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DIDS Increases K^+ Secretion through an I_{sK} Channel in Apical Membrane of Vestibular Dark Cell Epithelium of Gerbil

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Abstract. Vestibular dark cell epithelium secretes K^+ via I_{sK} channels in the apical membrane. The previous observation that disulfonic stilbenes increased the equivalent short circuit current (I_{sc}) suggested that these agents might be useful investigative tools in this tissue. The present experiments were conducted to determine if the increase in I_{sc} was associated with an increase in K^+ flux and if the effect was directly on the I_{sK} channel or indirectly via a cytosolic intermediary. Measurements of transepithelial K^+ flux with the K^+-selective vibrating probe and of changes in net cellular solute flux by measurements of epithelial cell height showed that 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) increased K^+ flux by a factor of 1.96 ± 0.71 and caused net solute efflux. The apical membrane was partitioned with a macropatch pipette and DIDS was applied either to the membrane outside the pipette, inside the pipette or to the entire apical membrane. DIDS inside the pipette increased the current across the patch, the membrane conductance, the slowly-inactivating (I_{sK}) component of the membrane current and shifted the reversal voltage toward the equilibrium potential for K^+. DIDS outside the patch decreased the patch current and conductance, consistent with shunting of current away from the membrane patch. These findings strongly support the notion that DIDS increases K^+ secretion through I_{sK} channels in the apical membrane of vestibular dark cell epithelium by acting directly on the channels or on a tightly colocalized membrane component.

Key words: I_{sK} potassium channel — Min K channel — Disulfonic stilbene — Inner ear — Ion-selective vibrating probe — Macropatch clamp technique

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Introduction

It has recently been shown that the isolated vestibular dark cell epithelium produces a lumen-positive transepithelial voltage (V_t) [10] and secretes K^+ [13] through a slowly-activating, voltage-dependent potassium channel (I_{sK} or min K) in the apical membrane [12]. Our previous studies showed that apical, but not basolateral, application of 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) increased V_t and decreased the transepithelial resistance (R_t) [10, 11]. Based on the known actions of DIDS as an anion transport inhibitor [3, 4, 19, 21], it was proposed that DIDS may have caused these effects by inhibition of Cl^-/HCO_3^- exchange. This would then have led to changes in intracellular pH and subsequent activation of another transport pathway. Alternatively, DIDS may have directly activated the apical I_{sK} channel responsible for K^+ secretion.

The present study was designed to discriminate between these hypotheses and utilized several experimental approaches. Experiments were conducted at the level of the epithelium with measurements of the equivalent short circuit current and of the relative transepithelial K^+ flux with the vibrating probe technique. At the cellular level, the cell volume was monitored as cell height in order to follow changes in the net flux of solutes. Properties of the apical membrane were measured with the on-cell macropatch technique. The results demonstrated that apical perfusion of DIDS directly activated either the I_{sK} channel in the apical membrane of vestibular dark cells or a tightly colocalized membrane component. Preliminary reports of some of these results have been made [15, 16].

Materials and Methods

Preparation

Gerbils (cared for and used under a protocol approved by the Boys Town National Research Hospital Animal Research Committee) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and decapitated. The temporal bone was removed and the dark cell epithelium with underlying connective tissue was dissected from the ampullae of semicircular canals, as described previously [23]. The epithelium was either transferred directly as a flat sheet to the micro-Ussing chamber or folded into a loop with the apical membrane facing outside of the loop and transferred to a recording chamber on the stage of an inverted microscope (IM, Zeiss, German; Diaphot, Nikon, Japan) for macropatch clamp experiments or for cell height measurements. All experiments were performed at 37°C except for the vibrating probe measurements which were performed at 20–22°C. Transepithelial transport by vestibular dark cells is qualitatively similar (lumen-positive and sensitive to bumetanide) at both temperatures, although the open circuit transepithelial voltage developed at room temperature is about half that at 37°C [9].

Micro-Ussing Chamber

The micro-Ussing chamber for inner ear tissue used here was described previously [10]. In brief, the diameter of the aperture between the apical and basolateral perfusion chamber was 80 μm. Transepithelial voltage (V_t) was measured with calomel electrodes connected to the chamber via agar bridges (4% agar in solution 1, Table). Transepithelial current pulses were passed via Ag/AgCl wires and bridges of 150 mM NaCl. Sample-and-hold circuitry was used to obtain a signal proportional to the transepithelial resistance (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 digitized directly with a 12-bit A/D converter. The transepithelial equivalent short circuit current (I_{sc}) was derived from V_t and R_t (V_t/R_t).

Vibrating Probe

The vibrating probe technique used here was the same as described previously [13], although the epithelium was mounted with the apical side against the aperture of the micro-Ussing chamber as for measurements of V_t and R_t. Briefly, the relative K^+ flux ($J_{K,probe}$) was monitored with a K^+-selective microelectrode vibrated with an excursion of 30 μm at 0.3 Hz along an axis normal to the plane of the tissue. The microelectrodes were constructed from 1.5 mm OD borosilicate glass capillary, pulled to a tip of about 4 μm OD, and silanized with dimethyldichlorosilane. The tip contained a column of K^+-selective ligand (cat #60398, Fluka Chemical) about 150 μm long, and the electrode was backfilled with 100 mM KCl and 0.5% agar. The reference was Ag/AgCl with a bridge of 3 M NaCl and 3% agar. Electrodes were only used if the slope was at least 56 mV/decade in 10 and 100 mM KCl solution. Data were recorded by digitizing with a 16-bit A/D converter. The contribution of the voltage gradient produced by the transepithelial electric current (ca 10 μV) was less than 6% of the voltage gradient observed at the K^+-selective electrode; no corrections were made for this component of the signal.

Cell Height

Cell height was used here as an index of cell volume as described previously [24, 25]. In brief, the microscope image (differential interference contrast) of an optical section of the folded tissue was viewed with a black/white video camera (Panasonic WV-1550), mixed with a time signal and displayed on a monitor (PVM-122, Sony, Park Ridge, NJ) and recorded (AG-1960, Panasonic, Secaucus, NJ) on videotape. A computer-generated image of two vertical cursors was mixed online with the microscope image (NTSC Recordable Videocard, USVideo, Stamford, CT). The two cursors were adjusted independently to overlay the apical and basal border of the epithelium. The calibrated distance between the two cursors (CH) was written into an ASCII file at a rate of 0.5 Hz.

Macropatch Clamp Technique

The cell-attached, macropatch clamp technique used here was described previously [12]. In brief, patch pipettes (3–5 μm ID) were manufactured from Corning 7052 glass capillary (1.5 mm OD, 0.86 mm ID: Garner Glass, Claremont, CA) with a 2-stage puller (PP-83, Narishige, Japan), and a microforge. Pipette tips were coated with a hydrocarbon consisting of a 2:1 mixture of α-tocopherol acetate and heavy mineral oil (Sigma, St. Louis, MO). Pipettes were connected to the patch clamp amplifier (Model 8900, Dagan, Minneapolis, MN, or Model 200A, Axon Instruments, Foster City, CA) via a Ag/AgCl wire. The reference was a Ag/AgCl electrode connected to the bath via a flowing 1 M KCl junction. High-resistance seals (>8 gigaohm) were made between the pipette and the apical membrane of vestibular dark cells within the untreated, native epithelium. Patch clamp data were collected via a 12-bit A/D converter and pClamp software (version 5.5.1, Axon Instruments, Foster City, CA) and analyzed with pClamp and Origin software (Microcal Software, Northampton, MA).

Two series of experiments were performed in which the apical membrane was partitioned with the patch pipette and the effects of DIDS in and outside of the pipette were observed. In the first series (Fig. 9), unpaired measurements were made in the presence and absence of DIDS in the pipette. The current was recorded in the presence and absence of DIDS in the bath. The electrical potential difference of the bath with respect to the pipette was clamped to zero continuously, except every 5 sec to + and −10 mV for 0.5 sec each (for measurement of apparent conductance, G_a [12]).

In the second series (Figs. 10, 11 and 12), paired measurements were made in the presence and absence of DIDS in the pipette by means of pipette perfusion. The current was recorded in the absence of DIDS in the bath. The electrical potential difference of the bath with respect to the pipette was clamped to zero continuously (for measurement of I_0) except every 15 sec the voltage was stepped briefly to three other levels for 10 msec each to obtain the "instantaneous" *I/V* relationships (Fig. 1; points 2, 3, 4, 5). The three voltages were −60, −90, and 0 mV for NaCl pipette solution and −20, 20, and 0 mV for KCl pipette solution. The *I/V* relationships were used to estimate the conductance (g_a) near the reversal voltage (V_r).

After the short steps, the voltage was hyperpolarized to −40 mV for 4 sec (Fig. 1) in order to ascertain that I_0 consisted of current passing through the I_{sK} channel. The I_{sK} channel is characterized by a slow activation (order of seconds) by depolarization > −20 mV and a slow inactivation (order of 100 msec) by hyperpolarization of −40 mV [12]. After inactivation of the apical I_{sK} channel, the command voltage was returned to 0 mV (point 7, Fig. 1) and 5 data points following the capacitive transient (samples #3–7) were averaged to obtain an estimate of the component of I_0 exclusive of I_{sK} ($I_0 - I_{sK}$). In each experiment, this value of $I_0 - I_{sK}$ was averaged over the last 5 min of both the control period and the period of perfusion of DIDS.

The current was low-pass filtered at 200 Hz for series 1 and 1000 Hz for series 2. Filtered currents were digitized to 12-bits resolution at

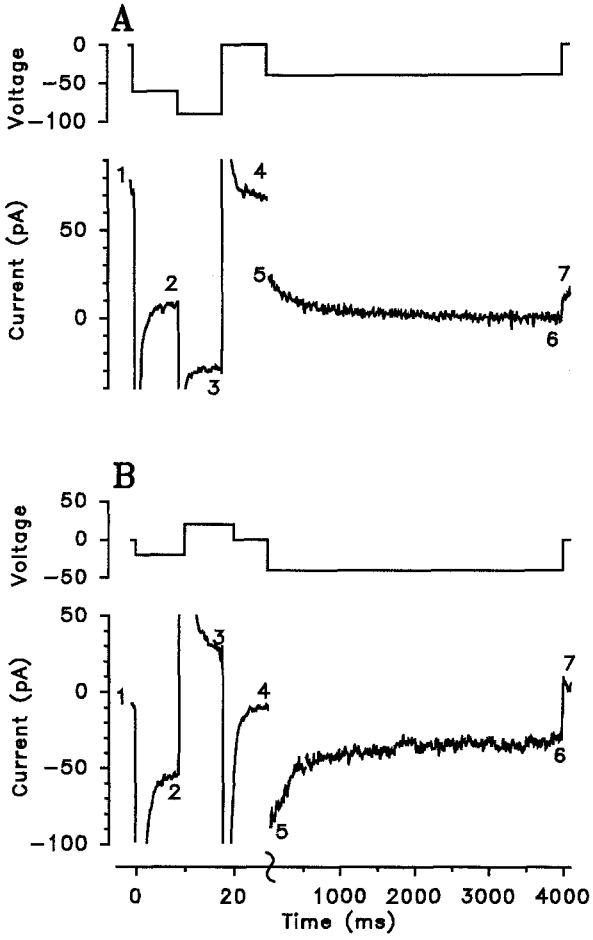

Fig. 1. Measurement of "instantaneous" currents and magnitude of $I_o - I_{sK}$ at a holding potential of 0 mV. (*A*) voltage protocol of the command voltage and representative macropatch recording of apical membrane current for NaCl pipette solution. (*B*) voltage protocol of the command voltage and representative macropatch recording of apical membrane current for KCl pipette solution.

a sampling rate of 400 Hz for protocol 1, 35.7 kHz for the 10 msec voltage steps and 250 Hz for the long hyperpolarization voltage step of series 2.

Pipette Perfusion Technique

We have developed and used in this study a pipette perfusion technique based on those of LaPoint and Szabo [7] and of Soejima and Noma [17]. The main features of this system are the use of positive pressure to enhance the rate of perfusion and the means to alternately switch between two perfusates in order to be able to conduct paired experiments. Basically, a modified pipette holder (model A003-1, E.W. Wright, Guilford, CT) was used which has an inlet for a perfusion line (Fig. 2). Perfusion pipettes were made from quartz tubing (100 μm ID, 165 μm OD) which was drawn to a tip of 50 μm ID and 75 μm OD in a hydrogen/oxygen flame. The quartz was tightly connected into a polyethylene tubing (PE-10; 0.28 mm ID, 0.61 mm OD) about 6 cm long which was connected to the outlet of a 4-way distribution valve (HVDP4-5, Hamilton, Reno, NV); only 2 inlets were used in the present study. Pipette solutions were contained in 1.5 ml plastic capsules (1.5 ml polypropylene microcentifuge tubes) which were shielded by grounded copper screen and which were held on the stage of the microscope near the distribution valve. Polyethylene tubing (about 4 cm) was connected between the distribution valve and the plastic capsules. A positive pressure was supplied by a water column of about 40 cm to both plastic capsules, so that no pressure difference existed between capsules during switching of the valve. A glass vial (20 ml) connected to both the source of pipette suction and to the pipette outlet (3 cm length of silicone tubing, 2 mm ID) was shielded by grounded copper screen. This collection vial was used both to collect the perfusate and to electrically limit the physical extent of the pipette electrolyte in order to make constant the capacitance associated with the pipette.

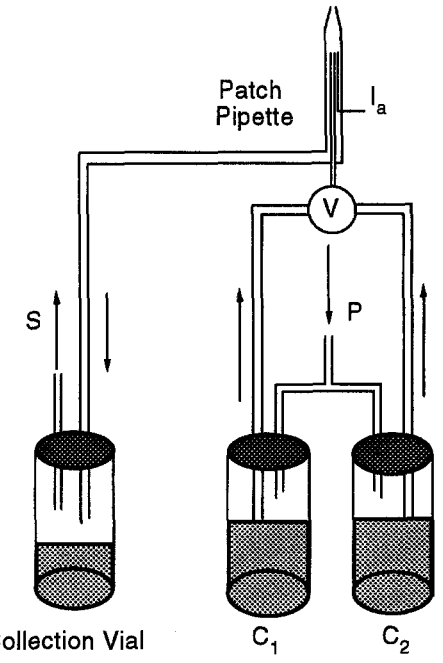

Fig. 2. Diagram of patch pipette perfusion system. Containers 1 and 2 (C_1 and C_2) had different pipette solutions and were sealed tightly. The containers were pressurized equally by a water column via an inlet (*P*). Perfusate from one container was selected by a valve (*V*) before flowing to the patch pipette through a quartz capillary. Pressure at the tip of the patch pipette was controlled by another water column and/or syringe attached (*S*) via a collection vial mounted close to the pipette, which served to limit the capacitance associated with the pipette.

Solutions

Solutions used are listed in the Table. Lidocaine and the three disulfonic stilbenes, DIDS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) and 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) were purchased from Sigma Chemical (St. Louis, MO). They were first dissolved in dimethylsulfoxide (DMSO) before adding to one of the solutions listed in the Table (final DMSO concentration 0.1%).

Liquid Junction Potentials

Data from the micro-Ussing chamber during experiments with ion substitutions were corrected for liquid junction potentials which were measured separately against a flowing saturated KCl electrode.

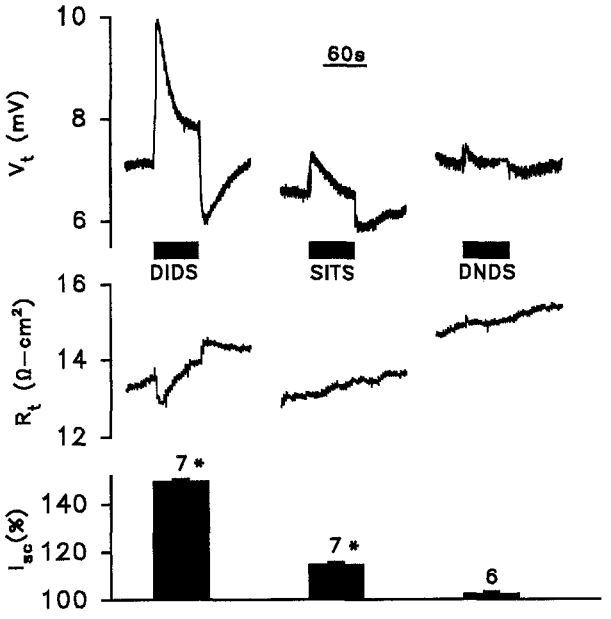

Fig. 3. Representative recordings of the effect of apical perfusions of the disulfonic stilbenes (200 μM) DIDS, SITS, and DNDS on (*A*) transepithelial voltage (V_t), (*B*) transepithelial resistance (R_t) and (*C*) mean peak equivalent short circuit current (I_{sc}) expressed as a percent of I_{sc} immediately prior to perfusion of DIDS; number near each bar is the number of experiments.

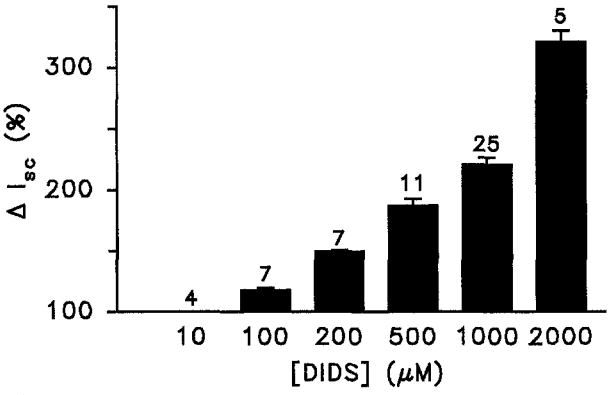

Fig. 4. The effect of apical perfusions of DIDS at concentrations between 10 and 2000 μM on I_{sc}. The mean peak I_{sc} expressed as a percent of I_{sc} immediately prior to perfusion of DIDS; number near each bar is the number of experiments.

Data Presentation and Statistics

Data are given as mean ± SEM (n = number of samples). Students' t-tests were used for paired and unpaired samples. Differences were assumed to be significant when $P < 0.05$.

Results

Micro-Ussing Chamber

The effects of apical perfusion of three disulfonic stilbenes DIDS, SITS and DNDS on V_t, R_t and I_{sc} were compared at $2 \cdot 10^{-4}$ M (Fig. 3). Apical DIDS significantly increased I_{sc} from 447 ± 26 to a peak of 667 ± 39 μA/cm² followed by a partial relaxation to a steady-state level of 498 ± 25 μA/cm² (n = 7), while SITS increased I_{sc} less, but significantly from 441 ± 11 to a peak of 505 ± 13 μA/cm² followed by a partial relaxation to a steady-state level of 457 ± 8 μA/cm² (n = 7), but DNDS had no significant effect on I_{sc} (440 ± 23 to 449 ± 23 μA/cm², n = 6). The changes in I_{sc} were due to a concurrent increase in V_t and a decrease in R_t, as observed earlier for SITS on the utricle and DIDS on both the ampullar dark cells and stria vascularis [10, 11, 22]. Basolateral perfusion of DIDS (10 μM, 100 μM and 500 μM) had no effect on V_t or R_t (*data not shown;* n = 6). DIDS was perfused on the apical side of the epithelium in all of the following experiments since it was found to be the most potent of the disulfonic stilbenes tested.

In the range 10^{-5} to $2 \cdot 10^{-3}$ M, 10^{-4} M DIDS was the lowest effective dose tested (Fig. 4) and the responses had apparently not saturated at $2 \cdot 10^{-3}$ M. 10^{-3} M DIDS increased I_{sc} from 569 ± 30 to a peak of 1237 ± 54 μA/cm² (N = 26) and was the concentration used in the remaining series of experiments.

If a Cl^-/HCO_3^- exchanger were present in the apical membrane, it could be expected that Cl^- would be transported into the cell in exchange for metabolically-produced HCO_3^- in the cytosol. Removal of apical Cl^- would then have an effect similar to that of DIDS and the superimposed application of DIDS would lead to a smaller response. Apical DIDS was perfused in the presence and absence of apical Cl^-. Removal of Cl^- was previously found to cause a small decrease of V_t and an increase in R_t [11]. In the present series, the decrease of V_t from 13.9 ± 1.6 to 11.2 ± 2.7 mV upon removal of Cl^- (solution 2, Table) did not reach statistical significance but R_t increased from 23.7 ± 2.7 to 32.8 ± 3.6 ohm-cm² (n = 7). In contrast to the prediction for a Cl^-/HCO_3^- exchanger, DIDS (10^{-3} M) produced a larger peak increase in V_t and decrease in R_t in the absence of apical Cl^- than in its presence (11.9 ± 1.0 *vs.* 9.1 ± 0.4 mV and 8.5 ± 1.8 *vs.* 3.7 ± 0.3 ohm-cm², n = 7; Fig. 5).

If DIDS produced its increase in V_t by activation of an apical K^+ conductance, it would be expected that reduction of the K^+ gradient across the apical membrane by elevation of the apical [K^+] would lead to a reduction of the effect of DIDS on V_t while DIDS would still decrease R_t. As found previously [10], raising the apical [K^+] to 150 mM (solution 3, Table; a solution similar to endolymph) decreased V_t from 8.9 ± 0.7 to –0.6 ± 0.4 mV and R_t from 16.9 ± 1.5 to 13.2 ± 1.2 ohm-cm² (n = 7). As predicted, DIDS (10^{-3} M) had no significant effect on V_t (–2.7 ± 0.3 *vs.* –2.4 ± 0.3 mV, n = 7) in the presence

Table. Composition of solutions (in mM)

Solution	1	2	3	4	5
KCl			146.4	3.6	150.0
NaCl	150.0			150.0	
NaGlu		150.0			
NMDG					
$MgCl_2$	1.0		1.0	1.0	1.0
$MgSO_4$		1.0			
$CaCl_2$	0.7		0.7	0.7	0.7
CaGlu		4.0			
K_2HPO_4	1.6	1.6	1.6		
KH_2PO_4	0.4	0.4	0.4		
HEPES				10.0	10.0
HCl					
Glucose	5.0	5.0	5.0		

Glu, gluconate; NMDG, N-methyl-D-glucamine; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; pH adjusted to 7.4.

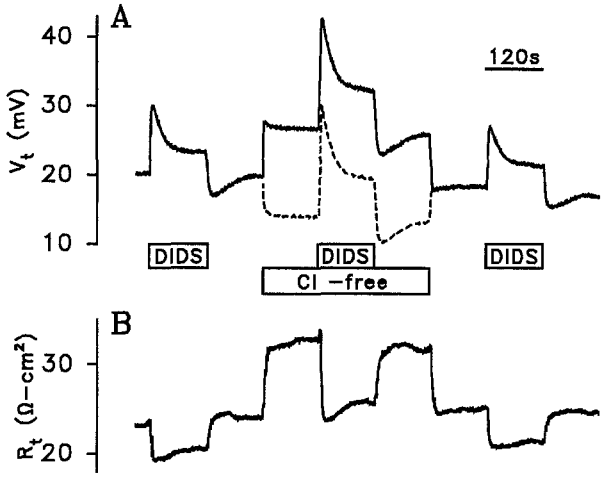

Fig. 5. Representative recording of the effect of apical perfusion of DIDS (1 mM) in the presence (solution 1) and absence (solution 2) of apical Cl^- (gluconate substitution) on V_t and R_t. Dotted line indicates V_t corrected for liquid junction potential.

of 150 mM K^+, while DIDS caused R_t to significantly decrease from 9.7 ± 1.3 to 6.8 ± 1.0 ohm-cm^2 (n = 7) in the presence of 150 mM K^+ (Fig. 6).

Vibrating Probe

The probe was located directly over the basolateral surface of the epithelium, and the magnitude of the K^+ gradient was recorded in the absence and presence of DIDS (Fig. 7). The negative values obtained indicated a K^+ flux directed from the basolateral to the apical side (solution 1, Table). Apical perfusion of 10^{-3} M DIDS increased the magnitude of $J_{K+,probe}$ by a factor of 1.96 ± 0.71 (n = 5).

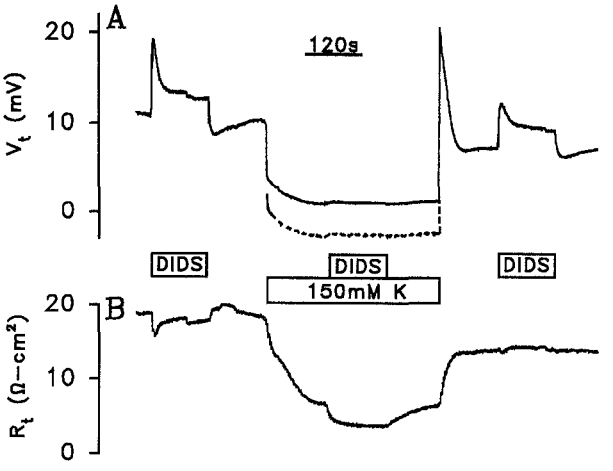

Fig. 6. Representative recording of the effects of apical perfusions of DIDS (1 mM) in the presence of 150 mM NaCl (solution 1) or 150 mM KCl (solution 3) in the apical perfusate on V_t and R_t. Dotted line indicates V_t corrected for liquid junction potential.

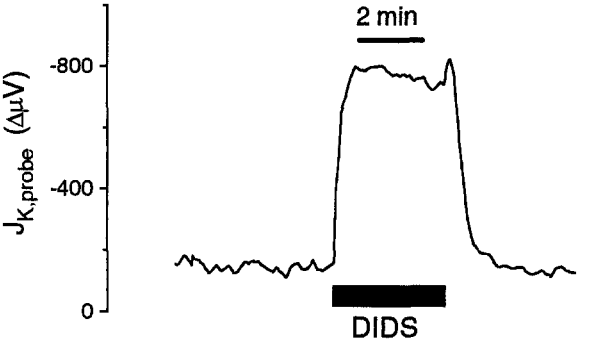

Fig. 7. Representative recording with the K^+-selective vibrating probe of the effect of DIDS (1 mM) on the K^+ gradient ($J_{K,probe}$) near the basolateral membrane.

Cell Height

Cell height was measured in order to monitor changes in net solute transport. Cells were swelled by exposure to elevated K^+ (25 mM) [24, 25] in the absence and presence of either 10^{-4} M DIDS, 10^{-3} M DIDS, 10^{-3} M SITS or 10^{-3} M DNDS (Fig. 8). During the initial 40 sec of disulfonic stilbene perfusion (interval 3, Fig. 8*A*), 10^{-3} M DIDS and SITS caused significant cell shrinking while 10^{-4} M DIDS or 10^{-3} M DNDS had no effect (Fig. 8*B*). Neither the rate of K^+-induced swelling (intervals 1 and 4) nor the rate of shrinking (intervals 2 and 5) was changed by any of the disulfonic stilbenes. The average rate of swelling was 100 ± 9 nm/sec and the average rate of shrinking was −85 ± 8 nm/sec (n = 27).

Macropatch Recordings

Apical membrane currents had characteristics as previously reported [12]. When the pipette contained NaCl

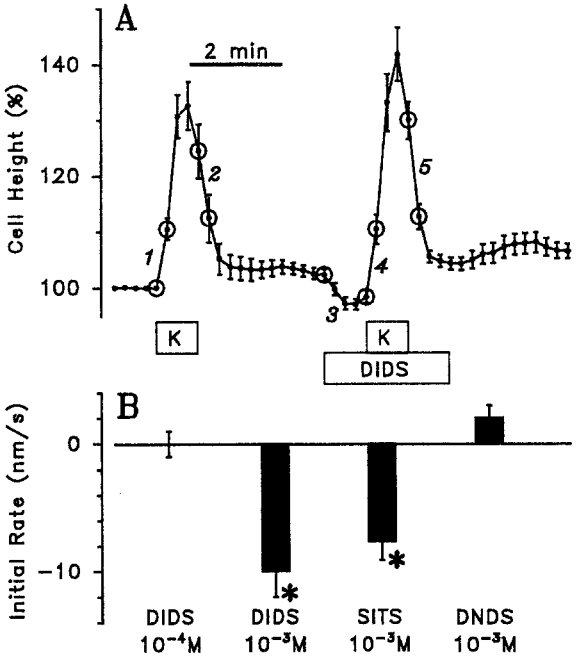

Fig. 8. Effect of DIDS (1 mM) on cell height. (*A*) summary of experiments in which DIDS was perfused during steady-state conditions prior to K^+-induced cell swelling. Numbers indicate intervals during which rates of cell swelling (1 and 4) and shrinking (2, 3 and 5) were measured. The rate of shrinking during interval 3 from this and similar experiments with SITS and DNDS are summarized in part (*B*).

solution (solution 4, Table), the currents were outward at holding potentials between −20 and +40 mV and these currents activated slowly over several seconds (*data not shown*), (Fig. 1) and inactivated about 10 times faster upon hyperpolarizing the membrane with a holding voltage of −40 mV. Similar observations were made when the pipette contained KCl solution (solution 5, Table), although the currents were inward as previously reported [12].

Voltage Protocol 1

With NaCl solution in the pipette, addition of 10^{-3} M DIDS to the bath caused the outward current across the apical membrane (I_a) at a holding potential of 0 mV to significantly decrease from 25.2 ± 3.2 to 1.3 ± 0.5 pA and return to 37.1 ± 9.8 pA upon washout of the DIDS (n = 11) (Fig. 9*A*). Concurrently, G_a was also significantly decreased from 449 ± 76 to 166 ± 43 pS and returned to 415 ± 50 pS upon washout of DIDS (n = 9) (Fig. 9*A*). In a separate series of experiments with 10^{-3} M DIDS in NaCl pipette solution (solution 4), I_a and G_a were initially significantly greater than in the previous series in the absence of DIDS in the pipette (Fig. 9*B*). Addition of 10^{-3} M DIDS to the bath significantly decreased I_a from 54.5 ± 9.0 to 14.8 ± 4.6 pA (n = 5) and G_a from 628 ± 28 to 186 ± 22 pS (n = 5). I_a with DIDS in both the

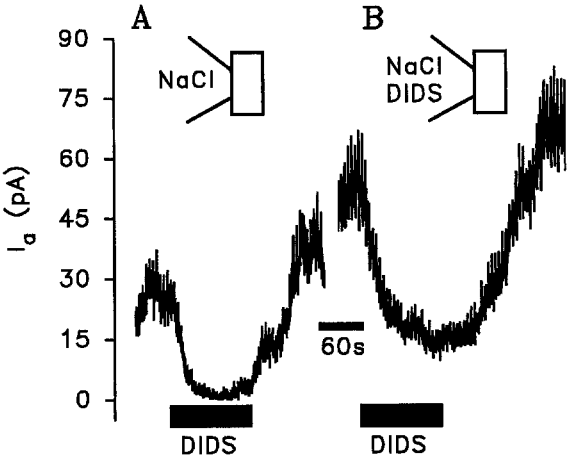

Fig. 9. Representative macropatch recordings of apical membrane current (I_a) and conductance (G_a) in the absence and presence (filled bar) of bath DDS (1 mM). NaCl pipette solution. (*A*) absence of DIDS (1 mM) in the pipette; (*B*) presence of DIDS (1 mM) in the pipette.

pipette and bath was significantly greater than with DIDS only in the bath; the difference in G_a did not reach statistical significance.

Voltage Protocol 2

This protocol provided more detailed information about the apical pathway stimulated by DIDS. I_0, g_a and V_r were obtained in 15-sec intervals during paired measurements in the absence and presence of DIDS in the pipette. With NaCl pipette solution, pipette perfusion of DIDS (10^{-3} M) for 8 min significantly increased I_0 from 24.1 ± 15.8 to 86.4 ± 22.6 pA, g_a from 377 ± 120 to 1366 ± 288 pS, and shifted V_r from −24.1 ± 6.1 to −61.4 ± 3.8 mV (n = 5) (Figs. 10 and 12). All effects were reversible. I_0-I_{sK} was not significantly different from 0 during the periods of control and DIDS perfusion (6.2 ± 2.8 and 12.8 ± 5.7 pA).

With KCl pipette solution, pipette perfusion of DIDS (10^{-3} M) for 8 min significantly increased I_0 from −5.9 ± 1.7 to −24.1 ± 6.9 pA and g_a from 989 ± 171 to 6003 ± 1637 pS (n = 5). V_r was not significantly changed (6.0 ± 1.6 vs 5.4 ± 1.1 mV, n = 5) (Figures 11 and 12). All effects were reversible. I_0-I_{sK} was not significantly different from 0 during the periods of control and DIDS perfusion (3.5 ± 1.8 and 5.9 ± 4.0 pA).

Discussion

It was a surprising observation that an agent which has mostly been used as a tool to inhibit anion transport processes [3, 4, 19, 21] activated a transport mechanism in a K^+ secretory epithelium. The site of action was apparently at the apical membrane and not at basolateral

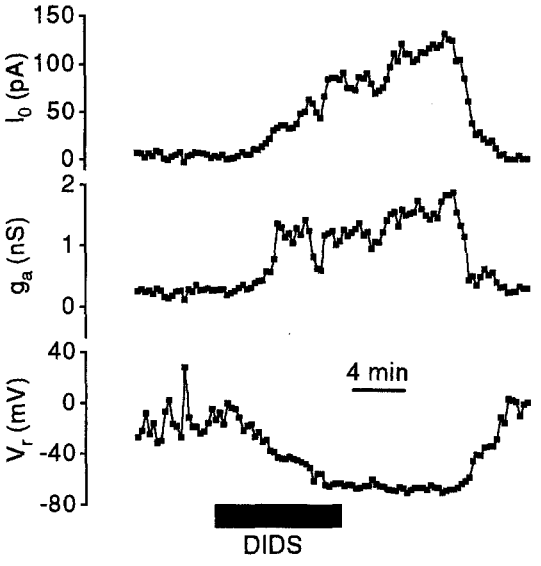

Fig. 10. Representative time course of the changes in the steady-state apical membrane current at a holding potential of 0 mV (I_0) and the "instantaneous" conductance (g_a) of the apical membrane at the reversal voltage (V_r). g_a and V_r were derived from the tail currents illustrated in Fig. 1. At the horizontal bar, DIDS (1 mM) was added to the NaCl pipette solution (solution 4, Table).

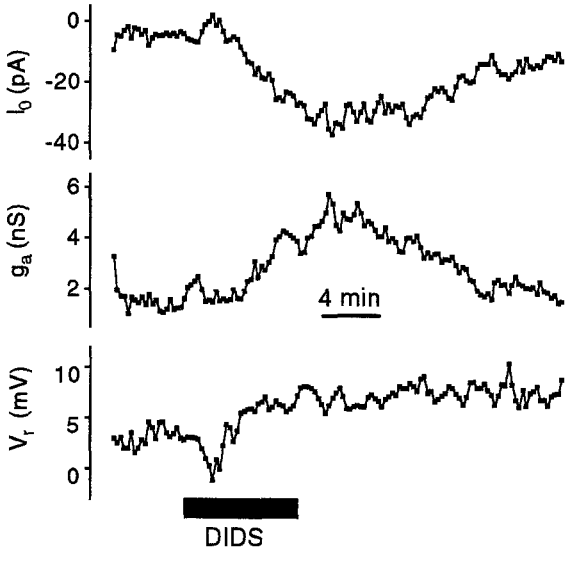

Fig. 11. Representative time course of the changes in the steady-state apical membrane current at a holding potential of 0 mV (I_0) and the "instantaneous" conductance (g_a) of the apical membrane at the reversal voltage (V_r). g_a and V_r were derived from the tail currents as obtained in Fig. 1. At the horizontal bar, DIDS (1 mM) was added to the KCl pipette solution (solution 5, Table).

or paracellular pathways since only apical perfusion caused the observed effects. The ability of DIDS and SITS to modify lysine residues might contribute to these effects because DNDS, which differs from DIDS and SITS in lacking a reactive thiocyanate group, was completely without effect [4].

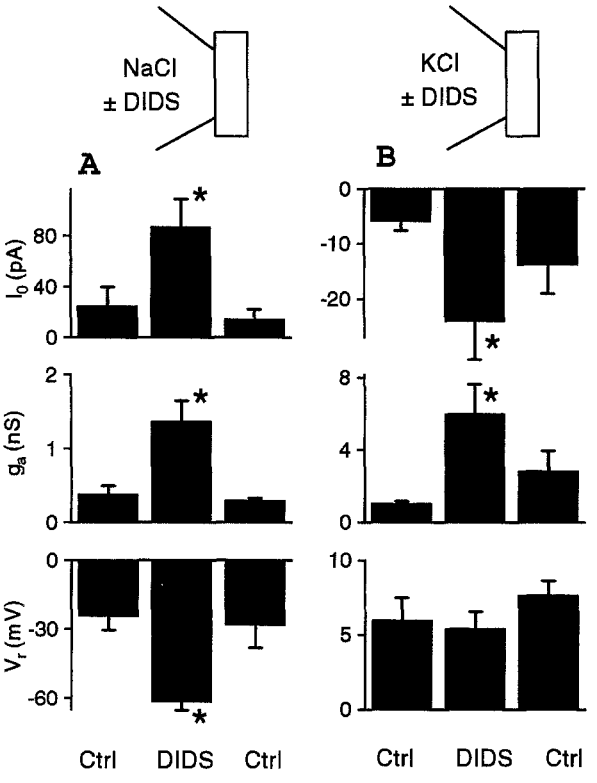

Fig. 12. Summary of experiments depicted in Figs. 10 and 11. (*A*) average values of I_0, g_a and V_r in the absence (Ctrl) and presence (DIDS) of 1 mM DIDS in NaCl pipette solution. (*B*) average values of I_0, g_a and V_r in the absence (Ctrl) and presence (DIDS) of 1 mM DIDS in KCl pipette solution. *, $P < 0.05$.

We had previously observed that stilbenes acted on the apical membrane of vestibular dark cells by increasing the transepithelial voltage and decreasing the resistance [10]. In the present study, we extended these finding by demonstrating that this increase in conductance also occurred in the presence of apical K^+ and in the absence of Cl^-. The reduction of the response of V_t to DIDS in the presence of high apical K^+ suggested that DIDS had acted by activation of an apical K^+ channel.

Two additional findings demonstrated that the increase in transepithelial conductance by DIDS had stimulated a transepithelial flux. It was found that DIDS caused a net solute efflux (decrease in cell height), consistent with an activated solute efflux pathway. Further, $J_{K,probe}$ was found to be stimulated by DIDS, showing that this increased rate of solute exit was indeed an activation of transepithelial K^+ secretion.

The known transport pathways in the apical membrane of vestibular dark cells are the I_{sK} channel [12], a nonselective cation channel [14] and a maxi-K^+ channel [20]. The nonselective cation channel and the maxi-K^+ channel are thought to not be involved in constitutive K^+ secretion due to their low density and to their activation by relatively high levels of intracellular Ca^{2+}. The non-

selective cation channel in vestibular dark cells was found to be insensitive to extracellular DIDS [14], although DIDS has been found in another cell type to activate from the cytosolic side a nonselective cation channel of similar conductance [6]. The maxi-K^+ channel is also known to be insensitive to DIDS from the extracellular side [20] although it can activate from the cytosolic side [5].

The sensitivity of the I_{sK} channel to DIDS has only recently been described for human I_{sK} protein expressed in Xenopus oocytes [2]. DIDS was found to activate the I_{sK} current in that preparation as in vestibular dark cells. To our knowledge, the only other direct activation of a K^+ channel by DIDS occurs in hepatocytes [26]. In contrast to vestibular dark cells, DIDS was found to be taken up in hepatocytes by the bilirubin transporter and DIDS exerted its effect from the cytosolic side.

Rather than a direct action on one of these types of channel, it is conceivable that DIDS exerted its effects indirectly by inhibiting another transporter which in turn altered the cytosolic composition in a way that activated one of these channels. In particular, it was hypothesized that there may be an apical Cl^-/HCO_3^- exchanger whose inhibition might have altered the cytosolic pH which subsequently would have activated conductive elements in the apical membrane. This hypothesis was tested with both transepithelial and patch clamp measurements.

To address the question of direct *vs.* indirect activation of the apical pathway as well as to show that the effect of DIDS was on the constitutive K^+ secretory pathway rather than on some other transport mechanism, we partitioned the apical membrane with a macropatch pipette and applied DIDS to the membrane outside the pipette, inside the pipette or to the entire apical membrane. If DIDS had acted via a cytosolic intermediary, it was expected that application of DIDS to the apical membrane outside the patch seal would have altered the cytosolic intermediary and thereby increased the current across the seal (as well as the current across the membrane outside the seal, which did not contribute to the measurements). By contrast, the membrane current decreased upon application of DIDS outside the patch pipette (Fig. 9), consistent with the notion that DIDS directly stimulated a K^+-secretory pathway. Further support was obtained from experiments in which addition of DIDS to the pipette solution increased the patch current and this increased current was also reduced by addition of DIDS to the bath. Inclusion of DIDS within the pipette always led to the expected increase in membrane current, while DIDS outside the seal always led to a decrease in membrane current across the patch.

These findings are most easily interpreted as indicating a limited capacity of the basolateral membrane to take up K^+. When DIDS was applied within the pipette, the I_{sK} channel was directly activated, as indicated by the parallel increase in I_0 and g_a. Activation of membrane outside of the patch by DIDS applied to that region of the membrane increased the current outside of the patch, shunting some of the limited available current away from the measured current passing into the pipette.

These results are strong evidence for the direct activation of the I_{sK} channel by DIDS and the resulting stimulation of K^+ secretion by vestibular dark cells. The findings do not, however, rule out the possibility that there is another membrane component which colocalizes with the I_{sK} channels and is affected by DIDS and which in turn activates the I_{sK} channels. One intriguing possibility is that the recently-discovered P_{2U} receptor on the apical membrane of these cells which mediate a decrease in I_{sc} [8] may be closely associated with the I_{sK} channels. DIDS might then be an inhibitor of this receptor, as found for some other P_2 receptors [1, 18], which would then relieve the negative effect of the presence of a putative local source of nucleotide at this receptor.

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