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Hypo-osmotic Challenge Stimulates Transepithelial K^+ Secretion and Activates Apical I_{sK} Channel in Vestibular Dark Cells

P. Wangemann[1], J. Liu[1,2], Z. Shen[2], A. Shipley[3], D.C. Marcus[2]

[1]Cell Physiology Laboratory, Boystown National Research Hospital, Omaha, NE
[2]Biophysics Laboratory, Boystown National Research Hospital, Omaha, NE
[3]National Vibrating Probe Facility, Marine Biological Laboratory, Woods Hole, MA

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Abstract. Volume regulation of vestibular dark cells from the gerbilline inner ear in response to a hypo-osmotic challenge depends on the presence of cytosolic K^+ and Cl^-. The present study addresses the questions: (i) whether and by what mechanism K^+ is released during volume regulation, (ii) whether the osmolarity of the basolateral medium has an effect on the steady-state rate of transepithelial K^+ transport and (iii) whether there is cross-talk between the basolateral membrane responsible for K^+ uptake and the apical membrane responsible for K^+ release. K^+ secretion ($J_{K^+,probe}$) and current density ($I_{sc,probe}$) were measured with vibrating probes in the vicinity of the apical membrane and the transepithelial potential (V_t) and resistance (R_t) were measured in a micro-Ussing chamber. The equivalent short-circuit current (I_{sc}) was calculated. The current (I_{IsK}), conductance (g_{IsK}) and inactivation time constant (τ_{IsK}) of the I_{sK} channel and the apparent reversal potential of the apical membrane (V_r) were obtained with the cell-attached macropatch technique. V_r was corrected (V_{rc}) for the membrane voltage (V_m) measured separately with microelectrodes. A hypo-osmotic challenge (294 to 154 mosM by removal of 150 mM mannitol) on the basolateral side of the epithelium increased $J_{K^+,probe}$ and $I_{sc,probe}$ by a factor of 2.7 and 1.6. When this hypo-osmotic challenge was applied to both sides of the epithelium V_t and I_{sc} increased from 5 to 14 mV and from 189 to 824 $\mu A/cm^2$ whereas R_t decreased from 27 to 19 Ω-cm^2. With 3.6 mM K^+ in the pipette I_{IsK} was outwardly directed, τ_{IsK} was 267 msec and the hypo-osmotic challenge caused I_{IsK} and g_{IsK} to increase from 14 to 37 pA and from 292 to 732 pS. V_{rc} hyperpolarized from −44 to −76 mV. With 150 mM K^+ in the pipette I_{IsK} was inwardly directed, τ_{IsK} was 208 msec and the hypo-osmotic challenge caused I_{IsK} and g_{IsK} to increase in magnitude from 0 to −21 pA and from 107 to 1101 pS. V_{rc} remained unchanged (−2 vs. 1 mV). These data demonstrate that a hypo-osmotic challenge stimulates transepithelial K^+ secretion and activates the apical I_{sK} channel. The hypo-osmotically-induced increase in K^+ secretion exceeded the estimated amount of K^+ release necessary for the maintenance of constant cell volume, suggesting that the rate of basolateral K^+ uptake was upregulated in the presence of the hypo-osmotic challenge and that cross-talk exists between the apical membrane and the basolateral membrane.

Key words: Regulatory volume decrease — Slowly activating K^+ channel — Vestibular Labyrinth — Vibrating probe — Micro-Ussing chamber — Patch clamp

Introduction

Vestibular dark cells transport K^+ electrogenically from the basolateral to the apical fluid compartment (Marcus & Shipley, 1994). K^+ is taken up across the basolateral membrane via the (Na^+ + K^+)-ATPase and the $Na^+/Cl^-/K^+$ cotransporter. K^+ release across the apical membrane occurs via the slowly activating K^+ channel (I_{sK} or minK channel) (Marcus & Shen, 1994) which is a protein of 129–130 amino acids (Takumi, Ohkubo & Nakanishi, 1988; Swanson et al., 1993). Cl^- recycles across the basolateral membrane via Cl^- channels (Marcus, Takeuchi & Wangemann, 1993). Vestibular dark cell epithelium bathed with symmetrical Na^+-rich solutions generates an apical-side positive transepithelial voltage which originates from the electromotive force associated with the K^+

Correspondence to: P. Wangemann

conductance in the apical membrane in series with the electromotive force associated with the Cl^- conductance in the basolateral membrane (Marcus, Liu & Wangemann, 1994).

Imbalances between K^+ uptake and release during transepithelial K^+ transport pose a threat to cell function necessitating mechanisms for volume regulation. Regulatory volume decrease has been shown to occur in vestibular dark cells in response to a hypo-osmotic challenge (Wangemann & Shiga, 1994*b*). Even though regulatory volume decrease was found to depend on the presence of cytosolic K^+ and Cl^- (Wangemann & Shiga, 1994*b*), a host of questions remained open. The present study addresses the questions of whether regulatory volume decrease involves K^+ release, whether K^+ release is electrogenic, across which membrane K^+ is released, which transport mechanism mediates K^+ release and whether the osmolarity of the basolateral medium has an effect on the steady state rate of transepithelial K^+ transport. Further, the present study explores whether there is cross-talk between the basolateral membrane responsible for K^+ uptake and the apical membrane responsible for K^+ release.

We report here that regulatory volume decrease involves activation of transepithelial K^+ secretion and stimulation of the apical I_{sK} channel. The hypo-osmotically-induced increase in K^+ secretion exceeded the estimated amount of K^+ release necessary for the maintenance of constant cell volume suggesting that the rate of basolateral K^+ uptake was upregulated in the presence of a hypo-osmotic challenge and that cross-talk exists between the apical membrane and the basolateral membrane.

Parts of this study have been presented at recent meetings (Wangemann et al., 1994*a,b*).

Materials and Methods

Preparation

The technique of dissection has been described previously (Wangemann & Marcus, 1989). Briefly, gerbils (4–10 weeks old) were anesthetized with pentobarbital sodium (50 mg/kg i.p.) and decapitated. Dark cell epithelium was dissected at 4°C from an ampulla of a semicircular canal and transferred to a bath chamber where experiments were conducted at 37°C with the exception of experiments involving the vibrating probe, which were carried out at room temperature (22°C). This difference in temperature has been shown to cause only a quantitative but not qualitative difference (Marcus, 1986). That finding is supported by the qualitative similarity between the measurements of $I_{sc,probe}$ at 22°C and I_{sc} at 37°C (*vide infra*). For measurements with the on-cell macropatch technique and for impalements with microelectrodes, the tissue was folded into a loop and the perfusate had simultaneous access to both sides of the epithelium (Wangemann & Marcus, 1989). For all other measurements the tissue was transferred as a flat sheet into a micro-Ussing chamber where the apical and basolateral side of the epithelium could be perfused independently (Marcus, Marcus & Greger, 1987; Marcus et al., 1994).

Solutions

The composition of solutions is listed in Table 1. All solutions were titrated to pH 7.4 and the osmolarity was measured by freezing point depression (Osmette A, Precision Systems, Natick, MA).

Data Acquisition and Analysis

Measurements with the Vibrating Probe

The K^+ gradient ($J_{K^+,probe}$) and the current density ($I_{sc,probe}$) in the vicinity of the apical membrane were measured with the vibrating probe as described previously (Marcus & Shipley, 1994). The tissue was placed in the micro-Ussing chamber with the connective tissue against the aperture which resulted in a partial seal such that $J_{K^+,probe}$ and $I_{sc,probe}$ were measured under short-circuit conditions. Solution changes were complete within 1 sec. The apical side of the tissue was not perfused but bathed with solution 1. The vibrating probe was located 20–240 μm over the apical membrane of the epithelium such that the signal under control conditions was >30 times the noise level at background obtained at a position >1 mm away from the tissue.

$J_{K^+,probe}$ was measured as voltage difference between two points near the apical membrane by vibrating (amplitude: 30 μm; 0.3 Hz) a K^+-selective microelectrode along the vertical axis normal to the plane of the tissue. Microelectrodes (O.D.: 4 μm) were pulled from borosilicate glass capillaries (O.D.: 1.5 mm) and silanized with dimethyldichlorosilane. Tips contained a column of K^+-selective ligand (#60398, Fluka Chemical, Ronkonkoma, NY) about 150 μm long. Electrodes were backfilled with 100 mM KCl in 0.5% agar. The reference was Ag/AgCl with a bridge of 3 M NaCl in 3% agar. Electrodes were only used if the slope was at least 56 mV/decade in 10 and 100 mM KCl solutions. The contribution of the voltage gradient produced by the transepithelial electric current was less than 8% of the voltage gradient observed at the K^+-selective electrode; as in previous studies, no corrections were made for this component of the signal (Marcus & Shipley, 1994). Effects in response to solution changes were expressed as relative changes since the signal was not calibrated in terms of absolute K^+ flux at the surface of the epithelium. The calibration procedure of the vibrating ion-selective probe in terms of absolute flux is not yet on firm ground because a variable and poorly understood "efficiency factor" must be employed (Kühtreiber & Jaffe, 1990). Data summarized in the text were normalized to the reading under control conditions (solution 1).

$I_{sc,probe}$ was derived from the voltage difference between two points near the apical membrane by vibrating (amplitude: 20 μm; 200–800 Hz) a stainless steel wire electrode with a platinum-black ball (diameter: 20 μm) along the vertical axis normal to the plane of the tissue. The bath reference was a 26-gauge platinum-black electrode. Calibration was performed using a glass microelectrode filled with 3 M KCl as a point current source yielding the current density at the position of the electrode tip (not the absolute current density at the surface of the epithelium). Data summarized in the text were normalized to the reading under control conditions (solution 1).

Transepithelial Measurements

For the measurement of the transepithelial voltage (V_t) and resistance (R_t) under open circuit conditions the epithelium was sealed with the apical membrane onto the aperture of the micro-Ussing chamber as

Table 1. Solutions (in mM)

Solution	1	2	3	4	5
KCl				3.6	150.0
NaCl	150.0	75.0	75.0	150.0	
$MgCl_2$	1.0	1.0	1.0	1.0	1.0
$CaCl_2$	0.7	0.7	0.7	0.7	0.7
K_2HPO_4	1.6	1.6	1.6		
KH_2PO_4	0.4	0.4	0.4		
HEPES				10	10
Glucose	5.0	5.0	5.0		
Mannitol		150.0			
mosM	298	298	154	298	298

The liquid junction potential between solution 1 and 2 or 1 and 3 was 2.3 mV.

described earlier (Marcus et al., 1987; Marcus et al., 1994). Briefly, V_t was measured with calomel electrodes connected to the chamber via agar bridges made with solution 1. Transepithelial current pulses were passed via Ag/AgCl wires. Sample-and-hold circuitry was used to obtain a signal proportional to R_t from the voltage response to the current pulses (50 nA for 34 msec at 0.3 Hz). V_t and R_t were recorded on a 2-pen chart recorder. Representative traces were digitized omitting, for clarity, the responses to the current pulses. Data were corrected for liquid junction potentials which were measured separately against a flowing 3 M KCl electrode (Table 1). When the epithelium was bathed with symmetrical solutions, the equivalent short circuit current (I_{sc}) was obtained according to Ohm's law from measurements of V_t and R_t ($I_{sc} = V_t/R_t$). I_{sc} and R_t were normalized for the area defined by the aperture of the micro-Ussing chamber (diameter of aperture: 80 μm). Solution changes in the apical and basolateral perfusate were complete within 1 sec.

Measurements in Cell-attached Apical Membrane Patches

The current through the I_{sK} channel at 0 mV holding voltage (I_{IsK}), the sum of the currents other than through the I_{sK} channel at 0 mV holding voltage ($I_0 - I_{IsK}$), the conductance of the I_{sK} channel (g_{IsK}), the sum of the conductance other than of the I_{sK} channel ($g_a - g_{IsK}$), the apparent reversal voltage (V_r) and the inactivation time constant of the I_{sK} channel (τ_{IsK}) were obtained with the cell-attached macropatch as described earlier (Marcus & Shen, 1994) in conjunction with a new voltage protocol. Briefly, the perfusate had simultaneously access to the apical and basolateral membrane and solution changes were complete within 15 sec. Patch pipettes (average I.D.: 3.8 μm) were manufactured from glass capillaries (Corning 7052; O.D.: 1.5 mm, I.D.: 0.86 mm; Garner Glass, Claremont, CA) with a 2-stage puller (PP-83, Narishige, Tokyo, Japan) and were cut with the help of a microforge. Tips were coated with a 2:1 mixture of α-tocopherol acetate and heavy mineral oil (Sigma, St. Louis, MO). Pipettes were connected to the patch clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA) via a Ag/AgCl wire. The reference was a Ag/AgCl wire connected to the bath via a flowing 1 M KCl junction. High-resistance seals of about 8 GΩ were made between the pipette and apical membrane of vestibular dark cells. Seals were generally made with light suction, resulting in a relatively flat membrane patch.

The voltage protocol consisted of three phases (Fig. 1). An 11-sec period during which the patch was held at the holding voltage of 0 mV (not fully shown in Fig. 1). A short period during which three 9

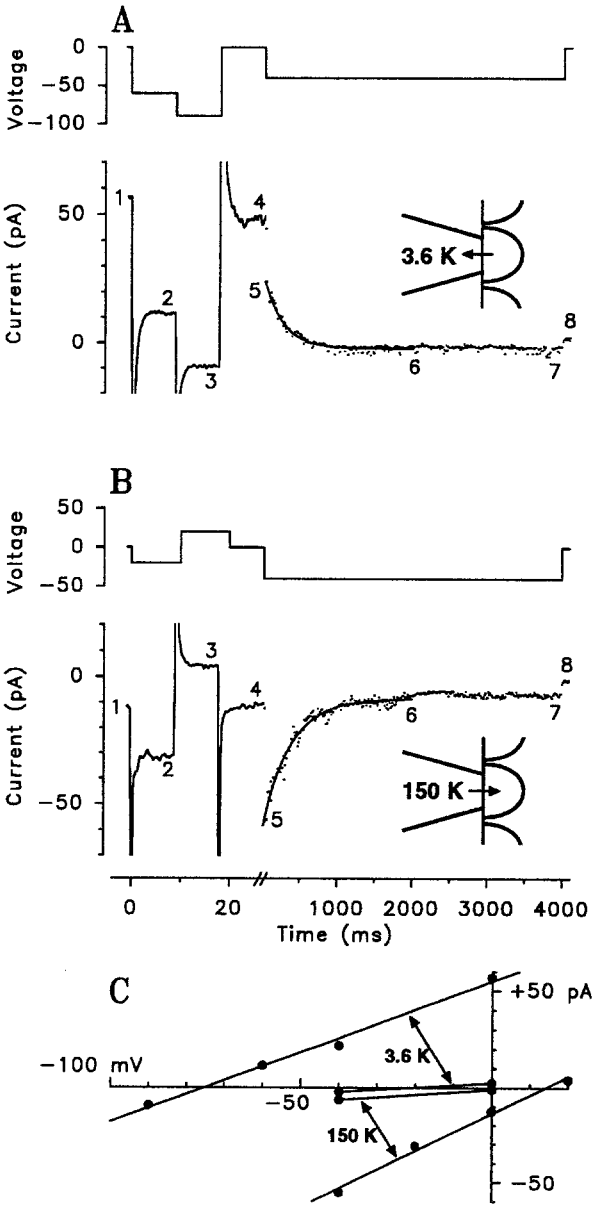

Fig. 1. Protocols for data acquisition and analysis of on-cell macropatch recordings obtained *A* with 3.6 mM K^+ and *B* with 150 mM K^+ in the pipette. These protocols were repeated every 15 sec such that data were obtained in 15-sec intervals throughout an experiment (*see* Figs. 5 and 7). The apical membrane current at 0 mV holding potential (I_0) was obtained at "1" and the sum of apical membrane currents other than through the I_{sK} channel ($I_0 - I_{IsK}$) was obtained at "8". The current through the I_{sK} channel was obtained by subtraction ($I_{IsK} = I_0 - (I_0 - I_{IsK})$). The conductance of the apical membrane (g_a) and the apparent reversal potential (V_r) were calculated from current measurements at points "2" and "3" in *A* and "3" and "4" in *B*. The apical membrane conductance other than of the I_{sK} channel ($g_a - g_{IsK}$) was calculated from current measurements at points "7" and "8". The conductance of the I_{sK} channel was obtained by subtraction ($g_{IsK} = g_a - (g_a - g_{IsK})$). The respective current/voltage relationships are shown in *C*. The inactivation time constant of the I_{sK} channel (τ_{IsK}) was obtained as a single exponential from a fit of the data between "5" and "6". For clarity only every 3rd data point is plotted between "5" and "8".

msec voltage steps were performed. When the patch pipette was filled with 3.6 mM K^+ (solution 4), this short period consisted of voltage steps to –60, –90, and 0 mV. Alternatively, when the patch pipette was filled with 150 mM K^+ (solution 5), the voltage was stepped to –20, +20 and 0 mV. Finally, the third phase of the protocol consisted of a 4-sec period during which the voltage was clamped to –40 mV. This protocol was continuously repeated such that parameters were obtained every 15 sec.

The total current through the apical membrane at 0 mV holding voltage (I_0) was obtained at the end of the 11-sec period (Fig. 1:"1"). $I_0 - I_{IsK}$ was obtained at the beginning of the 11-sec period (Fig. 1:"8") since the I_{sK} channel is known to be fully inactivated by a hyperpolarization of the holding voltage to –40 mV (Marcus & Shen, 1994). The total apical membrane conductance (g_a) was obtained as linear slope from tail-current *I/V* relationships constructed from currents at the end of the 9 msec voltage steps and at the beginning of the 4-sec voltage step (Fig. 1:"1"–"5"). V_r was obtained from these tail-current *I/V* relationships and was corrected ($V_{rc} = V_r + V_m$) for the membrane potential (V_m) which was measured separately (*see below*). $g_a - g_{IsK}$ was obtained as the linear slope from currents at the end of the 4-sec voltage step (Fig. 1:"7"–"8") and g_{IsK} was obtained by subtraction ($g_{IsK} = g_a - (g_a - g_{IsK})$).

The inactivation time constant of the I_{sK} channel (τ_{IsK}) was obtained by fitting the current measurements during the first 2 sec of the 4-sec period at –40 mV (Fig. 1:"5"–"6") with the single exponential equation $Current = A \cdot \exp(-t/\tau_{IsK}) + C$ where A is the amplitude, t the time and C the asymptote. Data were fitted by the method of Chebyshev (Clampfit, version 6.01, Axon Instruments). Values for τ_{IsK} were considered acceptable when the current difference between the first and last point used for fitting was bigger than 2 pA and when τ_{IsK} was smaller than 1000 msec. The mean and standard error (SEM) at each time point consisted of 2 to 7 measurements. Data summarized in the text were pooled from the last three time points at the end of the respective maneuver.

Measurements with Microelectrodes

V_m was measured with microelectrodes as previously described (Wangemann & Marcus, 1992; Wangemann & Shiga, 1994*a*). Briefly, the perfusate had simultaneous access to the apical and basolateral membrane. Solution changes were complete within 15 sec. Microelectrodes which had a tip resistance of 50–150 MΩ when filled with 1 M KCl were pulled from filament-containing borosilicate glass (O.D.: 1.0 mm, WPI, New Haven, CT) with a horizontal puller (Fredrick Haer, Brunswick, ME). V_m was measured with a single-ended electrometer (WPI) against the bath. The input resistance of the microelectrode was monitored throughout the experiments by current injections (0.2 nA at 0.1 or 0.2 Hz). The bath chamber was grounded via an agar bridge (4% in solution 1) and a calomel electrode. Data were averaged from 6 experiments and corrected for the liquid junction potential (Table 1).

Data Presentation and Statistics

Data in the text and in Figs. 4*C*, 5, 6 and 7 are given as average ± SEM. 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 or unpaired samples. Averages of normalized 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 (Snedecor & Cochran, 1954). Differences were assumed to be significant when $P < 0.05$.

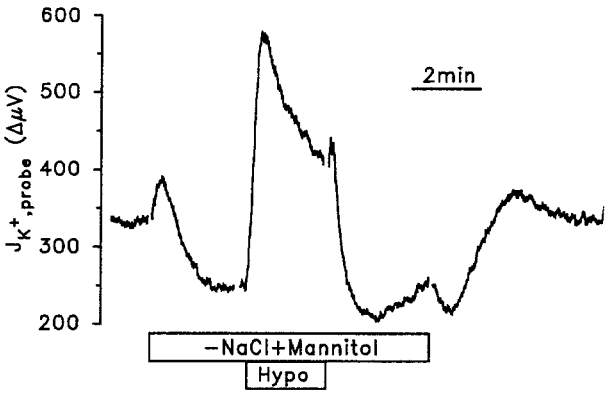

Fig. 2. Effect of the hypo-osmotic challenge on K^+ secretion measured as K^+ gradient ($J_{K^+,probe}$) in the vicinity of the apical membrane. Typical recording. The apical side of the epithelium was bathed with solution 1. Only the basolateral side was perfused. NaCl was isosmotically replaced by mannitol (*–NaCl + Mannitol*) and the osmolarity was reduced from 298 to 154 mosM by removal of mannitol (*Hypo*).

Results

Effect of the Hypo-osmotic Challenge on Transepithelial K^+ Secretion

Transepithelial K^+ secretion during a hypo-osmotic challenge was expected to increase if cytosolic K^+ is released across the apical membrane. To test this possibility we measured $J_{K^+,probe}$. Prior to the hypo-osmotic challenges 75 mM NaCl was isosmotically replaced by 150 mM mannitol (solutions 1 and 2). The hypo-osmotic challenge consisted of the subsequent removal of 150 mM mannitol (solution 3). This protocol was chosen in order to avoid changes in the ionic composition during the hypo-osmotic challenge.

$J_{K^+,probe}$ under control conditions (solution 1 on both sides of the epithelium) was 180 ± 40 μV (n = 7). Isosmotic replacement of NaCl with mannitol in the basolateral perfusate caused a transient increase of $J_{K^+,probe}$ by a factor of 1.21 ± 0.08 and a subsequent decrease by a factor of 0.72 ± 0.06 (n = 7) of control. A hypo-osmotic challenge by removal of mannitol from the basolateral perfusate caused a significant increase in $J_{K^+,probe}$ initially by a factor of 2.67 ± 0.48 which relaxed to a factor of 1.72 ± 0.28 (n = 7; solution 3 with respect to solution 2; Fig. 2). These observations demonstrate that the hypo-osmotic challenge increased transepithelial K^+ secretion significantly suggesting that K^+ is released at least partially across the apical membrane.

Effect of the Hypo-osmotic Challenge on $I_{sc,probe}$, V_t, R_t and I_{sc}

Constitutive K^+ secretion across vestibular dark cell epithelium involves an apical electrogenic mechanism, the

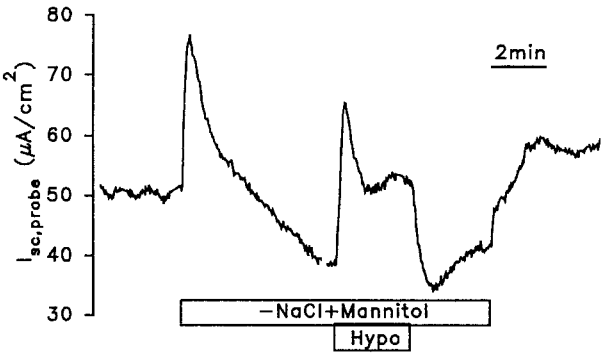

Fig. 3. Effect of a hypo-osmotic challenge on the transepithelial current measured as current density ($I_{sc,probe}$) in the vicinity of the apical membrane. Typical recording. The apical side of the epithelium was bathed with solution 1. Only the basolateral side was perfused. NaCl was isosmotically replaced with mannitol (*–NaCl + Mannitol*) and the osmolarity was reduced from 298 to 154 mosM by removal of mannitol (*Hypo*).

I_{sK} channel, and generates a transepithelial current directed from the basolateral to the apical side and an apical-side positive transepithelial voltage. If K^+ release during regulatory volume decrease occurred via this or another apical electrogenic mechanism it would be expected that the transepithelial current and voltage would increase during the hypo-osmotic challenge. Alternatively, if K^+ were released via an electroneutral KCl symport, no change or a decrease in the transepithelial current and voltage would be expected. To distinguish between these two possibilities, we measured $I_{sc,probe}$, V_t, and R_t and obtained I_{sc}.

$I_{sc,probe}$ under control conditions (solution 1 on both sides of the epithelium) was 54 ± 3 μA/cm² (n = 4). Isosmotic replacement of NaCl with mannitol in the basolateral perfusate caused a decrease of $I_{sc,probe}$ by a factor of 0.76 ± 0.03 (n = 4) of control (Fig. 3). A hypo-osmotic challenge by removal of mannitol from the basolateral perfusate caused a significant increase in $I_{sc,probe}$ initially by a factor of 1.58 ± 0.09 which relaxed to a factor of 1.29 ± 0.08 (n = 4; solution 3 with respect to solution 2).

V_t, R_t and I_{sc} under control conditions (solution 1 on both sides of the epithelium) were 9 ± 1 mV, 15 ± 1 Ω-cm² and 590 ± 26 μA/cm² (n = 19). Isosmotic replacement of NaCl with mannitol in the apical perfusate caused a significant increase of V_t to 10 ± 1 mV and a significant increase of R_t to 20 ± 1 Ω-cm² (n = 19; Fig. 4). Subsequent isosmotic replacement of NaCl with mannitol in the basolateral perfusate caused a transient increase of V_t from 10 ± 1 to 22 ± 1 mV which relaxed to 4.2 ± 0.4 mV and a sustained increase of R_t from 19 ± 1 to 32 ± 2 Ω-cm² (n = 19). I_{sc} in the presence of mannitol on both sides of the epithelium was 139 ± 16 μA/cm² (n = 19). Hypo-osmotic removal of mannitol from the apical perfusate caused no significant change in V_t, R_t and I_{sc} (5 ± 1 *vs.* 5 ± 1 mV, 27 ± 7 *vs.* 29 ± 7 Ω-cm²

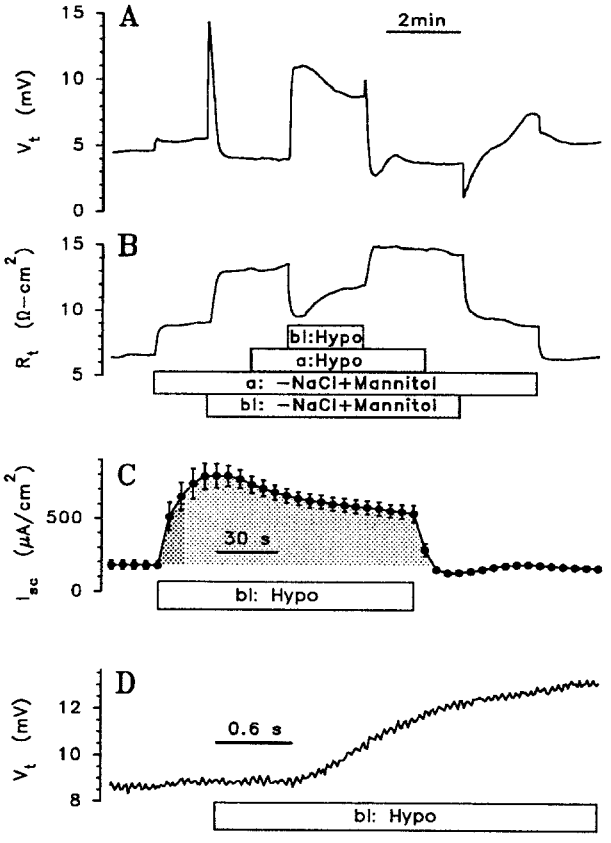

Fig. 4. Effect of a hypo-osmotic challenge on *A* the transepithelial voltage (V_t) and *B* the transepithelial resistance (R_t). Typical recordings. NaCl was isosmotically replaced with mannitol (*–NaCl + Mannitol*) in the apical (*a:*) and basolateral (*bl:*) perfusate. The osmolarity was reduced from 298 to 154 mosM by removal of mannitol (*Hypo*). V_t has been corrected for the liquid junction potential. The time base given in *A* applies also to *B*. (*C*) The equivalent short circuit current (I_{sc}) was calculated in 6-sec intervals from measurements of V_t and R_t (n = 4). The integral of the increase in I_{sc} during the hypo-osmotic challenge represents the increase in K^+ secretion and is highlighted by light shading. The integral of the increase in I_{sc} during the first 15 sec of the hypo-osmotic challenge which represents the K^+ release necessary to maintain constant cell volume is highlighted by dark shading. (*D*) Effect of a basolateral hypo-osmotic challenge on V_t plotted at a faster time scale. Note, that there was a 0.6-sec delay between the onset of arrival of the hypo-osmotic solution at the connective tissue and the onset of the response of V_t.

and 189 ± 30 *vs.* 188 ± 27 μA/cm², respectively, n = 4). However, subsequent hypo-osmotic removal of mannitol from the basolateral perfusate caused significant changes which began with a transient peak followed by relaxation to a sustained level. V_t increased significantly from 5 ± 1 to 14 ± 2 mV and relaxed to 10 ± 1 mV, R_t decreased significantly from 29 ± 7 to 19 ± 5 Ω-cm² and relaxed to 21 ± 5 Ω-cm² and I_{sc} increased significantly from 180 ± 26 to 824 ± 85 μA/cm² and relaxed to 597 ± 87 μA/cm² (n = 4). The onset of the response of V_t occurred with less than 1 sec (Fig. 4*D*).

Similar results were obtained when the hypo-

osmotic challenge was applied by removal of NaCl rather than of mannitol. These experiments were conducted in order to demonstrate that the above results were not a function of the lower NaCl concentration. A hypo-osmotic challenge by removal of NaCl (solution 3) from the apical perfusate significantly increased V_t from 9 ± 1 to 12 ± 1 mV and R_t from 16 ± 3 to 25 ± 5 Ω-cm^2 (n = 11). A hypo-osmotic challenge by removal of NaCl from the basolateral perfusate caused a significant increase of V_t from 10 ± 1 to 26 ± 2 mV which relaxed to 13 ± 1 mV, and a decrease of R_t from 23 ± 3 to 16 ± 2 which relaxed to 21 ± 2 Ω-cm^2 (n = 10). Taken together, these observations demonstrate that K^+ release during a hypo-osmotic challenge involved an electrogenic mechanism.

Effect of the Hypo-osmotic Challenge on I_{IsK}, $I_0 - I_{IsK}$, g_{IsK}, $g_a - g_{IsK}$, V_{rc}, V_m and τ_{IsK} Under Conditions of an Outwardly Directed K^+ Gradient

If K^+ release occurred via an apical electrodiffusive pathway it would be expected that the hypo-osmotic challenge would cause an increase in the current and the conductance of the apical membrane. If K^+ release involved in particular activation of the I_{sK} channel, an increase in the magnitude of I_{IsK} and g_{IsK} would be expected as well as a shift of V_{rc} toward the K^+ equilibrium potential.

Under control conditions (solution 1 in the bath and 3.6 mM K^+ in the pipette, solution 4) I_{IsK} was 22 ± 8 pA, $I_0 - I_{IsK}$ was 1.0 ± 0.2 pA, g_{IsK} was 323 ± 116 pS, $g_a - g_{IsK}$ was 127 ± 34 pS, τ_{IsK} was 267 ± 51 ms, V_{rc} was −51 ± 8 mV, and V_m was −8 ± 2 mV (each n = 6). Isosmotic replacement of 75 mM NaCl with mannitol (solution 2) caused a transient increase of I_{IsK} to a peak of 42 ± 14 pA and a transient increase of g_{IsK} to a peak of 709 ± 243 pS after which a new steady state was reached (each n = 6; Fig. 5). $I_0 - I_{IsK}$ was significantly larger and g_{IsK} was significantly smaller at this new steady state than under control conditions. Presenting a hypo-osmotic challenge by removal of mannitol (solution 3) increased I_{IsK} from 14 ± 8 pA to 37 ± 8 pA and g_{IsK} from 292 ± 104 pS to 732 ± 193 pS (each n = 6). V_{rc} shifted from −44 ± 7 mV to −76 ± 4 mV and V_m hyperpolarized from 2 ± 3 mV to −20 ± 5 mV (each n = 6; Fig. 6). There was no significant change in $I_0 - I_{IsK}$ (2.0 ± 0.6 pA *vs.* 2.6 ± 0.8 pA), $g_a - g_{IsK}$ (109 ± 31 pS *vs.* 126 ± 36 pS) and τ_{IsK} (242 ± 73 msec *vs.* 260 ± 38 msec). These observations demonstrate that the I_{sK} channel is activated during a hypo-osmotic challenge.

Effect of the Hypo-osmotic Challenge on I_{IsK}, $I_0 - I_{IsK}$, g_{IsK}, $g_a - g_{IsK}$, V_{rc}, V_m and τ_{IsK} under Conditions of an Inwardly Directed K^+ Gradient

It has been shown that the I_{sK} channel is only minimally rectifying and that the direction of the apical membrane

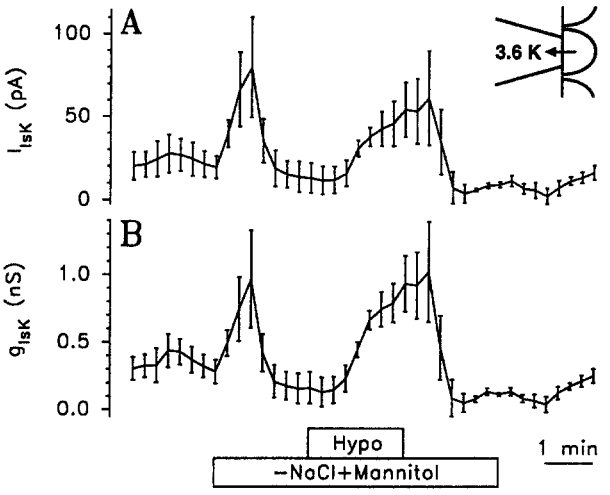

Fig. 5. Effect of a hypo-osmotic challenge on *A* the current (I_{IsK}) and *B* the conductance (g_{IsK}) of the I_{sK} channel. The pipette was filled with 3.6 mM K^+ such that the current through the I_{sK} channel was outwardly directed. In the bath, NaCl was isosmotically replaced with mannitol (*–NaCl + Mannitol*) and the osmolarity was reduced from 298 to 154 mosM by removal of mannitol (*Hypo*).

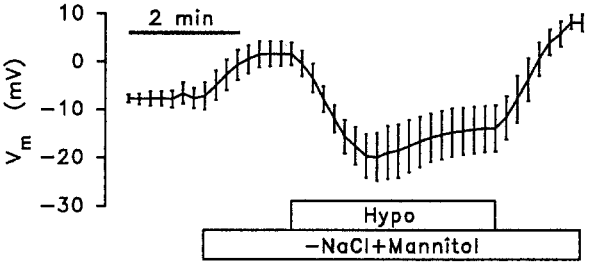

Fig. 6. Effect of a hypo-osmotic challenge on the membrane voltage (V_m). Average of 6 recordings. NaCl was isosmotically replaced with mannitol (*–NaCl + Mannitol*) and the osmolarity was reduced from 298 to 154 mosM by removal of mannitol (*Hypo*).

current can be inverted by inversion of the K^+ gradient (Marcus & Shen, 1994). This finding was confirmed in the present study. If the hypo-osmotic challenge indeed would cause activation of the I_{sK} channel, it would be expected that V_{rc} stays near the K^+ equilibrium potential and that the magnitude of the inwardly-directed I_{IsK} would increase.

Under control conditions (solution 1 in the bath and 150 mM K^+ in the pipette, solution 5) I_{IsK} was −2.5 ± 0.7 pA, $I_0 - I_{IsK}$ was −0.8 ± 0.3 pA, g_{IsK} was 157 ± 57 pS, $g_a - g_{IsK}$ was 158 ± 38 pS, τ_{IsK} was 208 ± 52 msec, and V_{rc} was 1 ± 3 mV (n = 7). Isosmotic replacement of 75 mM NaCl with mannitol (solution 2) caused a transient increase of $I_0 - I_{IsK}$ to a peak of 0.4 ± 0.1 pA (n = 7) after which a new steady state was reached. I_{IsK} and $I_0 - I_{IsK}$ were significantly smaller at this new steady state than under control conditions. Presenting a hypo-osmotic challenge by removal of mannitol (solution 3) increased

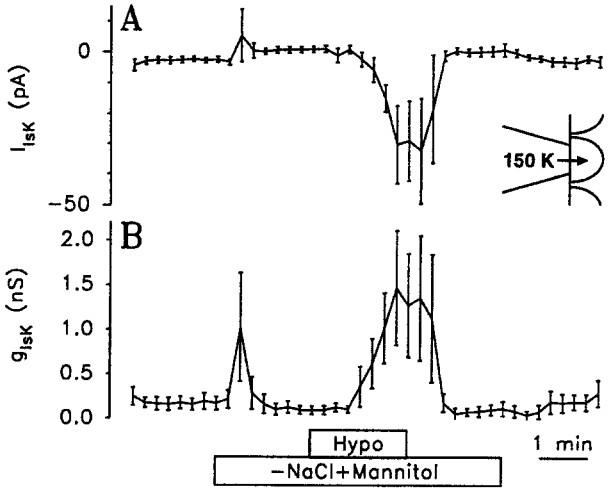

Fig. 7. Effect of a hypo-osmotic challenge on A the current (I_{IsK}) and B the conductance (g_{IsK}) of the I_{sK} channel. The pipette was filled with 150 mM K^+ such that the current through the I_{sK} channel was inwardly directed. In the bath, NaCl was isosmotically replaced with mannitol (*-NaCl + Mannitol*) and the osmolarity was reduced from 298 to 154 mosM by removal of mannitol (*Hypo*).

the magnitude of I_{IsK} from 1.0 ± 1.0 pA to −21 ± 7 pA, $I_0 - I_{IsK}$ from 0.1 ± 0.3 pA to −2.2 ± 0.7 pA, and g_{IsK} from 107 ± 45 pS to 1101 ± 384 pS (each n = 7; Fig. 7). There was no significant change in V_{rc} (−2 ± 4 mV *vs.* 1 ± 6 mV), $g_a - g_{IsK}$ (126 ± 28 pS *vs.* 149 ± 25 pS) and τ_{IsK} (319 ± 93 msec *vs.* 184 ± 33 msec). These observations support the conclusion that the I_{sK} channel is activated during a hypo-osmotic challenge and that activation of the I_{sK} channel is independent of the direction of K^+ flux. No evidence for hypo-osmotic activation of an apical conductive pathway other than the I_{sK} channel was obtained.

Discussion

Hypo-osmotic Challenge Activates Transepithelial K^+ Secretion

It was shown in a previous study that regulatory volume decrease was reduced when vestibular dark cells were depleted of cytosolic K^+ or Cl^- (Wangemann & Shiga, 1994*b*). This observation demonstrated that regulatory volume decrease depended on the presence of cytosolic K^+ and Cl^- and suggested that it involved release of these osmolytes. The present data confirm this suggestion and localize K^+ release during a hypo-osmotic challenge to the apical membrane (Fig. 2). Observations made during isosmotic replacement of NaCl with mannitol, the first solution change, are discussed below with respect to membrane cross-talk.

The response of $J_{K^+,probe}$, $I_{sc,probe}$, V_t and R_t to a hypo-osmotic challenge consisted of an initial peak followed by an elevated plateau (Figs. 2–4). The initial peak is most likely a reflection of K^+ release in conjunction with regulatory volume decrease. The observation of an elevated steady state, however, suggests that the hypo-osmotic challenge caused an increase in the rate of transepithelial K^+ secretion, necessitating a stimulation of the basolateral K^+ uptake mechanisms. Support for this hypothesis comes from a comparison of the increase in K^+ secretion observed during a hypo-osmotic challenge and the estimated K^+ efflux necessary to maintain constant cell volume during a hypo-osmotic challenge. The increase in K^+ secretion during a hypo-osmotic challenge was obtained from the integral of the increase in I_{sc} divided by the Faraday constant (Fig. 4*C*). The integrated current increase during the 2 min hypo-osmotic challenge was 59.8 ± 5.5 mA · s/cm^2 which equals a release of 6.3 · 10^{-7} mol/cm^2 of K^+ under the assumption that I_{sc} equals K^+ flux under hypo-osmotic conditions as under isosmotic conditions (Marcus & Marcus, 1987). The amount of K^+ release necessary for maintaining constant cell volume during a hypo-osmotic challenge was estimated to be maximally 6.2 · 10^{-8} mol/cm^2 calculated from an average cell height of 8.2 μm (Wangemann & Shiga, 1994*b*), an estimated cytosolic K^+ concentration of maximally 150 mM and the assumption that for constant volume half of cytosolic K^+ content was released. The observation that the estimated K^+ released during the hypo-osmotic challenge was a factor of 10 larger than that estimated to be necessary for regulatory volume decrease supports the conclusion that the hypo-osmotic challenge stimulated transepithelial K^+ transport in excess of that necessary to maintain constant cell volume. In fact, after the first 15 sec of the hypo-osmotic challenge enough K^+ (based on the integral of I_{sc}; Fig. 4*C*) was released to maintain a constant volume. The cellular mechanism by which the hypo-osmotic challenge triggered stimulation of transepithelial K^+ transport remains unknown. Hypo-osmotic stimulation of transepithelial ion transport, however, has also been observed in other epithelia (Ussing, 1965; Crowe & Wills, 1991).

The observation that hypo-osmotically-induced activation of the I_{sK} channel observed in individual cells was paralleled by an increase in I_{sc}, which was measured across a sample of about 100 cells, demonstrates that activation of the I_{sK} channel is a significant response of the dark cell epithelium rather than a feature of a few cells selected by the patch clamp technique. The observation that a basolateral hypo-osmotic challenge caused stimulation of transepithelial K^+ secretion is expected to be of physiological significance for the homeostasis of inner ear fluids during depression of the serum osmolarity. Even though the basolateral membrane of vestibular dark cells is bathed by perilymph (a fluid similar to solution 1) and not by serum, it has been shown that the osmolarity of perilymph follows the osmolarity of serum

with some time delay (Juhn & Rybak, 1981). The observations that the barrier between perilymph and endolymph is water permeable (Sterkers et al., 1982) but that dilutions of the serum had no effect on inner ear function (Jefferis & Johnstone, 1987) suggest that homeostatic mechanisms are present. It remains to be demonstrated whether stimulation of transepithelial K^+ transport during a basolateral hypo-osmotic challenge occurs also under in vivo-like conditions, when the apical membrane of vestibular dark cells is bathed with a KCl-rich solution.

The Onset of Regulatory Volume Decrease Occurs with Little Delay

Most cells which regulate their volume respond to a hypo-osmotic challenge with an initial increase in cell volume to values near those expected for a perfect osmometer. This initial peak in cell volume suggests that regulatory volume decrease occurred in those cells with a delay of 30 sec or more (Völkl & Lang, 1988; Tauc et al., 1990; Farahbakhsh & Fain, 1987). In contrast, regulatory volume decrease in vestibular dark cells occurred as fast as solutions could be changed such that no initial peak in cell volume was observed (Wangemann & Shiga, 1994*b*). The present data demonstrate that the onset of cellular responses toward the hypo-osmotic challenge occurred within 1 sec (Fig. 4*D*). Rapid osmotically-induced changes in V_t have been attributed in other epithelia to streaming potentials rather than to cellular responses (Frömter & Gessner, 1974; Reuss et al., 1992). The magnitude of streaming potentials, however, is independent of the side, apical or basolateral, on which the osmotic challenge is applied. The observed response in V_t to a basolateral hypo-osmotic challenge was considered a cellular response since no significant change in V_t was observed in response to an apical hypo-osmotic challenge (Fig. 4). The rapid onset of the cellular responses in conjunction with the absence of an initial peak in cell volume suggests that mechanisms underlying regulatory volume decrease were activated during very small increases in cell volume. The hypothesis that very small increases in cell volume are sufficient to activate transepithelial K^+ transport is supported by the observation that a small hypo-osmotic challenge (298 to 270 mosM) on the basolateral side under conditions similar to those of the present study caused a significant increase in V_t and I_{sc} and a significant decrease in R_t (Wangemann et al., 1995). The time resolution of the present experiments, however, is not sufficient to exclude the involvement of a second messenger system in the transduction mechanism between the hypo-osmotic challenge and the cellular response. The onsets of responses observed during cell-attached macropatch recordings (Figs. 5 and 7) or during cell impalements (Fig. 6) were significantly slower than those of parameters measured in the micro-Ussing chamber. This discrepancy is not contradictory but merely the result of lower perfusion rates employed during cell-attached macropatch and microelectrode recordings (*see* Materials and Methods).

Hypo-osmotic Challenge Activates the Apical I_{sK} Channel

Many cells activate a K^+ channel in response to a hypo-osmotic challenge (Eveloff & Warnock, 1987; Lang et al., 1993). The observation that a hypo-osmotic challenge caused a significant increase in the magnitude of I_{IsK} and g_{IsK} demonstrates that regulatory volume decrease involved activation of the I_{sK} channel in the apical membrane (Figs. 5 and 7). Consistent with the activation of the I_{sK} channel is the observation that V_{rc} either shifted toward or stayed at the K^+ equilibrium potential. The coincidence of a hyperpolarization of V_m and an increase in I_{IsK} and g_{IsK} suggests that hypo-osmotically-induced activation involved a modification of the voltage-dependency of the I_{sK} channel since the I_{sK} channel has been shown under isosmotic conditions to be inactivated by a hyperpolarization of the holding voltage rather than activated (Marcus & Shen, 1994). Hypo-osmotic activation of the I_{sK} channel, however, was independent of the direction of the K^+ gradient and did not affect τ_{IsK} although small changes in τ_{IsK} might have gone undetected. The molecular mechanism of activation of the I_{sK} channel by a hypo-osmotic challenge in gerbilline vestibular dark cells is as yet unknown, however, some data are available from the rat kidney I_{sK} channel expressed in *Xenopus* oocytes. In that preparation a similar alteration of the voltage activation was observed during a hypo-osmotic challenge and evidence suggested that activation of the I_{sK} channel encompassed Ca^{2+} entry and changes in the actin network (Busch et al., 1992). Whether a similar Ca^{2+} and actin-network dependent mechanism is present in vestibular dark cells remains to be determined.

The observation that $I_0 - I_{IsK}$ and $g_a - g_{IsK}$ did not change throughout the present experiments suggests that no conductive pathway other than the I_{sK} channel was activated in the apical membrane by the hypo-osmotic challenge. Thus, K^+ secretion under isosmotic conditions (Marcus & Shen, 1994) and K^+ efflux during regulatory volume decrease both involve the apical I_{sK} channel.

Identification of the I_{sK} channel was aided in the present study by monitoring τ_{IsK}. No significant change in τ_{IsK} was observed throughout the present experiments suggesting that the hypo-osmotic challenge did not alter the voltage-inactivation of the I_{sK} channel. Values for τ_{IsK} obtained from human, mouse and rat I_{sK} channels expressed in *Xenopus* oocytes and presumably studied at room temperature were of similar magnitude (Hice et al.,

1994). This similarity, however, was not necessarily expected based on the difference in temperature and the finding that at least the activation time constant of the I_{sK} channel is strongly temperature dependent (Busch & Lang, 1993).

A quantitative comparison between I_{IsK} and K^+ flux based on the measurements of $J_{K^+,probe}$ is not possible since data obtained with the vibrating probe could not be calibrated (*see* Materials and Methods). An indirect comparison, however, can be made since present measurements of I_0 (when normalized to the surface area) are quantitatively similar to measurements of I_{sc} and because I_{sc} is at least under isosmotic conditions quantitatively accounted for by the transepithelial flux of K^+ (Marcus & Marcus, 1987). The area of the apical membrane, across which I_0 was measured, was taken to be 11 μm^2 based on the assumption that the membrane isolated by the macropatch pipette was flat and that the area was defined by the inner diameter of the patch pipette. Under isosmotic conditions in the presence of mannitol I_0 was 17 pA corresponding to 155 $\mu A/cm^2$ which is similar to the I_{sc} of 139 $\mu A/cm^2$ and during the hypo-osmotic challenge I_0 was 73 pA corresponding to 664 $\mu A/cm^2$ which is similar to the I_{sc} of 824 $\mu A/cm^2$. The correlation between I_0 and I_{sc} was found to be poorer under control conditions when I_0 was 20 pA corresponding to 182 $\mu A/cm^2$ and I_{sc} was 590 $\mu A/cm^2$. This discrepancy might be due to the fact that I_0 was obtained under short-circuit conditions whereas I_{sc} was obtained under open circuit conditions. This difference in experimental conditions might be more significant in the presence of solutions with low resistivity such as under control conditions (150 mM NaCl, solution 1) than in solutions with a higher resistivity due to the presence of only 75 mM NaCl ± mannitol.

It is conceivable that $g_a - g_{IsK}$ which did not change significantly during the present experiments is comprised of the nonselective cation channel found with low density in the apical membrane of vestibular dark cells (Marcus et al., 1992) or of some yet unknown channel with a reversal potential more positive than the K^+ equilibrium potential. Alternatively, this apparent additional conductance could merely be the result of the nonselective nature of the seal. Regardless, the observation that $g_a - g_{IsK}$ did not increase during a hypo-osmotic challenge demonstrates that no apical membrane conductance other than the I_{sK} channel was activated by the hypo-osmotic challenge.

Estimation of the Upper Limit of the Water Permeability Ratio

Nothing is currently known about the relative water permeability of the apical and basolateral membrane of vestibular dark cells. The finding that there was no significant change in I_{sc} in response to an apical hypo-osmotic challenge cannot be taken as evidence for a water impermeable apical membrane since the difference in membrane area must be considered (Spring, 1983). In fact, the present data are consistent with an up to 4-fold larger water permeability of the apical membrane compared to that of the basolateral membrane. A basolateral hypo-osmotic challenge caused an increase of I_{sc} by 685 $\mu A/cm^2$ (from 139 to 824 $\mu A/cm^2$). Considering that the area of the apical membrane is 40 times smaller than that of the basolateral membrane (W. ten Cate, *personal communication*) it can be assumed that the response of I_{sc} would be 40 times less if the absolute water permeability of both membranes would be equal. Under this condition, I_{sc} would have increased by 17 $\mu A/cm^2$ (685 $\mu A/cm^2/40$) which would not be detectable assuming that a detectable increase in I_{sc} must be larger than 70 $\mu A/cm^2$ (one standard error). From this detection limit it can be calculated that no significant increase in I_{sc} in response to an apical hypo-osmotic challenge would have been detected in the presence of an up to 4-fold larger absolute water permeability of the apical membrane (70 $\mu A/cm^2$/17 $\mu A/cm^2$). This estimate only places an upper limit and does not rule out the possibility that the absolute water permeability of the apical membrane is equal or smaller than that of the basolateral membrane.

Verification of Measurements of V_m

Contrary to other inner ear epithelia (Wangemann & Shiga, 1994*a*), V_m in vestibular dark cells is very small (about −8 mV) since it is dominated by the large basolateral Cl^- conductance (Wangemann & Marcus, 1992) and since the cytosolic Cl^- concentration was estimated to be 100 mM or more (Marcus et al., 1993). Verification of the microelectrode measurements of V_m comes from the measurements of V_r obtained with the less invasive cell-attached macropatch technique. V_r of a cell-attached patch is expected to equal $-V_m$ when the membrane under the patch is solely K^+ selective and the K^+ gradient is zero. These conditions were approximated when the patch pipette contained 150 mM K^+ and the membrane patch contained the K^+ selective I_{sK} channel. Under these conditions V_r was +10 ± 3 mV, which verifies the microelectrode measurement of V_m of −8 ± 2 mV.

The cytosolic Cl^- concentration can be estimated from the Nernst equation throughout the present experiments under the assumption that V_m remained close to the Cl^- equilibrium potential. During isosmotic replacement of NaCl with mannitol the cytosolic Cl^- concentration was estimated to decline from 100 to 80 mM and during the hypo-osmotic challenge to decline further to 35 mM. The assumption that V_m remained close to the Cl^- equilibrium potential also during the hypo-osmotic challenge is not conflicting with the finding of an increased apical K^+ conductance under these conditions

since the ratio of the apical and basolateral membrane area is about 1:40. The apical membrane potential can therefore be expected to contribute little to V_m under the present conditions.

Evidence for Cross-talk Mechanisms between the Basolateral K^+ Uptake Mechanisms and the Apical I_{sK} Channel

A reduction of the extracellular Cl^- concentration caused a depolarization of V_m which was expected to consist of a fast depolarization due to a transient increase in the outward Cl^- gradient across the basolateral membrane followed by a slower partial repolarization due to loss of cytosolic Cl^- and the resulting decrease of the basolateral Cl^- gradient. Even though these two phases were clearly observed as a transient increase and subsequent decrease of V_t (Fig. 4*A*: "bl: –NaCl + Mannitol"), the transient depolarization was not distinguishable in the recording of V_m (Fig. 6), most likely due to an insufficient perfusion rate. Similar effects on V_t were observed during basolateral replacement of Cl^- with gluconate$^-$ (Marcus & Marcus, 1989). The presumed loss of cytosolic Cl^- during the depolarization was accompanied by a transient release of K^+ which was observed as a transient increase in $J_{K^+,probe}$ and $I_{sc,probe}$ (Figs. 2 and 3). This transient electrogenic K^+ release occurred via the apical I_{sK} channel as indicated by the transient increase in I_{IsK} (Fig. 5). Consistent with this interpretation is the observed increase in g_{IsK} since the apical I_{sK} channel is known to be activated by a depolarization of V_m (Marcus & Shen, 1994). Thus, V_m which is dominated by the basolateral Cl^- conductance (Wangemann & Marcus, 1992) mediated cross-talk to the apical I_{sK} channel (Fig. 8). In addition, the transient increase in I_{IsK} and g_{IsK} might be due to a cytosolic acidification which may have occurred via the basolateral Na^+/H^+ exchanger when the extracellular Na^+ concentration was lowered. Indeed, cytosolic acidification has been shown to cause a transient activation of transepithelial K^+ secretion via the I_{sK} channel and a subsequent inhibition of transepithelial K^+ secretion due to inhibition of the Na,K-ATPase (Wangemann, Liu & Shiga, 1995; Kuijpers & Bonting, 1969). Thus, the cytosolic pH which is controlled by the basolateral Na^+/H^+ exchanger mediated cross-talk to the apical I_{sK} channel (Fig. 8).

The observation that the increase in I_{IsK} and g_{IsK} was transient rather than sustained in the presence of a sustained depolarization of V_m and presumably sustained acidification of the cytosolic pH might be related to another cross-talk mechanism. This cross-talk mechanism between the basolateral mechanisms for K^+ uptake and the apical I_{sK} channel is necessary in order to limit cell shrinkage which has been observed during the isosmotic replacement of NaCl with mannitol (Wangemann & Shiga, 1994*b*) (Fig. 8). In order for the cell to limit the extend of shrinkage, it is necessary to reduce the release

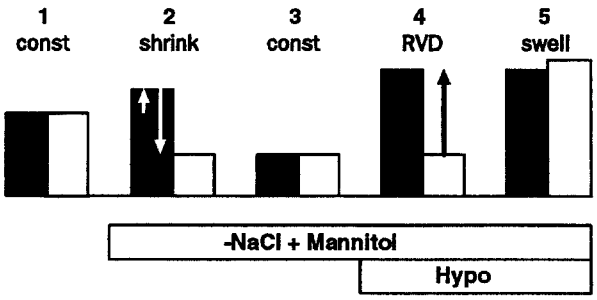

Fig. 8. Diagram illustrating cross-talk in vestibular dark cells. (1) cell volume under control conditions was constant since the rate of basolateral K^+ influx (*white bar*) matches the rate of apical K^+ efflux (*black bar*). (2) when NaCl was isosmotically replaced with mannitol (*–NaCl + Mannitol*), cell shrinking occurred due to a voltage and pH-mediated increase in the rate of apical K^+ release and decrease in the rate of basolateral K^+ uptake. The reduction in the extracellular Na^+ and Cl^- concentration caused a cytosolic acidification and a depolarization of the V_m which as cross-talk mechanisms (*upward arrow*) caused activation of the apical pH and voltage-dependent I_{sK} channel and an increase in K^+ release. Subsequently, the acidification of the cytosolic pH caused inhibition of the basolateral Na,K-ATPase and a decrease in K^+ uptake. Cell shrinking was limited by cross-talk (*downward arrow*) which permitted readjustment of the rate of apical K^+ release to the rate of basolateral K^+ uptake. (3) The rate of apical K^+ release was reduced and cell volume was constant again. Compared to control conditions, however, the rate of transepithelial K^+ secretion was reduced. (4) the hypo-osmotic challenge caused influx of water. Regulatory volume decrease (*RVD*) was due to stimulation of the apical I_{sK} channel and an increase in the rate of K^+ efflux. In addition, the hypo-osmotic challenge caused upregulation (*arrow*) of the rate of basolateral K^+ uptake to allow for an elevation of steady state K^+ secretion. (5) basolateral K^+ uptake slightly exceeded apically K^+ efflux resulting in the observed slow rate of cell swelling.

of K^+, which was in fact observed as a sustained reduction of $J_{K^+,probe}$ (Fig. 2), $I_{sc,probe}$ (Fig. 3), I_{sc} (Fig. 4), I_{IsK} and g_{IsK} (Figs. 5 and 7). Thus, the reduction of the transport rate of the basolateral K^+ uptake mechanism resulted in cross-talk to the apical membrane and reduction of K^+ release. This sustained reduction was not merely the result of a limited availability of cytosolic K^+ since the cells were able to respond subsequently to a hypo-osmotic challenge with a large increase in K^+ secretion.

The finding that the rate of K^+ release across the apical membrane was stimulated during a hypo-osmotic challenge is consistent with the observed regulatory volume decrease (Wangemann & Shiga, 1994). The observation, however, that the increase in K^+ secretion exceeded that necessary for the maintenance of constant cell volume (Fig. 4*C*) suggests that the hypo-osmotic challenge caused not only stimulation of the apical I_{sK} channel but also of the basolateral K^+ uptake mechanisms (Fig. 8). Consistent with this view is the observation that cell swelling occurred during a hypo-osmotic challenge at a very slow rate more likely being due to K^+ uptake slightly exceeding K^+ secretion (Wangemann & Shiga, 1994*b*). The nature of this regulatory mechanism mediating upregulation of both the apical I_{sK} chan-

nel and the basolateral K^+ uptake mechanisms, however, remains unknown.

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