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Nonselective Cation and BK Channels in Apical Membrane of Outer Sulcus Epithelial Cells

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Abstract. The outer sulcus epithelium was recently shown to absorb cations from the lumen of the gerbil cochlea. Patch clamp recordings of excised apical membrane were made to investigate ion channels that participate in this reabsorptive flux. Three types of channel were observed: (i) a nonselective cation (NSC) channel, (ii) a BK (large conductance, maxi K or K_{Ca}) channel and (iii) a small K^+ channel which could not be fully characterized. The NSC channel found in excised inside-out patch recordings displayed a linear current-voltage (*I-V*) relationship (27 pS) and was equally conductive for Na^+ and K^+, but not permeable to Cl^- or N-methyl-D-glucamine. Channel activity required the presence of Ca^{2+} at the cytosolic face, but was detected at Ca^{2+} concentrations as low as 10^{-7} M (open probability (P_o) = 0.11 ± 0.03, n = 8). Gadolinium decreased P_o of the NSC channel from both the external and cytosolic side (IC_{50} ~ 0.6 μM). NSC currents were decreased by amiloride (10 μM – 1 mM) and flufenamic acid (0.1 mM). The BK channel was also frequently (38%) observed in excised patches. In symmetrical 150 mM KCl conditions, the *I-V* relationship was linear with a conductance of 268 pS. The Goldman-Hodgkin-Katz equation for current carried solely by K^+ could be fitted to the *I-V* relationship in asymmetrical K^+ and Na^+ solutions. The channel was impermeable to Cl^- and N-methyl-D-glucamine. P_o of the BK channel increased with depolarization of the membrane potential and with increasing cytosolic Ca^{2+}. TEA (20 mM), charybdotoxin (100 nM) and Ba^{2+} (1 mM) but not amiloride (1 mM) reduced P_o from the extracellular side. In contrast, external flufenamic acid (100 μM) increased P_o and this effect was inhibited by charybdotoxin (100 nM). Flufenamic acid inhibited the inward short-circuit current measured by the vibrating probe and caused a transient outward current. We conclude that the NSC channel is Ca^{2+} activated, voltage-insensitive and involved in both constitutive K^+ and Na^+ reabsorption from endolymph while the BK channel might participate in the K^+ pathway under stimulated conditions that produce an elevated intracellular Ca^{2+} or depolarized membrane potential.

Key words: BK channel — Nonselective cation channel — Cation absorption — Inner ear epithelium — Patch clamp — Vibrating probe — Gerbil

Introduction

The response of the cochlea to sound depends on the modulation of a standing current through the hair cells. This current is predominantly carried by K^+ ions from the luminal compartment, which has a content of about 160 mM K^+ and 1 mM Na^+. Maintenance of these cation levels is important for normal cochlear function (Marcus, 1997). K^+ is secreted into the lumen from the lateral wall by the stria vascularis while cations leave the lumen through the hair cells and the parasensory outer sulcus epithelium (Marcus & Chiba, 1999).

Absorptive transport of cations was demonstrated in the outer sulcus by measurements of relative short-circuit current with a vibrating current-density probe. This current was inhibited by gadolinium (Gd^{3+}) and by ouabain, the latter demonstrating its dependence on Na^+,K^+-ATPase activity. Nonselective cation (NSC) channels were found in the apical membrane that were also sensitive to high concentrations of Gd^{3+}, suggesting that these channels participated in the transepithelial absorption of cations (Marcus & Chiba, 1999).

In the present study, we looked in some detail at the characteristics of the NSC channels and investigated other channels also present in the apical membrane. The

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Table. Composition of bath and pipette solutions (mM)

Solution	Bath								Pipette		
	B1	B2	B3	B4	B5	B6	B7	B8	P1	P2	P3
NaCl	150	150						115	150		
KCl	3.6		150		150			35		150	150
$MgCl_2$	1	1	1	1	1			1	1	1	1.04
$Mg(SO_4)_2$						1	1				
$CaCl_2$	0.7	0.7	0.7	0.7				0.7	0.7	0.7	0.272
Glucose	5										
HEPES	10	10	10	10	10	10	10	10	10	10	10
EGTA					1						1
Na-gluconate						150					
K-gluconate							150				
Ca-gluconate							4				
NMDG-Cl				150							
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.2

NMDG: N-methyl-D-glucamine

NSC channels were found to be sensitive to Gd^{3+} from both sides of the membrane, insensitive to voltage and stimulated by cytosolic Ca^{2+}. Another channel permeable to K^+ was observed that had the characteristics of a large-conductance BK channel.

Materials and Methods

Preparation

Outer sulcus epithelium varied in its features according to location along the place-frequency map of the cochlea (Spicer & Schulte, 1996). In the 0.5–2 kHz frequency region of the cochlea, the Claudius cells and spiral prominence epithelium are separated by outer sulcus epithelial cells that border the luminal (endolymphatic) surface. Gerbils were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and sacrificed using a protocol approved by the Institutional Animal Care and Use Committee. The temporal bone was removed and spiral ligament from the 2nd and 3rd turn was dissected at 4°C as described previously (Marcus & Chiba, 1999). A piece of spiral ligament containing outer sulcus cells and stria vascularis was folded into a loop such that the outer border was formed by the apical membrane of the outer sulcus cells and stria vascularis. This tissue was mounted in a bath chamber on an inverted microscope (Nikon, Diaphot). The edge of the fold was brought into focus so that gigohm seals could be formed between the patch pipette and the apical membrane under visual control. The stria vascularis was removed for measurements of short-circuit current with the vibrating probe.

Data Acquisition and Analysis

Patch clamp recordings were made using methods previously established in this laboratory (Marcus et al., 1992; Takeuchi, Marcus & Wangemann, 1992*a*). Patch pipettes were pulled in two stages from borosilicate glass capillaries (TW150-4, WPI, Sarasota), heat polished, filled with electrolyte solution (P1, P2 or P3, Table) and had a resistance of around 10 MΩ. High-resistance seals (2–10 GΩ) were formed between the patch pipette and the apical membrane of outer sulcus epithelial cells. The pipette was connected to a patch amplifier (Dagan 3900 or Axon Instruments 200A). Recordings were made at room temperature or at 37°C, as indicated in the Results, with continuous perfusion at a rate which exchanged the bath 0.8 times/sec.

The polarity of the voltage of excised membrane patches was that of the cytosolic face with respect to the extracellular face. Outside-out patches were formed by first establishing the whole-cell configuration followed by excision of the membrane. Since the procedure for forming inside-out patches can occasionally lead to the formation of an outside-out patch (e.g., if the cell membrane ruptures spontaneously just prior to excision), the configuration was checked by application of low-Ca^{2+} solution in the bath prior to testing of putative inhibitors. Both NSC and BK channels were found to be activated by Ca^{2+} and inactivated by low Ca^{2+} at the cytosolic face (vide infra).

The conductance (g) of single channel current-voltage (I-V) relationships was derived from the slope of a least squares fit to linear I-V functions. The corresponding permeabilities were estimated as described previously (Marcus et al., 1992). The permeabilities under conditions leading to nonlinear I-V relationships were determined by fitting the data using a nonlinear regression algorithm to the Goldman-Hodgkin-Katz (GHK) current equation. Corrections were applied to the measured voltages during ionic replacement experiments to compensate for liquid junction potentials that were obtained by measurements against a flowing saturated KCl electrode. Open probabilities were calculated as fractional open times.

Vibrating probe measurements were carried out as before (Marcus & Chiba, 1999). Briefly, current density ($I_{sc,probe}$) was monitored by vibrating a platinum-iridium wire microelectrode with a Pt black tip for an excursion of about 20 μm along both a horizontal (X) and vertical (Z) axis. The X-axis was perpendicular to the face of the epithelium. The probe was positioned ~10 μm from the apical surface of the epithelium with computer-controlled, stepper-motor manipulators (Applicable Electronics, Forestdale, MA) and specialized probe software (ASET version 1.0, Science Wares, East Falmouth, MA). The signal from the probe was led to the input of a dual channel phase-sensitive detector. The bath references were 26-gauge Pt-black electrodes.

Calibration was performed in control solution (B1, Table) using a glass microelectrode (tip <1 μm OD) filled with 3 M KCl as a point source of current (Scheffey, Shipley & Durham, 1991). The frequencies of vibration were in the range of 200–800 Hz and were well

separated for the 2 orthogonal directions. The signals from the two oscillators driving the probe served as references for the dual detectors. The quadrature signal of each channel was minimized by adjusting the driving frequency to be near resonant. Asymmetry of the probe design yielded different resonant frequencies for the two directions of vibration. The signals of the *X* and *Z* detectors were connected to a 16-bit analog-to-digital converter (CIO-DAS1602/16, ComputerBoards, Mansfield, MA) in a 233 MHz pentium computer. Recordings were made at room temperature by averaging samples taken for 200 msec at 200 Hz with 20 msec between averaging periods. The electrode was positioned near the center of the fold in the epithelium such that the *Z* component was typically small compared to the *X* component. The *X* component, rather than the magnitude of the vector, is shown in order to preserve the sign of the current.

SOLUTIONS AND DRUGS

Tissues were initially bathed in solution B1 (Table). Solutions were made with different free Ca^{2+} concentration in order to determine the sensitivity of the NSC and BK channels to Ca^{2+} at their cytosolic face. For NSC channels, Ca^{2+} and Mg^{2+} concentrations were varied in solution B2 in order to obtain the free Ca^{2+} concentrations desired while maintaining Mg^{2+} at 1 mM (Schoenmakers et al., 1992). For the determination of the dependence of the BK channel on the intracellular free calcium concentration, the bath solutions contained 90 mM KCl, 10 mM HEPES, 120 mM mannitol, were buffered by either 1 mM NTA (for Ca^{2+} concentrations from 10^{-6} to 10^{-5} M) or 1 mM EGTA (for Ca^{2+} concentrations from 10^{-8} to 10^{-7} M), pH 7.4 (Takeuchi et al., 1992*a*). The final Ca^{2+} activity was checked in each solution with a Ca^{2+}-selective microelectrode (WPI, Sarasota, FL). The monovalent cation concentration was lower than that used in other solutions in order to reduce interference with the Ca^{2+} electrode measurements, thereby increasing our confidence in the calibration procedure. Amiloride and flufenamic acid were predissolved in dimethylsulfoxide (DMSO, Sigma, St. Louis, MO) to a final concentration of 0.1% DMSO. Barium, charybdotoxin, gadolinium (Gd^{3+}) and tetraethylammonium chloride (TEA-Cl) were dissolved directly into aqueous solution. Charybdotoxin, amiloride, flufenamic acid and TEA-Cl were purchased from Sigma and barium chloride from Fisher Scientific (Fair Lawn, New Jersey). All other chemicals were purchased from Sigma or Fluka (Ronkonkoma, NY).

DATA PRESENTATION AND STATISTICS

All data in the text are given as mean ± SEM. The number of observations (*n*) is equal to the number of cells. Student's *t*-test of paired and unpaired samples, as appropriate, was applied and a level of $P < 0.05$ taken as statistically significant.

Results

Single channel events were observed in both the on-cell and excised patch configuration. Previously, nonselective cation (NSC) channels were found to carry much of the transepithelial current and an example recording with resulting *I-V* relationship was shown (Marcus & Chiba, 1999). In the present study, on-cell recordings of NSC channels with NaCl pipette solution (P1, Table) showed a single-channel conductance of 28.2 ± 0.7 pS ($n = 18$). The NSC channel was characterized more fully in excised patches. It was also observed that large conductance K^+ channels were in many of the excised patches. The large K^+ channel was found to be of the BK (maxi K; K_{Ca}) channel type from its voltage, Ca^{2+} and drug sensitivities.

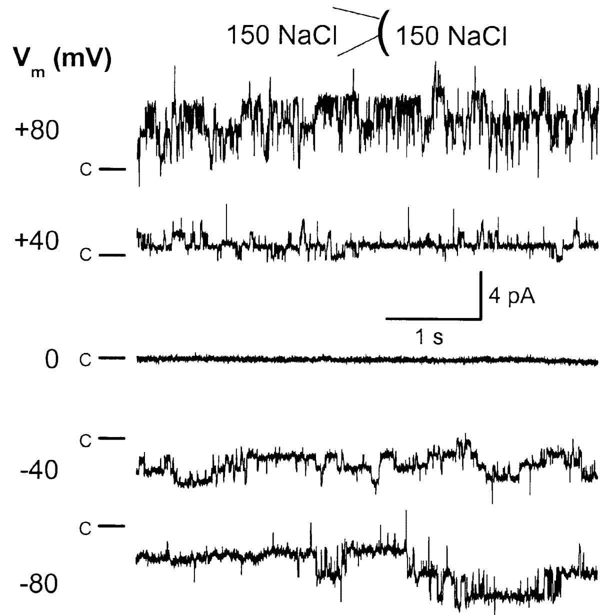

Fig. 1. Single channel recordings of the nonselective cation (NSC) channel in excised inside-out patches. Recordings were made from apical membrane of an outer sulcus epithelial cell under symmetrical NaCl conditions at 37°C. V_m, membrane potential = $-V_p$ (pipette potential); C, closed channel level.

NONSELECTIVE CATION CHANNEL

In 52 out of 70 (74%) excised apical membrane patches, single-channel activity of NSC channels was observed. Figure 1 shows a typical recording of NSC channel activity in an excised inside-out patch with 150 mM NaCl in both the pipette and the bath (P1, B2; Table). Current transitions indicated that the patch contained at least two individual channels. Patches often exhibited more than one current level (3.0 ± 0.2 channels/patch, $n = 52$), although this number was derived from traces in which distinct current level transitions could be discerned. There also were many recordings which appeared to be a relatively smooth continuum due to a larger number of channels in the patch. The *I-V* relationships of single channels in outside-out patches were linear with NaCl in the pipette and either NaCl, KCl or Na^+-gluconate in the bath (Marcus & Chiba, 1999). The P_{Na}/P_K ratio was 0.96 ± 0.02 ($n = 6$), indicating that the channel did not discriminate between K^+ and Na^+.

The channel was found to be impermeable to the large cation N-methyl-D-glucamine$^+$ (NMDG). *I-V* rela-

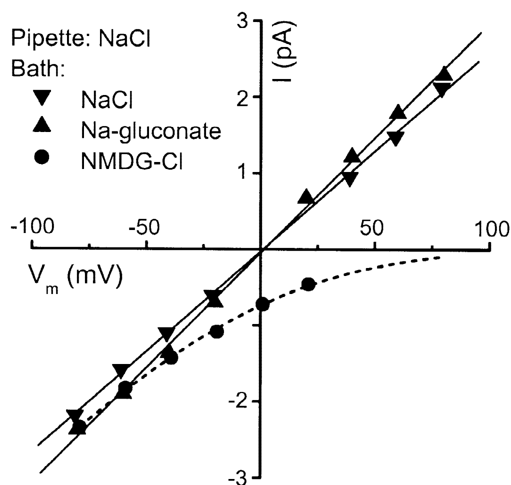

Fig. 2. Representative *I-V* relationships of NSC channel in excised inside-out patch recordings. The pipette contained 150 mM NaCl (P1, Table) and the bath NaCl (B2, Table), Na-gluconate (B6, Table) or NMDG-Cl (B4, Table). The *I-V* relationship was linear with Na^+ in the bath (28 pS) but nonlinear with NMDG. The dotted curve is the best fit to the Goldman-Hodgkin-Katz current equation for Na^+.

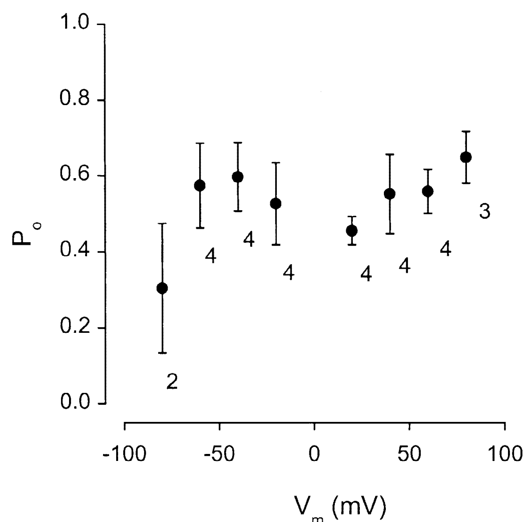

Fig. 3. Effect of membrane voltage (V_m) on the open probability (P_o) of the NSC channel. Mean ± SEM (*n*).

tionships obtained at 37°C when Na^+ in the bath was substituted by NMDG (P1, B4; Table) could be closely fitted by the Goldman-Hodgkin-Katz (GHK) equation under the assumption that Na^+ was the only conducting ion species (Fig. 2). When the assumption was relaxed to include NMDG as a permeant species, the GHK equation yielded a permeability ratio $[P_{NMDG})/P_{Na}]$ of 0.09 ± 0.03 (n = 7), confirming the very low permeability of NMDG.

The open probability of the NSC channel was measured in symmetrical NaCl (P1, B2; Table) at 37°C over a range of applied voltages and found to not be correlated with the voltage (Fig. 3). This finding is consistent with the on-cell observations that this channel is active under near-physiologic conditions of a highly polarized membrane.

By contrast to voltage, cytosolic Ca^{2+} was found to be a strong activator of the NSC channel. Eight excised inside-out patches at 37°C with symmetrical NaCl (P1, B2; Table) were highly active when excised into a bath of 0.7 mM Ca^{2+}. The channel activity was decreased when the Ca^{2+} concentration at the cytosolic face (bath) was reduced to 10^{-5} M or less (Fig. 4). Strikingly, significant channel openings (P_o = 0.11 ± 0.03, n = 8) were still observed at 10^{-7} M, a level typical of resting cells. These observations demonstrate that intracellular Ca^{2+} activated this NSC channel, but that the Ca^{2+} threshold for channel opening was in the physiologic range.

Our previous report (Marcus & Chiba, 1999) demonstrated that high levels (1 mM) of Gd^{3+} inhibited both cation reabsorption by the outer sulcus epithelial region measured with the vibrating probe technique and NSC channel currents. It was of interest to learn the sensitivity of the current to Gd^{3+} and if Gd^{3+} blocked more strongly from one side of the membrane or the other.

The effect of Gd^{3+} was tested (P1, B2; Table) at 37°C on excised outside-out patches with a high NSC channel activity. Gd^{3+} inhibited NSC channel current in a concentration-dependent manner within the range from 10^{-7} M to 10^{-3} M (Fig. 5*A*). The half-maximal inhibitory concentration (IC_{50}) of Gd^{3+} on the NSC channel from the extracellular side was estimated from a fit to the Hill equation to be 5.8 × 10^{-7} M (n = 8). Gd^{3+} also inhibited NSC channel current from the cytosolic side in a concentration-dependent manner (Fig. 5*B*). The IC_{50} of Gd^{3+} on the NSC channel from the cytosolic side was estimated to be 6.0 × 10^{-7} M (n = 12). The data were from patches held between −40 to −80 mV; the mean current in the presence of Gd^{3+} was normalized to that in the absence of Gd^{3+} for each patch.

Amiloride is known to inhibit a variety of cation transport processes and was tested for its effect on the NSC channels in the apical membrane of outer sulcus epithelial cells. In symmetrical NaCl (P1, B2; Table) at 37°C, amiloride reduced the NSC current although not as strongly as Gd^{3+}; the effects were reversible (Fig. 6*A*). Amiloride at 10^{-5} M inhibited the mean current of outside-out patches to 75.1 ± 5.9% (n = 11) of the control level at membrane potentials between −60 and +60 mV. Amiloride at 1 mM inhibited the current to 59.0 ± 8.4% (n = 11) of the control level while 100 μM Gd^{3+} in the same series of experiments reduced the current to 35.8 ± 10.5% of control (Fig. 6*B*).

The effect of flufenamic acid on NSC channel currents was investigated in outside-out patches devoid of BK channels or in which BK channels had been inacti-

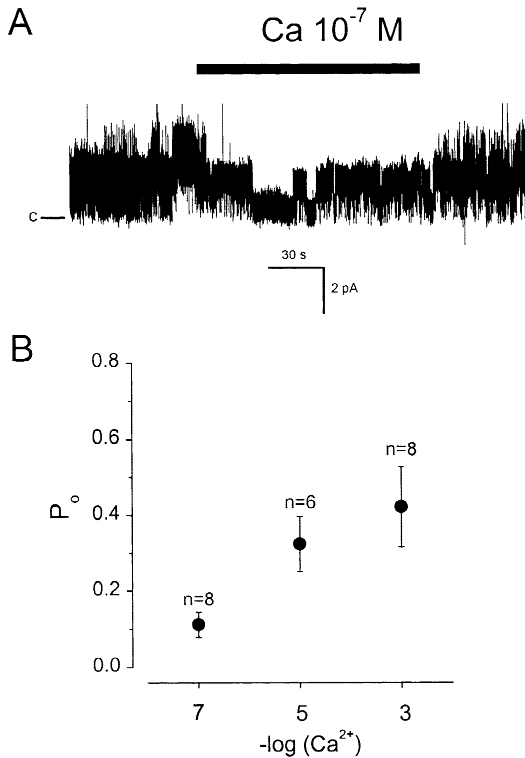

Fig. 4. Activation of NSC channel by cytosolic calcium. (*A*) Representative recording was made from inside-out excised patch under symmetrical NaCl conditions at a membrane potential of +40 mV. Three channels were open under initial conditions of 0.7×10^{-3} M cytosolic Ca^{2+} but 10^{-7} M cytosolic Ca^{2+} decreased the open probability. (*B*) Summary of Ca^{2+}-dependence of P_o. Channel activity was still significant at a calcium activity of 10^{-7} M. Mean ± SEM (*n*).

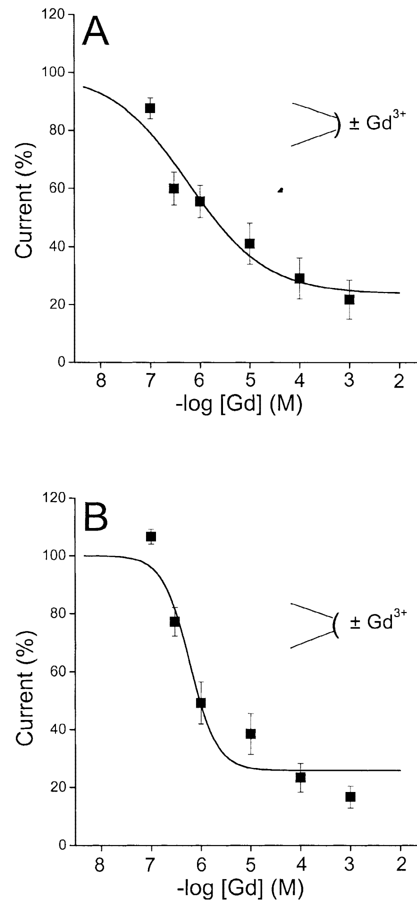

Fig. 5. Inhibition of NSC current by bath gadolinium (Gd^{3+}). *Top panel,* outside-out patch (*n* = 8); *bottom panel,* inside-out patch, *n* = 12. Membrane voltage −40 to −80 mV; currents normalized to value before addition of Gd^{3+}. Curves are best fit to Hill equation with an offset term to account for leak currents. Mean ± SEM.

vated by a combination of low [Ca^{2+}] at the cytosolic face (P3, Table) and negatively polarized membrane potentials. A recording from a membrane patch with low Ca^{2+} at the cytosolic face is shown in Fig. 7*A*. The currents at all membrane voltages between −90 and +90 mV were reduced by flufenamic acid (100 μM). The augmented current seen at large positive voltages (Fig. 7*A* and *B*) is likely due to an additional K^+ conductance in the membrane which is also inhibited by flufenamic acid. Although the slow kinetics of the additional component are suggestive of the IsK/KvLQT1 K^+ channel complex (Marcus & Shen, 1994), that channel, by contrast, is stimulated by fenamates (Busch et al., 1994).

The ionic species responsible for the inward current inhibited by flufenamic acid was investigated by holding the membrane at −90 mV with either Na^+, K^+ or NMDG as the primary cation in the bath. In the absence of flufenamic acid, the mean current at room temperature was 26.1 ± 5.6 pA (*n* = 5) when the current was carried by Na^+ (B2, Table), 18.9 ± 4.6 pA (*n* = 6) when the current was carried by K^+ (B3, Table; no significant difference to Na^+) and reduced to 4.8 ± 1.9 pA (*n* = 6) when NMDG (B4, Table) was the primary cation in the bath. Flufenamic acid (0.1 mM) reduced the mean current when the current was carried by Na^+ or K^+ from the bath but not when the bath contained NMDG as the primary cationic species (Fig. 7*C*).

BK Channel

BK channels were identified by their large single-channel amplitude when there was a high concentration of K^+ available to carry the current. In 58 excised apical membrane patches, BK channel activity was observed in 22 patches (38%) at 37°C. An average of 1.9 ± 0.2 (*n* = 22) channel levels were found per active patch. No channel activity was observed for the BK channel in on-cell patch recordings with the pipette potential in the range of ±60 mV.

Excised Patch Recordings

Patches often exhibited more than one current level of equal magnitude, which most likely represented multiple

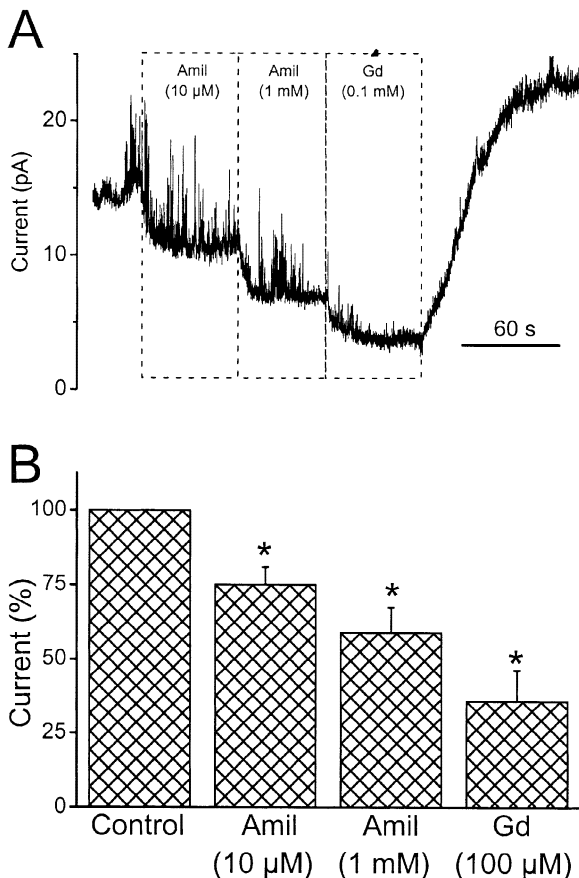

Fig. 6. Summary of effect of amiloride (10^{-5} and 10^{-3} M) at the extracellular face on NSC channel currents. Outside-out membrane patches were exposed to symmetrical NaCl (P1, B2, Table). The effects of amiloride were compared to that of gadolinium (Gd^{3+}, 100 μM). (*A*) Recording of patch current at +60 mV. Effects were reversible during washout after Gd^{3+}. (*B*) Summary of results from 11 patches held at −60 mV (n = 5), −40 mV (n = 4) and +60 mV (n = 4); mean ± SEM, n = 11. *, $P < 0.05$.

channels per patch rather than multiple-state channels. However, distinct subconductance states were occasionally observed. Channel activities in inside-out patches observed at every membrane potential between −40 and +40 mV in the presence of 0.7 mM Ca^{2+} at the cytosolic face (P2, B3, Table) were abolished when the bath was perfused with low Ca^{2+} solution (B5, Table), demonstrating that the BK channel is Ca^{2+} dependent (Fig. 8).

The ion selectivity of these channels was determined by measurement of *I-V* relationships with K^+ on one side of the membrane and various monovalent cations on the other side (P2, B2/3/4/7/8, Table). When membrane patches were exposed to KCl on one side and either KCl or K-gluconate solution on the other side, the *I-V* relationship was linear (conductance 268 ± 20 pS (n = 9) and 294 ± 15 pS (n = 4) respectively, not significantly different) and passed nearly through the origin (reversal voltage 0.1 ± 1.2 mV (n = 9) and 2.5 ± 1.0 mV (n = 4), respectively, not significantly different; Fig. 9). The average permeabilities to K^+ under these conditions were found to be 6.6 ± 0.5 × 10^{-13} cm^3/sec and 7.3 × 10^{-13} cm^3/sec, respectively, which were not significantly different. The channel is therefore highly permeable to K^+ and does not significantly conduct anions.

To test for permeability to Na^+, the other prevalent monovalent cation, K^+ was replaced with Na^+ or NMDG. When the bath contained NaCl solution and the pipette KCl solution, the *I-V* relationship was described by the GHK current equation under the assumption that K^+ was the sole conductive ion (Fig. 9). The permeability of the channel to K^+ under these conditions averaged 7.1 ± 0.5 × 10^{-13} cm^3/sec (n = 9) which was not significantly different from the value obtained under symmetrical K^+ conditions. To get a good estimate of the permeability ratio of K^+ to Na^+, an intermediate condition was tested, 35 mM K^+ + 115 mM Na^+ in the bath (B8, Table) (Fig. 9). The mean permeabilities obtained by fitting the curve to the GHK current equation for K^+ and Na^+ were 7.0 ± 0.6 × 10^{-13} cm^3/sec (n = 5) and 6.0 ± 0.2 × 10^{-14} cm^3/sec, respectively, and permeability ratios of K^+ to Na^+ averaged 17.4 ± 5.8.

The observation that the channel was highly K^+ selective was further supported by substituting the large cation NMDG in the bath; the *I-V* relationship was indistinguishable from that with NaCl in the bath (Fig. 9). The average permeability to K^+ under these conditions was 6.4 ± 0.5 × 10^{-13} cm^3/sec (n = 4) which is not significantly different from those found for NaCl in the bath.

The open probability of the channel in inside-out patches was found to increase with membrane depolarization and with increasing free Ca^{2+} concentration at the cytosolic face. The free Ca^{2+} concentration in the bath was changed in decade steps from 10^{-8} M to 10^{-3} M in 4 patches. At every tested Ca^{2+} concentration the open probability increased monotonically with depolarization (Fig. 10). For membrane voltages between −60 and +40 mV the channel was regulated by a free Ca^{2+} concentration in the range of 10^{-6} to 10^{-5} M (Fig. 10).

Several agents known to inhibit BK channels were tested for their effect on the large conductance channel (Fig. 11). Addition of TEA (2 × 10^{-2} M) on the extracellular face of the membrane significantly reduced the open probability reversibly (n = 4; Fig. 11). No effect was observed when TEA (2 × 10^{-2} M) was applied to the cytosolic side (n = 4). Charybdotoxin (10^{-7} M), a known BK channel blocker, significantly decreased the open probability (n = 4; Fig. 11) from the extracellular side of the membrane but had no effect when added to the cytosolic side (P_o = 0.92 to 0.89, n = 1). The K^+ channel blocker barium (10^{-3} M) significantly reduced the open probabilities from both the cytosolic and extracellular sides (Fig. 11).

We further tested amiloride (10^{-3} M), which is known to cause a reduction of the basolateral K^+ conductance of outer sulcus cells (T. Chiba and D.C. Mar-

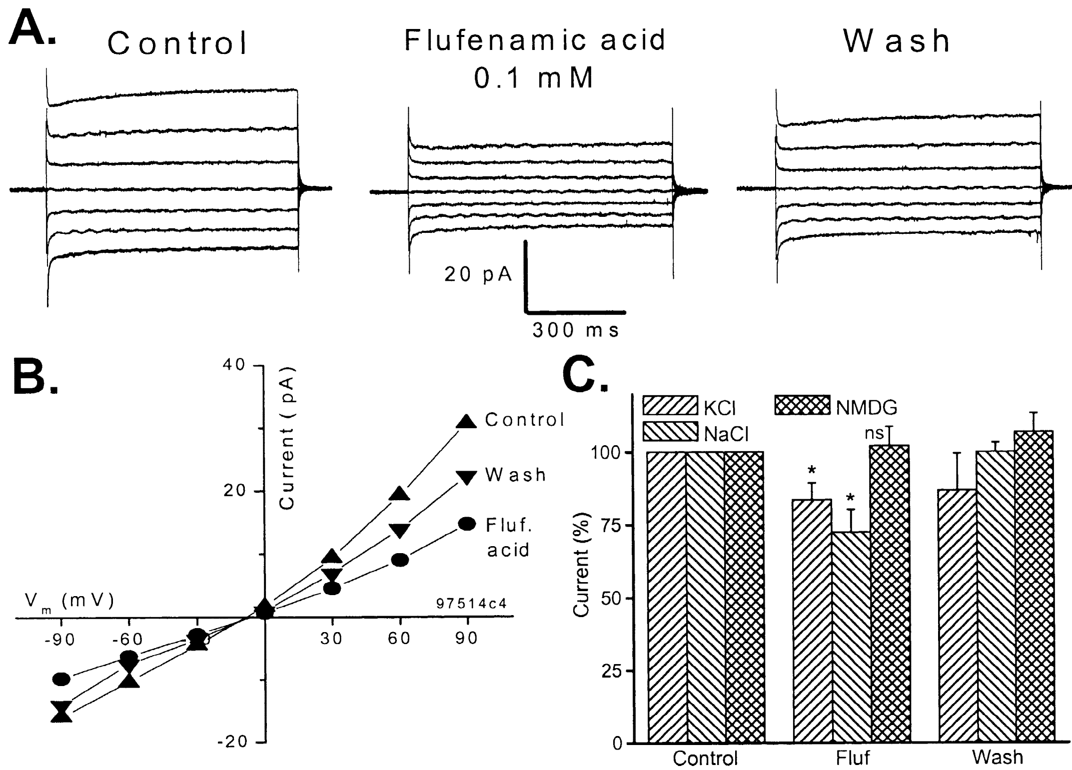

Fig. 7. Inhibition of NSC current by 0.1 mM flufenamic acid (Fluf). (*A*) recording of currents from an outside-out patch in the presence and absence of flufenamic acid. Membrane voltage was stepped from −90 to +90 mV in 30 mV increments for 800 msec each. (*B*) *I-V* relationships for steady-state currents in *A*. (*C*) Excised outside-out patches of apical membrane were held at −90 mV and the current recorded in the presence and absence of flufenamic acid with various cations in the bath. Currents expressed as percent of control, mean ± SEM (n = 5–6); *, $P < 0.05$; *ns*, not significant; not corrected for leak currents.

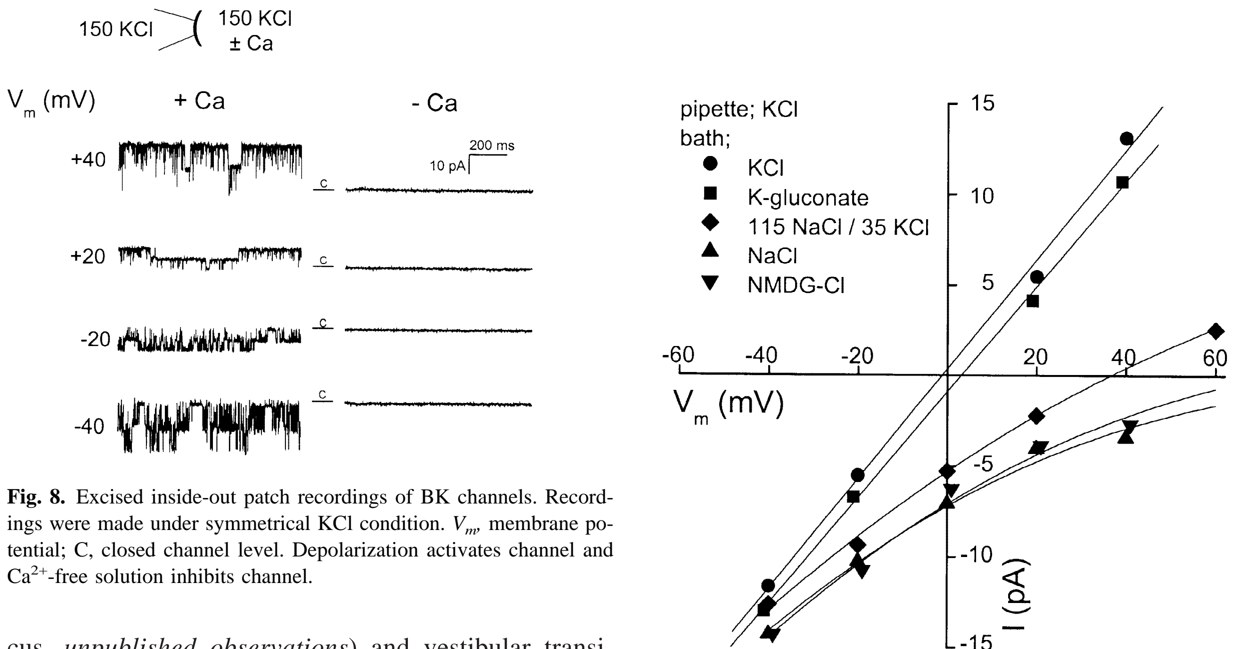

Fig. 8. Excised inside-out patch recordings of BK channels. Recordings were made under symmetrical KCl condition. V_m, membrane potential; C, closed channel level. Depolarization activates channel and Ca^{2+}-free solution inhibits channel.

cus, *unpublished observations*) and vestibular transitional cells (Wangemann & Shiga, 1994), on the extracellular side of the membrane. Amiloride (10^{-3} M) had no effect on the activity of the apical BK channels (n = 4; Fig. 11).

Fig. 9. *I-V* relationship of the BK channel with KCl in the pipette. Bath contained different ion compositions as indicated (*see* Table). Lines are best fit to Goldman-Hodgkin-Katz (GHK) current equation. All data shown from one representative membrane patch.

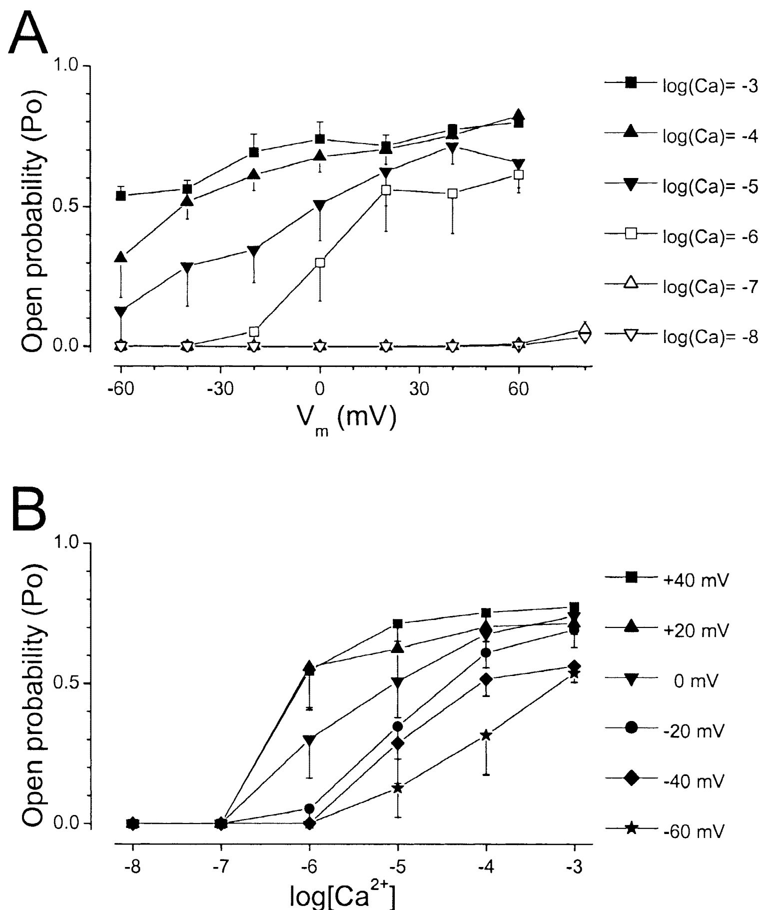

Fig. 10. Dependence of open probability (P_o) on (*A*) the membrane voltage or (*B*) the Ca^{2+} activity on the cytosolic side of the patch; mean ± SEM, $n = 4$.

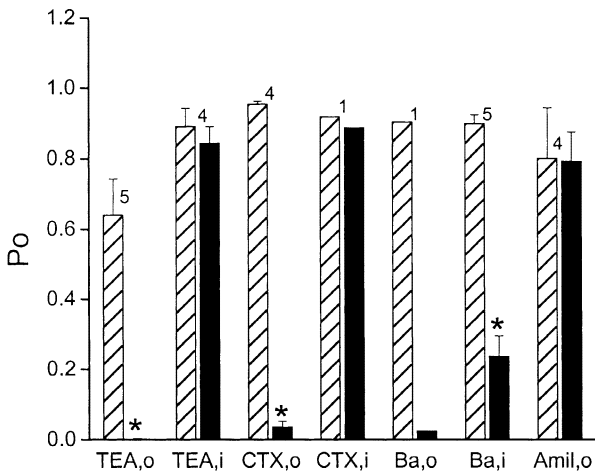

Fig. 11. Summary of pharmacological effects on BK channel. Hashed bars, control; solid bars, drug; *o,* outside; *i,* inside. Tetraethylammonium (TEA, 20 mM); charybdotoxin (CTX, 100 nM); Ba^{2+} (1 mM); Amiloride (amil, 1 mM). All recordings were made at 0 mV, NaCl pipette (P1, Table) and KCl bath (B3, Table). *, $P < 0.05$.

Flufenamic acid was found to decrease the NSC channel current (above) and was therefore expected to reduce the transepithelial short circuit current, I_{sc}. $I_{sc,probe}$ was measured with the vibrating probe and flufenamic acid (100 μM) (B1, Table) was found to abruptly decrease the absorptive current but continued on to make an "overshoot" which led to transient secretion, followed by abolition of the current (Fig. 12).

This multiphasic response was further investigated by patch clamp recordings from outside-out patches of apical membrane from outer sulcus epithelial cells. Flufenamic acid (100 μM) in solution B2 (Table) was applied to the extracellular side while the pipette contained KCl and 10^{-7} M Ca^{2+} (P2, Table). A representative recording of the effect of flufenamic acid on BK channels is shown in Fig. 13. The recording was made at a holding membrane potential of +20 mV. Application of flufenamic acid increased the P_o of BK channels and subsequent addition of charybdotoxin (CTX), a potent blocker of this channel, decreased P_o. The effect of flufenamic acid was reversible. These data demonstrated that external application of flufenamic acid reversibly activated BK channels.

A small K^+ channel was clearly observed in 8 excised patches. In one patch where the single-channel currents were clearly present at both positive and negative membrane voltages and with asymmetric K^+ at the membrane (B1, P3; Table), the *I-V* relationship was quasilinear in the range ±60 mV (Fig. 14). The single-channel conductance was 6.6 pS and the extrapolated

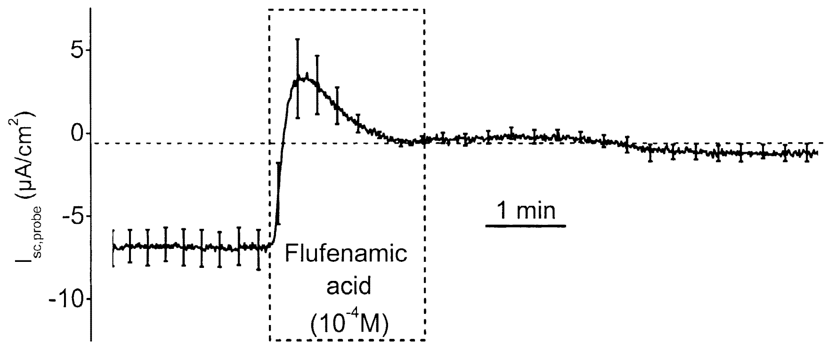

Fig. 12. Effect of flufenamic acid (0.1 mM) on absorptive current ($I_{sc,probe}$) at the apical surface of outer sulcus epithelium. Inward current changes transiently to an outward current, followed by total inhibition. (mean ± SEM, $n = 9$; room temperature).

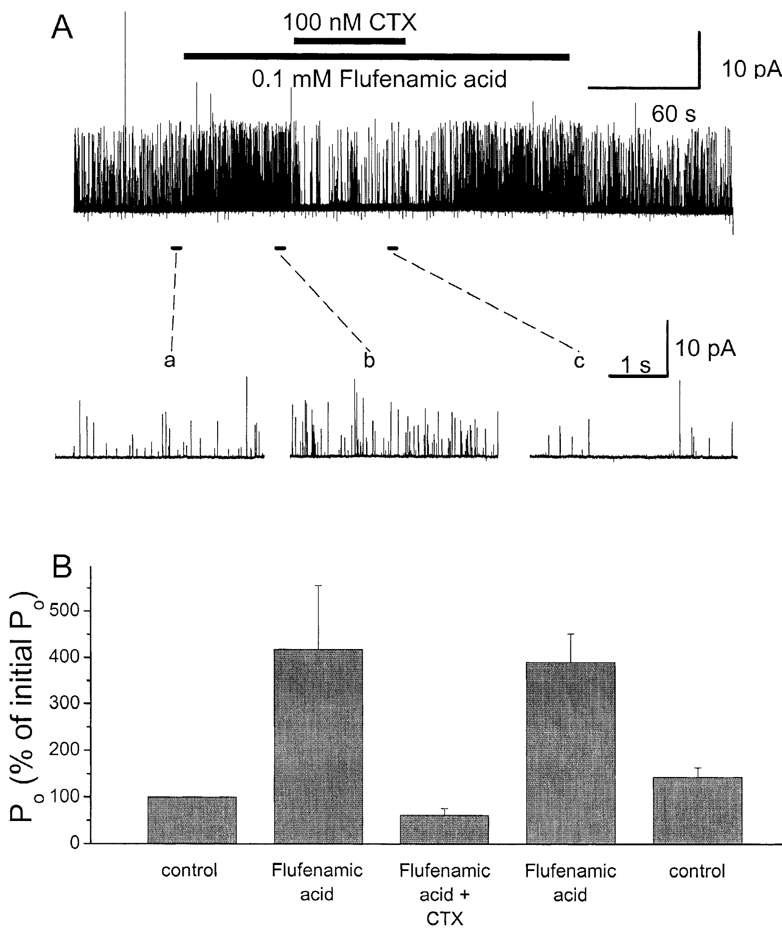

Fig. 13. (*A*) Effect of flufenamic acid on open probability (P_o) of BK channels in a representative recording. Recording was made from an excised outside-out patch at +20 mV, KCl, 10^{-7} M Ca^{2+} pipette (P3, Table), NaCl bath (B2, Table). Lower horizontal bar indicates application of flufenamic acid (0.1 mM), and upper horizontal bar indicates addition of charybdotoxin (CTX, 100 nM). *Lower traces* (*a, b, c*) show the details of single channel activities on an expanded time scale during (*a*) control, (*b*) flufenamic acid and (*c*) flufenamic acid + CTX. (*B*) *Bar graph,* summary of the effect on open probability (P_o) of flufenamic acid in the absence and presence of CTX ($n = 6$) at 37°C.

reversal voltage was –118 mV. The equilibrium potential for K^+ was about –94 mV, suggesting that the channel would rectify slightly at the more polarized voltages seen under physiologic conditions *in situ.* The channel was not characterized further.

Discussion

The outer sulcus epithelial cells directly border the cochlear lumen in the upper cochlear turns, as described previously (Duvall, 1969; Spicer & Schulte, 1996). Several morphologic observations led to speculations that these cells participated in cation reabsorption from endolymph (*see* review: (Kucuk & Abe, 1990)). Our previous study (Marcus & Chiba, 1999) was the first to directly demonstrate cation reabsorption in this region of the cochlear duct using the vibrating probe technique. The purpose of this study was to investigate ion channels of the apical membrane of outer sulcus epithelial cells in the upper cochlear turns that could account for the observed cationic reabsorption. This report describes the characterization of NSC channels and of BK channels in the apical membrane.

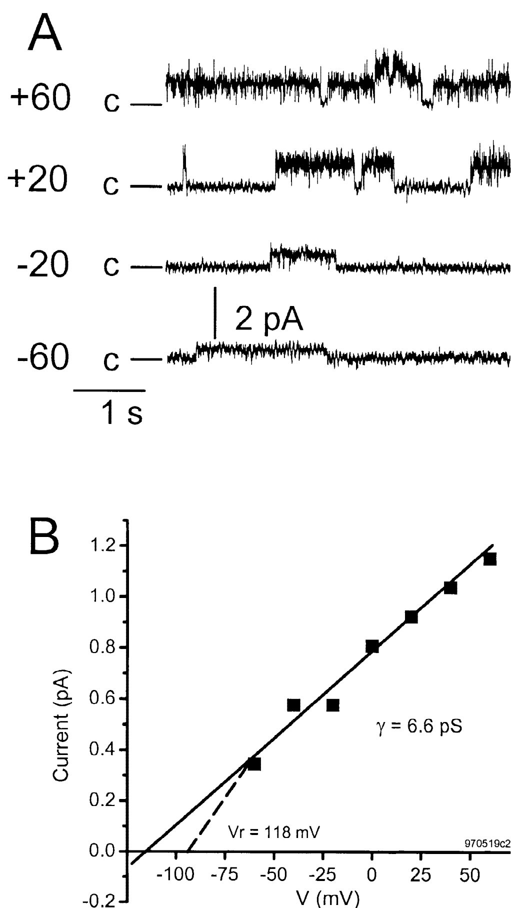

Fig. 14. Single channel recordings of a small K^+ channel in an excised inside-out patch. Recording was made from apical membrane of an outer sulcus epithelial cell under asymmetrical KCl conditions (B1, P3; Table) at room temperature. (*A*) Representative traces at ±60 mV; C, closed channel level. (*B*) *I-V* relationship of single channel currents. Solid line is least squares best fit to the data; dashed line shows intersection at equilibrium potential for K^+.

Properties of the Nonselective Cation Channel

The single-channel current events were identified as originating from NSC channels by the observations that (i) the *I-V* relationships were linear and passed through or near the origin in all cases in which monovalent cations such as K^+ and Na^+ were present on both sides of the membrane in equal concentrations, (ii) the *I-V* relationship was not altered by replacement of bath Cl^- with gluconate but was altered by replacement of NaCl by NMDG-Cl. The single channel conductance of 27 pS, Ca^{2+} sensitivity from the cytosolic side, linear *I-V* relationship and poor discrimination between Na^+ and K^+ are similar to those properties of the family of Ca^{2+}-activated nonselective cation channel (CAN) as reported previously (Partridge & Swandulla, 1988). Similar observations of NSC channels were reported in various cells of the inner ear (Marcus et al., 1992; Takeuchi et al., 1992*b;* Sunose et al., 1993; Van den Abbeele et al., 1994; Yeh et al., 1998) and in other preparations (Ling & O'Neill, 1992; Popp & Gogelein, 1992; Nonaka et al., 1995; Korbmacher et al., 1995; Vandorpe et al., 1997).

A wide range of sensitivity to the cytosolic Ca^{2+} activity has been described for the activation of NSC channels. Some NSC channels do not require Ca^{2+} for activation (Yeh et al., 1998) while others require at least 10^{-6} M cytosolic Ca^{2+} for observable activation (Marcus et al., 1992; Van den Abbeele et al., 1994), suggesting that those channels requiring high cytosolic Ca^{2+} levels make little or no significant contribution to membrane conductance under undisturbed conditions. In our preparation, channel activity was significant with cytosolic Ca^{2+} as low as 10^{-7} M. This activity at low Ca^{2+} levels coupled with the high density of this channel in the apical membrane suggests a major physiological significance.

The NSC channel reported here was insensitive to membrane voltage, similar to NSC channels in the antiluminal membrane of rat cerebral capillary endothelial cells (Popp & Gogelein, 1992) and in the luminal membrane of vestibular dark cells (Marcus et al., 1992). By contrast, the activity of NSC channels in rat inner medullary collecting duct cells and in the apical membrane of epithelial cells of Reissner's membrane were suppressed by hyperpolarization (Nonaka et al., 1995; Yeh et al., 1998) while NSC channels in bovine aortic endothelial cells were stimulated by membrane hyperpolarization (Ling & O'Neill, 1992).

Gadolinium (Gd^{3+}) is known as a blocker of NSC channels (Ling & O'Neill, 1992; Popp & Gogelein, 1992; Marunaka et al., 1994; Nonaka et al., 1995; Hoyer et al., 1997). Although Gd^{3+} was an effective inhibitor of NSC channels at submicromolar levels (IC_{50} 0.6 μM), the slope of the concentration-response curve was relatively shallow so that the full effect required mM levels of Gd^{3+}. This is in contrast to some NSC channels such as stretch-activated channels found in *Xenopus* oocytes (Yang & Sachs, 1989) and aortic endothelial cells (Ling & O'Neill, 1992) which are fully inhibited by 10 μM Gd^{3+}, but similar to others such as stretch-insensitive NSC channels in *Xenopus* oocytes (Reifarth, Clauss & Weber, 1999).

Amiloride fully inhibits epithelial Na^+ channels at 10 μM and Na^+/H exchange at 1 mM (Frelin et al., 1987). The effect on NSC channels is quite variable among types and therefore constitutes a distinguishing characteristic. The NSC current in outer sulcus epithelial cells was only slightly inhibited by 10 μM amiloride and more strongly inhibited by 1 mM amiloride. This sensitivity to high concentrations of amiloride is characteristic of the stretch-activated NSC channels of A6 cells (Marunaka et al., 1994) and *Xenopus laevis* oocytes (Reifarth et al.,

1999), but contrasts to the amiloride-insensitive NSC channels of mouse cortical collecting duct cells (Korbmacher et al., 1995) and rat inner medullary collecting duct cells (Nonaka et al., 1995). On the other hand, an NSC channel in fetal lung epithelium was inhibited by amiloride with an IC50 of <1 μM. This channel, however, was not operant at physiological intracellular Ca^{2+} concentrations (Marunaka, 1996).

Flufenamic acid caused a partial inhibition of NSC currents in outer sulcus epithelial cells. Such a block was also found in M-1 mouse cortical collecting duct cells where the inhibition was found to be voltage-dependent (Korbmacher et al., 1995). By contrast, the stretch-activated NSC channels in Reissner's membrane were insensitive to fenamates (Yeh et al., 1998).

Properties of the BK Channel

The conductance of the BK channel in symmetrical KCl solution was 268 pS and occasionally showed a subconductance state as has been reported for BK channels in vestibular dark cells (Takeuchi et al., 1992*a*) and in cochlear efferent nerve terminals (Wangemann & Takeuchi, 1993). The characteristics of the large-conductance K^+ channels observed in the apical membrane of the outer sulcus epithelial cells are similar to those of BK channels in other cells including those of the inner ear (Takeuchi et al., 1992*a;* Wangemann & Takeuchi, 1993; Takeuchi & Irimajiri, 1996; Gribkoff et al., 1996): (i) high selectivity for K^+ over Na^+, (ii) open probability (P_o) increased with depolarization, (iii) P_o was dependent on cytosolic-free Ca^{2+} concentration, (iv) P_o was reduced by Ba^{2+}, external TEA and low concentrations of charybdotoxin. It was not possible in this study to determine which alpha and beta subunit isoforms of BK channels are present in this tissue (Fettiplace & Fuchs, 1999).

In addition to these features, large K^+ channels observed in the apical membrane of the outer sulcus epithelial cells were stimulated by flufenamic acid, which is known as an inhibitor of Cl^- channels (Weber et al., 1995*a, b*) and nonselective cation channels (vide infra and (Popp & Gogelein, 1992; Van den Abbeele et al., 1994; Korbmacher et al., 1995)). Ottolia and Toro (1994) suggested the hypothesis that large conductance calcium-activated K^+ channels possess at least one fenamate receptor based on the observations that external niflumic, flufenamic and mefenamic acid application activated large calcium-activated K^+ channels in coronary smooth muscle membrane and external niflumic acid caused left-shifting of both voltage- and calcium-activation curves without major changes in the slopes of the curves. Further, stimulation of cloned K_{ca} by flufenamic acid was also reported (Gribkoff et al., 1996). In rabbit portal vein smooth muscle cells, fenamates including flufenamic acid activated Ca^{2+}-activated potassium currents with both perforated patch and conventional whole-cell recording (Greenwood & Large, 1995), and the authors proposed that fenamates would interact directly with calcium-activated potassium channels to activate IK(Ca) and their action might modify the binding of Ca^{2+} to the channel protein.

The finding that flufenamic acid initially reversed the direction of I_{sc}, followed by a decline to zero (Fig. 12) can most simply be explained by the following. In the absence of flufenamic acid, the cytosolic $[K^+]$ is kept slightly above electrochemical equilibrium by the activity of the basolateral Na^+,K^+-ATPase, with K^+ leaving the cell across the basolateral membrane. The basolateral membrane is known to be dominated by a K^+ conductance of a type different from the BK channel (*unpublished observations*).

Addition of flufenamic acid to the bath had two simultaneous effects. The apical K^+ conductance was greatly increased via stimulation of the BK channels and the Na^+ conductance was substantially diminished by inhibition of the apical NSC channels. The large increase in apical K^+ conductance led to the transient net secretion (reversal of I_{sc} to positive values). The diminished influx of Na^+ deprived the Na^+,K^+-ATPase of a requisite transport substrate, reducing its transport activity. With the cells now nearly symmetric in permeability to K^+ on the apical and basolateral membranes and the Na^+ pump inhibited, the cells lost their ability to support vectorial transport. This interpretation is supported by the observation of increased channel P_o in the presence of flufenamic acid and the prevention of that effect by the BK channel blocker charybdotoxin.

Physiological Significance

Maintenance of the unusual ionic composition of endolymph in the cochlea is now known to be the net result of secretory and absorptive processes contributed by several cell types. The strial marginal cells secrete K^+ while the sensory hair cells and the parasensory outer sulcus epithelial cells absorb cations. Na^+ absorption by the hair cells is thought to be relatively low compared to that by the outer sulcus cells (Marcus & Chiba, 1999), and the overall Na^+ absorption rate by the cochlear duct is only about 1% of the K^+ secretion rate (Sellick & Johnstone, 1975).

The electrodiffusive entry pathways for cations across the apical membrane of outer sulcus epithelial cells were identified as NSC channels and BK channels. The apical membrane sustains an electrical potential difference *in vivo* of about 170 mV, cell negative, due to the transepithelial voltage of +80 mV (Marcus, 1997) and the basolateral potential difference of about −90 mV (T. Chiba & D.C. Marcus, *unpublished results*). The cyto-

solic [K^+] is thought to be about the same as that in endolymph (based on the highly negative basolateral potential) and the cytosolic [Na^+] is thought to be about one decade higher than that of endolymph, corresponding to 60 mV chemical driving force outward for Na^+. There are therefore large electrochemical driving forces for the entry of both Na^+ (110 mV) and K^+ (170 mV) from the lumen into the cell across the apical membrane. The NSC and BK channels described here are therefore poised to participate in carrying and regulating the transepithelial absorption of Na^+ and K^+.

The NSC channels are likely to be important players in the absorptive processes since they occur at a high density, as manifest in the many membrane patches containing multiple channels, and since their sensitivity to cytosolic Ca^{2+} occurs in a physiologically relevant range. Further, these channels may have provided the epitopes for antibodies against amiloride binding sites which were found to bind to the apical membrane of these cells (Mizuta et al., 1995) since we found a weak inhibition of NSC channel activity by amiloride.

The significance of the BK channels in the apical membrane is more difficult to assess. Our voltage- and Ca^{2+}-dependence data suggest that if the BK channels are to become active *in vivo,* that another modulating factor must come into play. It is likely that such a modulating factor exists in these cells since the channels are present in a relatively high density as judged by their high frequency of occurrence compared to BK channels in other inner ear epithelial cells (Takeuchi et al., 1992*a, b*).

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