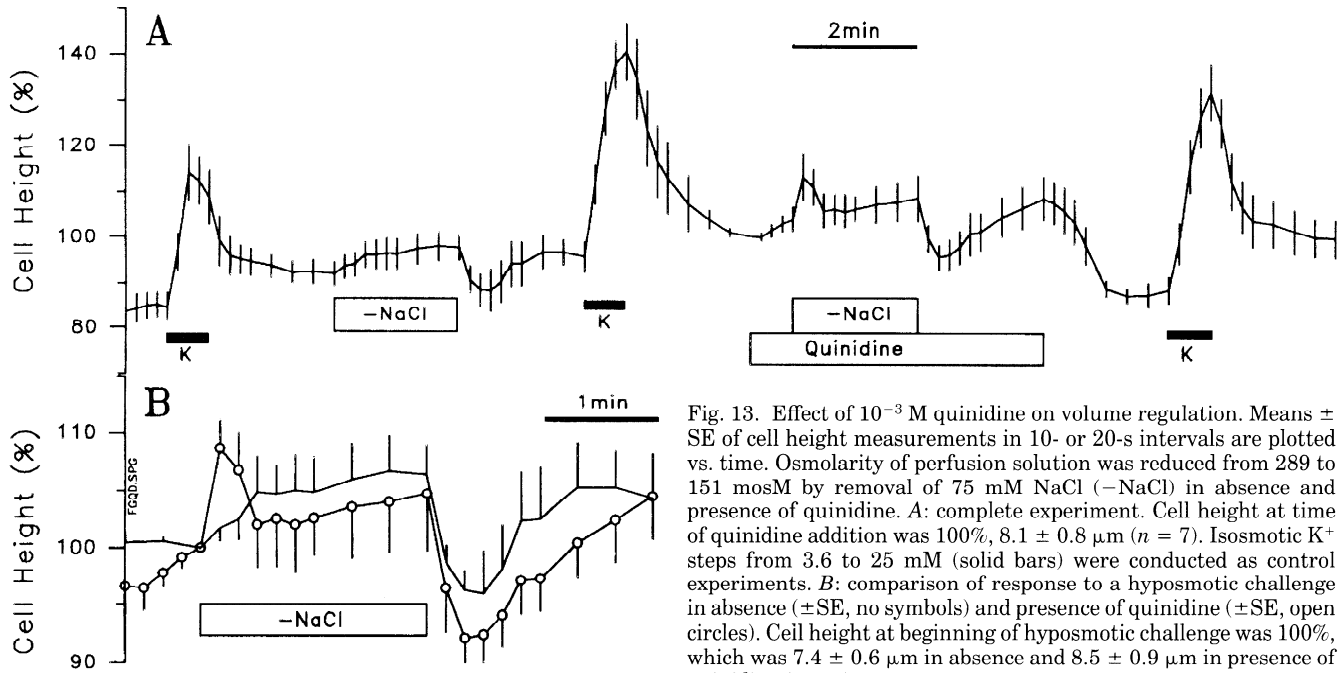

Fig. 13. Effect of 10^{-3} M quinidine on volume regulation. Means ± SE of cell height measurements in 10- or 20-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl) in absence and presence of quinidine. *A*: complete experiment. Cell height at time of quinidine addition was 100%, 8.1 ± 0.8 μm (n = 7). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: comparison of response to a hyposmotic challenge in absence (±SE, no symbols) and presence of quinidine (±SE, open circles). Cell height at beginning of hyposmotic challenge was 100%, which was 7.4 ± 0.6 μm in absence and 8.5 ± 0.9 μm in presence of quinidine (n = 7).

plained by the fact that cell height and width rather than cell volume were measured. It is conceivable that the extracellular spaces within the basolateral infoldings or between cells would decrease during the hyposmotic challenge such that cell volume doubled while no measurable increase in cell height and width occurred. Extracellular spaces within a cross section of fixed dark cell epithelium have been found to be only 15% of the area (30); however, dimensions of extracellular spaces in living tissue are not available.

Under unimpaired conditions, dark cells swelled to 107% when the hyposmotic challenge was achieved by removal of 75 mM NaCl (Fig. 2*A*) and to 119% when the hyposmotic challenge was achieved by removal of 150 mM mannitol (Fig. 2*B*). We observed that a hyposmotic challenge in conjunction with partial NaCl removal caused significantly less swelling at least partially due to the superimposition of cell shrinking caused by the partial removal of NaCl and cell swelling caused by the reduction of the osmolarity (Fig. 2, *A* and *C*). Further-

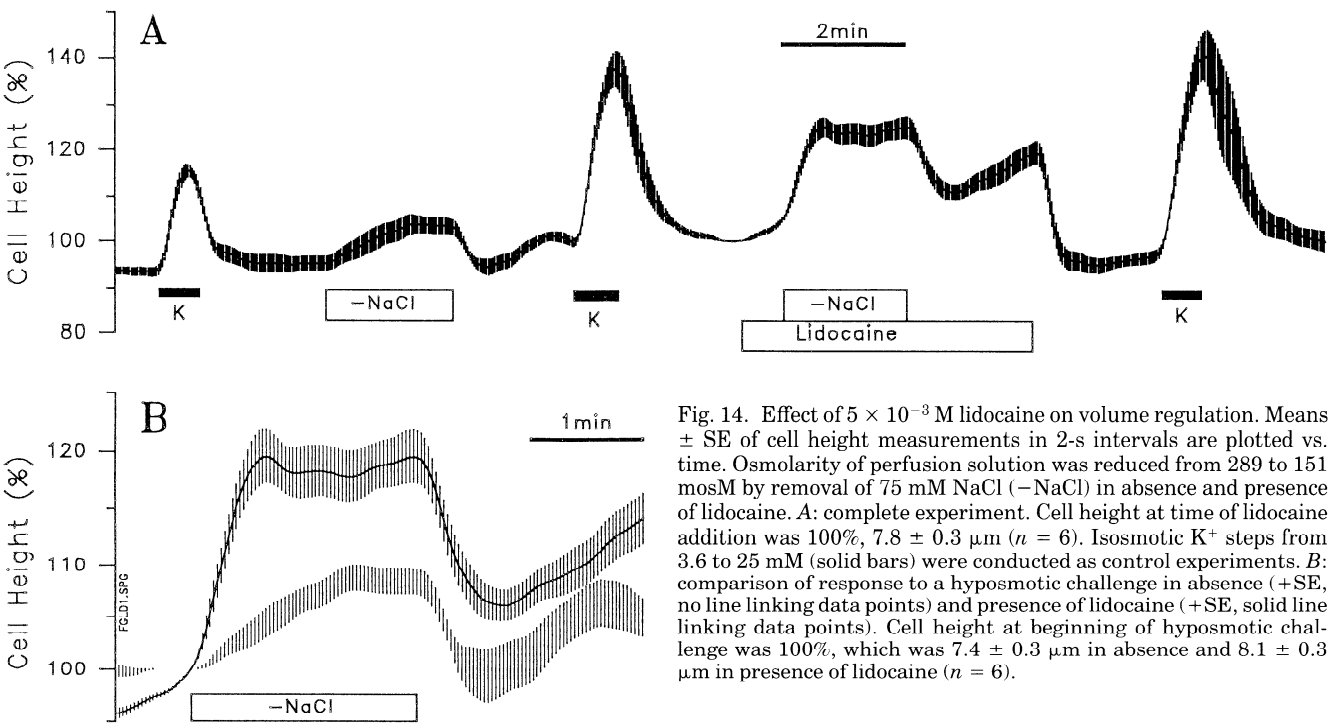

Fig. 14. Effect of 5×10^{-3} M lidocaine on volume regulation. Means ± SE of cell height measurements in 2-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 75 mM NaCl (−NaCl) in absence and presence of lidocaine. *A*: complete experiment. Cell height at time of lidocaine addition was 100%, 7.8 ± 0.3 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: comparison of response to a hyposmotic challenge in absence (+SE, no line linking data points) and presence of lidocaine (+SE, solid line linking data points). Cell height at beginning of hyposmotic challenge was 100%, which was 7.4 ± 0.3 μm in absence and 8.1 ± 0.3 μm in presence of lidocaine (n = 6).

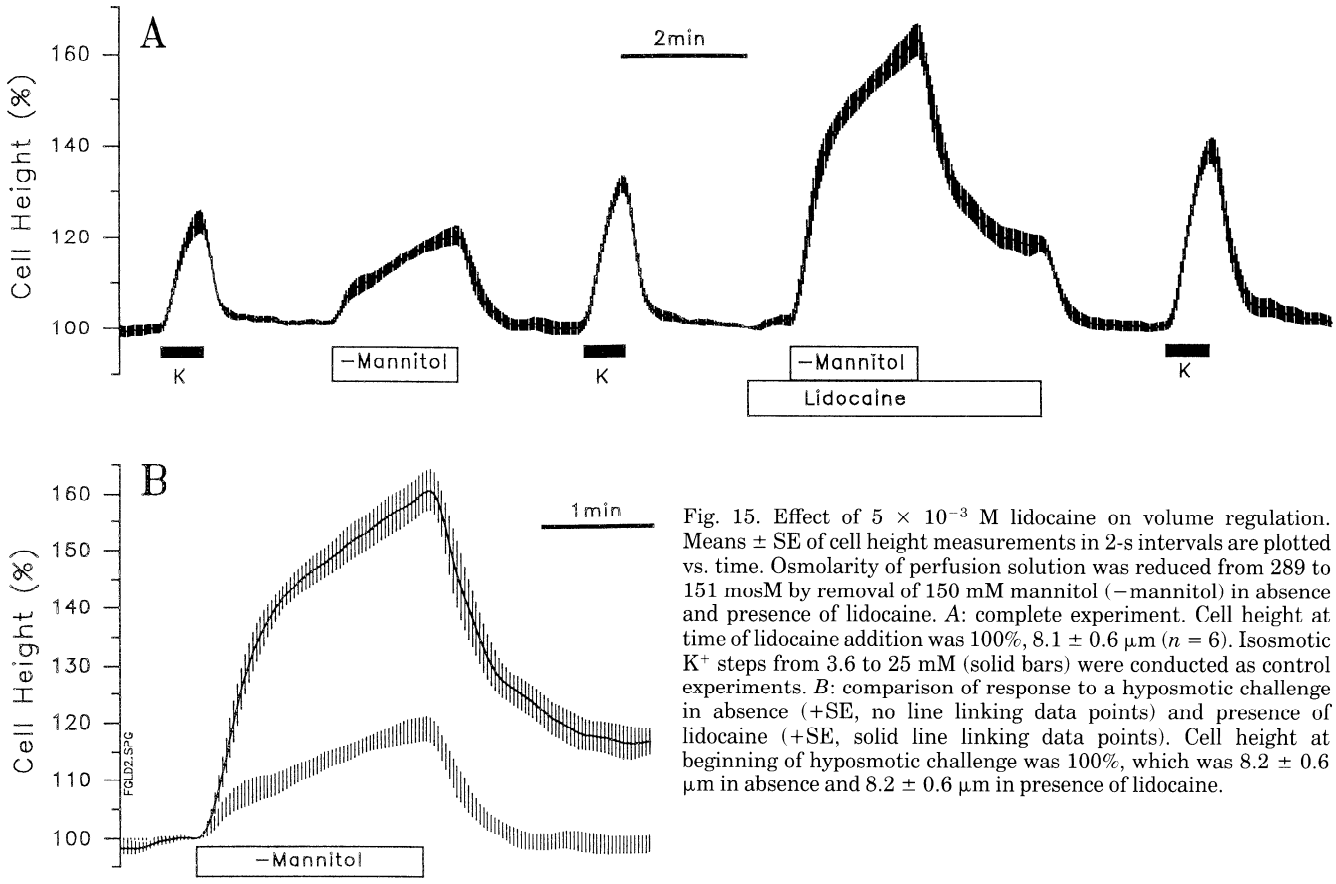

Fig. 15. Effect of 5×10^{-3} M lidocaine on volume regulation. Means ± SE of cell height measurements in 2-s intervals are plotted vs. time. Osmolarity of perfusion solution was reduced from 289 to 151 mosM by removal of 150 mM mannitol (−mannitol) in absence and presence of lidocaine. *A*: complete experiment. Cell height at time of lidocaine addition was 100%, 8.1 ± 0.6 μm (n = 6). Isosmotic K^+ steps from 3.6 to 25 mM (solid bars) were conducted as control experiments. *B*: comparison of response to a hyposmotic challenge in absence (+SE, no line linking data points) and presence of lidocaine (+SE, solid line linking data points). Cell height at beginning of hyposmotic challenge was 100%, which was 8.2 ± 0.6 μm in absence and 8.2 ± 0.6 μm in presence of lidocaine.

more, it is conceivable that the greater cell swelling observed upon removal of mannitol than of NaCl is due to an impairment of volume control mechanisms that depended directly or indirectly on a higher extracellular NaCl concentration.

During the hyposmotic challenge, dark cells did not display the transient cell swelling that characteristically precedes regulatory volume decrease in numerous other epithelial cells (for review, see Ref. 4). The absence of this transient response is most likely due to cell volume regulation occurring simultaneously with dilution of the cytosol, as it has been observed in fused Madin-Darby canine kidney cells (9).

Ionic Requirements for Volume Regulation During a Hyposmotic Challenge

In a variety of tissues it has been found that regulatory volume decrease involves loss of K^+ together with either Cl^- or HCO_3^- (for review, see Ref. 4). Regulatory volume decrease in many tissues was inhibited, and cells swelled like ideal osmometers when *1*) cells were depleted of cytosolic Cl^- or HCO_3^- by incubation in Cl^-- or HCO_3^--free solutions (6, 8, 21, 29, 31, 38) and *2*) when depleted of intracellular K^+ by incubation in K^+-free or ouabain-containing solutions (3, 5, 10, 11, 13, 21).

Cell height of dark cells increased in response to a hyposmotic challenge in the presence of Na^+, K^+, and Cl^- to 107% (Fig. 2*A*). Significantly larger increases in response to hyposmotic challenges were observed *1*) when the cytosolic Cl^- concentration presumably was reduced in Cl^--free solutions (Fig. 6), *2*) when the cytosolic K^+ concentration was presumably reduced in K^+-free solutions (Fig. 7), *3*) when the cytosolic K^+ concentration was presumably decreased and the cytosolic Na^+ concentration was presumably increased in the presence of ouabain (Fig. 9), and *4*) when enzymatic activity in general was reduced by cooling (Fig. 4) or fixation (Fig. 5). These observations suggest that dark cells employed a volume regulatory mechanism during a hyposmotic challenge that *1*) depended on cytosolic K^+ and Cl^-, *2*) depended on the ouabain-sensitive Na^+-K^+-ATPase that might be involved in maintaining a sufficiently high cytosolic K^+ concentration, and *3*) was independent of HCO_3^-, since volume regulation was not inhibited in the virtual absence of HCO_3^- and presence of the membrane-permeable carbonic anhydrase inhibitor ethoxyzolamide (Fig. 11).

The observation that no significantly different increase in cell height was observed during a hyposmotic challenge in the presence of 150 mM Na^+ and in the virtual absence of Na^+ is not necessarily inconsistent with the effect of ouabain. It is not known whether the local cytosolic Na^+ concentration under nominally Na^+-free conditions fell below the concentration necessary for the Na^+-K^+-ATPase. Furthermore, it would be conceivable that dark cells contained Na^+ uptake mechanisms with extremely high affinities, since they are thought to reabsorb Na^+ from endolymph, which contains only 9 mM Na^+ (19). A high affinity for Na^+ has been observed for some Na^+ uptake mechanisms, e.g.,

the Na^+ requirement of the apically located Na^+-Cl^--K^+ cotransporter in the thick ascending limb of the loop of Henle could only be demonstrated when extreme precautions against Na^+ contamination of solutions were taken (7).

Mechanism for Volume Control During a Hyposmotic Challenge

Volume regulatory decrease in many epithelial cells depends on the extrusion of cytosolic KCl either via parallel K^+ and Cl^- channels, a KCl symporter, or parallel K^+-H^+ and Cl^--HCO_3^- exchangers (for review, see Ref. 4). In many of these epithelia, regulatory volume decrease is inhibited by K^+ channel blockers, and cells in the presence of these blockers swelled during a hyposmotic challenge like ideal osmometers (5, 10, 29, 31). However, among several K^+ channel blockers, only lidocaine caused a sustained inhibition of volume control, whereas quinidine and barium caused only a transient inhibition (Figs. 12–15). These findings suggest that more than one mechanism was involved in volume control of dark cells. During the first 10–20 s of the hyposmotic challenge, volume control was mediated by a barium-, quinidine-, and lidocaine-sensitive mechanism, whereas subsequently a barium- and quinidine-insensitive but lidocaine-sensitive mechanism seemed to be involved. The nature of these mechanism remains unclear due to the fact that none of the blockers is very specific.

Comparison Between Solute Exit Mechanisms Under Isosmotic and Hyposmotic Conditions

Under isosmotic conditions, the effects of K^+ channel blockers on cell volume have been investigated under two conditions. *1*) Barium, quinidine, and lidocaine have been shown to cause significant cell swelling (4). With regard to barium and lidocaine, these observations have been confirmed by the present study (Figs. 12 and 14); however, the effect of quinidine did not reach significance (Fig. 13), which remains unexplained. *2*) Cell shrinking after K^+-induced swelling has been shown to be completely inhibited by quinidine and lidocaine and partially inhibited by barium (36). The latter observations suggested that dark cells contain under isosmotic swollen conditions K^+ channels that are equally sensitive to quinidine and lidocaine and less sensitive to barium. If volume control during a hyposmotic challenge depended on release of K^+ via these K^+ channels, it would have been expected that quinidine and lidocaine would have had a similar effect and that barium had a lesser inhibitory effect. In contrast, barium and quinidine inhibited volume control in a similar fashion, whereas lidocaine had a more sustained effect (Figs. 12–15). These findings suggest that different mechanisms are involved in solute exit after K^+-induced cell swelling and during a hyposmotic challenge.

Mechanism for RVI

In a variety of tissues it has been found that RVI involves uptake of NaCl via parallel Na^+-H^+ and Cl^--HCO_3^- exchangers or via the bumetanide-sensitive Na^+-Cl^--K^+ cotransporter (for review, see Refs. 1, 4).

The observation that RVI in dark cells was inhibited in the absence of Na^+ or Cl^- and in the presence of ouabain is consistent with the hypothesis that RVI involves NaCl uptake, which requires an inwardly directed Na^+ gradient maintained by the ouabain-sensitive Na^+-K^+-ATPase. The observation that RVI in dark cells was present in the virtual absence of HCO_3^- suggests that it is unlikely that parallel Na^+-H^+ and Cl^--HCO_3^- exchangers were involved. Most likely RVI in dark cells was mediated via the Na^+-Cl^--K^+ cotransporter, since RVI depended on the presence of Na^+, Cl^-, and K^+ and was inhibited by bumetanide, as it has been observed in other epithelia that are known to employ the Na^+-Cl^--K^+ cotransporter for RVI (e.g., Ref. 12).

The Na^+-Cl^--K^+ cotransporter in vestibular dark cells is not only involved in RVI but also in isosmotic K^+-induced cell swelling (34, 36) and transepithelial K^+ secretion (14–16).

In summary, the data suggest that cell volume control during a hyposmotic challenge depended on cytosolic KCl and the Na^+-K^+-ATPase and that RVI after the hyposmotic challenge involved bumetanide-sensitive Na^+-Cl^--K^+ cotransporter.

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