Potassium Secretion by Vestibular Dark Cell Epithelium Demonstrated by Vibrating Probe

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ABSTRACT Detection of motion and position by the vestibular labyrinth depends on the accumulation of potassium within a central compartment of the inner ear as a source of energy to drive the transduction process. Much circumstantial evidence points to the vestibular dark cell (VDC) epithelium as being responsible for concentrating K^+ within the lumen. We have used the vibrating probe technique to directly observe voltage and ion gradients produced by this tissue to put this assumption on a solid experimental footing. Relative current density ($I_{sc,probe}$) over the apical membrane of VDC epithelium was measured with the vibrating voltage-sensitive probe, and this technique was validated by performing maneuvers known to either stimulate or inhibit the transepithelial equivalent short circuit current. Basolateral bumetanide (5×10^{-5} M) and ouabain (1×10^{-3} M) caused a decrease in $I_{sc,probe}$ by 55 ± 6% and 39 ± 3%, respectively while raising the basolateral K^+ concentration from 4 to 25 mM caused an increase by 35 ± 8%. A K^+ gradient directed toward the apical membrane was detected with the vibrating K^+-selective electrode, demonstrating that, indeed, the VDC epithelium secretes K^+ under control conditions. This secretion was inhibited by bumetanide (by 94 ± 7%) and ouabain (by 52 ± 8%). The results substantiate the supposition that dark cells produce a K^+ flux and qualitatively support the correlation between this flux and the transepithelial current.

INTRODUCTION

The transduction of static position and motion of the body into nervous information is the result of modulation of a standing K^+ current through neuroepithelial hair cells. This K^+ current is driven by the unusually high K^+ concentration difference across these cells; approximately 145 mM K^+ in the luminal fluid, endolymph, versus approximately 4–6 mM in the basolateral fluid, perilymph (Marcus and Marcus, 1985; Salt and Ohyama, 1993). Several lines of indirect evidence, including histochemical, electrophysiological, and flux studies, have strongly suggested that it is the dark cell epithelium that is responsible for secretion of K^+ into the lumen of the vestibular labyrinth (Bernard et al., 1986; Kimura, 1969; Marcus et al., 1994; Marcus and Marcus, 1987; Schulte and Adams, 1989).

To put the earlier inferences of K^+ secretion by dark cells on a solid experimental footing, we measured relative current density and K^+ gradients in the direct vicinity of vestibular dark cells (VDC) from the mammalian ampulla under several conditions known to alter electrogenic ion transport by this epithelium. The ion-selective vibrating probe was the most appropriate method available with which to detect the K^+ flux from the small region of dark cells (approximately 100-μm diameter).

We found both an electric current and a K^+ flux directed out of the apical surface of the VDCs, which was stimulated by elevation of basolateral K^+ and inhibited by basolateral bumetanide and ouabain. These responses are consistent with previous measurements of equivalent short circuit current in the same preparation (Marcus et al., 1994).

MATERIALS AND METHODS

Gerbils 4–10 weeks old (cared for and used under a protocol approved by the Marine Biological Laboratory Animal Care and Use Committee) were anesthetized with ethyl carbamate (2 g/kg, i.p.) and decapitated. The temporal bones were removed, and the dark cell regions on the canal side of the ampullae were dissected in chilled control solution. The dark cell epithelium was identified by the melanocytes present in the underlying connective tissue. The isolated dark cell tissue was placed into a micro-Ussing chamber such that the entire aperture was covered by this epithelium. The fluid composition at the apical and basolateral sides could be independently controlled (Marcus et al., 1994). The basolateral side of the epithelium was continuously perfused at a rate of about 2 ml/min and the apical side was in contact with a static bath to which the vibrating electrodes had access. Experiments were conducted at room temperature (25–26°C).

The control solution consisted of (in mM): 150 NaCl, 1.6 K_2HPO_4, 0.8 KH_2PO_4, 0.7 $CaCl_2$, 1 $MgSO_4$, and 5 glucose, pH 7.4. 25 K solution was made by substituting 21 mM KCl for an equimolar amount of NaCl. Ouabain and bumetanide were purchased from Sigma Chemical (St. Louis, MO) and first dissolved in dimethylsulfoxide (DMSO; final DMSO concentration 0.1%). All other chemicals were obtained either from Sigma or Fluka Chemical (Ronkonkoma, NY).

The vibrating probe techniques used were identical to those previously described (Kühtreiber and Jaffe, 1990; Scheffey et al., 1991). Briefly, current density ($I_{sc,probe}$) was monitored by vibrating a stainless steel wire electrode with a Pt black ball (20-μm diameter) for an excursion of about 20 μm along both a horizontal (X) and vertical (Z) axis. The bath reference was a 26-gauge Pt black electrode. Calibration was performed using a glass microelectrode filled with 3 M KCl as a point source of current (Scheffey et al., 1991). The frequencies of vibration were in the range of 200–800 Hz and were well separated for the 2 directions. The signals from the oscillators driving the probe were also fed to a dual channel phase-sensitive detector. The quadrature signal of each channel was minimized by adjusting the reference phase of the detector. The in-phase signal components of the X and Z detectors were connected to a 16-bit analog-to-digital converter (Data

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Translation Model DT2801/5716A, Marlboro, MA) in an IBM-compatible 80486 processor-based computer. The 2 in-phase signals were used to construct vectors whose magnitudes were proportional to $I_{sc,probe}$ in the plane defined by the 2 modes of vibration. The electrode was positioned over the epithelium, such that the X component was typically <5% of the Z component except when a scan was made over the tissue.

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 in the Z direction (Kochian et al., 1992; Kühtreiber and Jaffe, 1990). The microelectrodes were constructed from 1.5-mm OD borosilicate glass capillary, were pulled to a tip of about 4 μm OD, and were silanized with dimethyldichlorosilane. The tips 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 solutions. 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.

Data are expressed as current density at the voltage-sensitive electrode tip or voltage difference detected by the K^+-selective electrode and plotted with Origin software (MicroCal Software, Northampton, MA). In both techniques, the probe was located between 20 and 240 μm over the apical surface of the epithelium, such that the signal under control conditions was >30 times the noise level.

Data summaries of effects of solution changes are given as a percentage of the reading under control conditions immediately before the solution change. The signals were not calibrated in terms of absolute current density or 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 and Jaffe, 1990).

RESULTS

Current density

With identical solutions on both sides of the epithelium, current was found to flow from the tissue into the apical bath ($I_{sc,probe}$: 43 ± 1 $\mu A/cm^2$, N = 15). Two-dimensional scans (Fig. 1) of the tissue revealed current vectors with the maximum vertical component over the dark cell epithelium (N = 4). The apparent asymmetric distribution reflects the presence of additional ampullar tissue to the right of the aperture.

The probe was located then at a stationary position directly over the center of the apical surface of the epithelium, and the vector magnitude was recorded over time. Elevating the basolateral K^+ concentration from 4 to 25 mM caused a reversible increase in the $I_{sc,probe}$ by 35 ± 8% (N = 6; Fig. 2). Basolateral perfusion of bumetanide (5×10^{-5} M) led to a partially reversible decrease in the $I_{sc,probe}$ by 55 ± 6% (N = 8; Fig. 3). Similarly, basolateral perfusion of ouabain (1×10^{-3} M) led to a partially reversible decrease in the $I_{sc,probe}$ by 39 ± 3% (N = 6; Fig. 4).

K^+ gradient

With identical solutions on both sides of the epithelium, the K^+-selective microelectrode was at a higher potential ($J_{K+,probe}$: 180 ± 6 μV, N = 16) when closer to the tissue, indicating an upwardly directed K^+ flux. One-dimensional scans (Fig. 5) of the tissue revealed vertical components of the K^+ flux with a maximum over the dark cell epithelium (N = 2).

In the following experiments, the probe was located directly over the apical surface of the epithelium, and the magnitude of the K^+ gradient was recorded over time. Basolateral perfusion of bumetanide (5×10^{-5} M) led to a decrease in $J_{K+,probe}$ by 94 ± 7% (N = 8; Fig. 6). Similarly, basolateral perfusion of ouabain (1×10^{-3} M) led to a decrease in $J_{K+,probe}$ by 52 ± 8% (N = 8; Fig. 7). The percentage decrease of $J_{K+,probe}$ due to bumetanide was greater ($P < 0.05$) than the percentage decrease of $I_{sc,probe}$, whereas the decreases of both

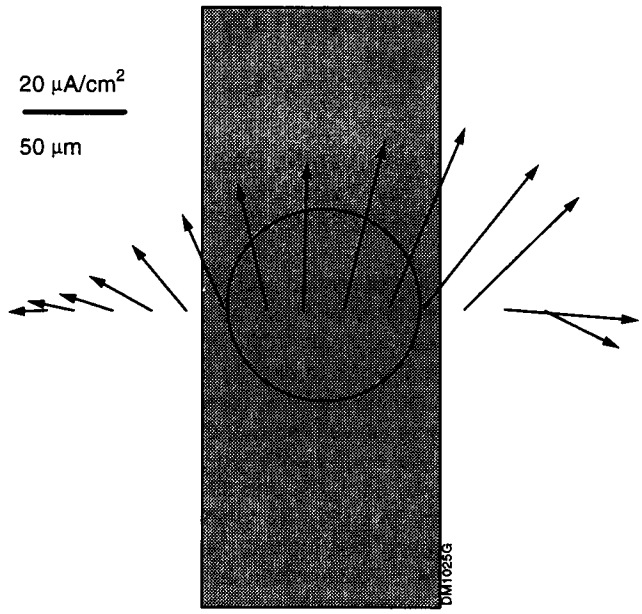

FIGURE 1 Diagram of the VDC epithelium (*shaded area*) covering the aperture (*circle*) in a micro-Ussing chamber. Vectors represent two-dimensional current density ($I_{sc,probe}$) at the position of the base of each arrow and were obtained by passing the tip of the vibrating electrode at a constant height over the epithelium. The vertical component represents the Z signal (out of the plane of the figure), and the horizontal component represents the X signal. The scale bar indicates the current density of vectors and distance.

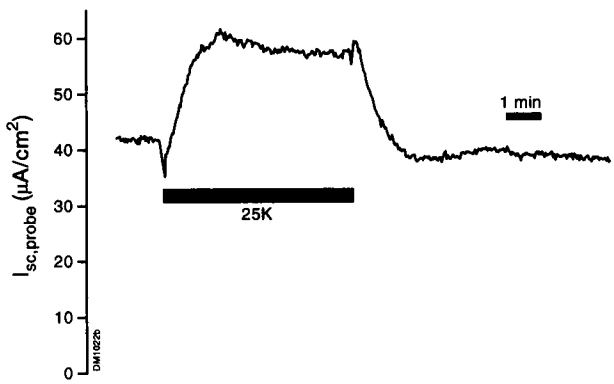

FIGURE 2 Representative record of the effect of raising basolateral K^+ from 4 to 25 mM on $I_{sc,probe}$ at a fixed position over the apical surface of the dark cell epithelium.

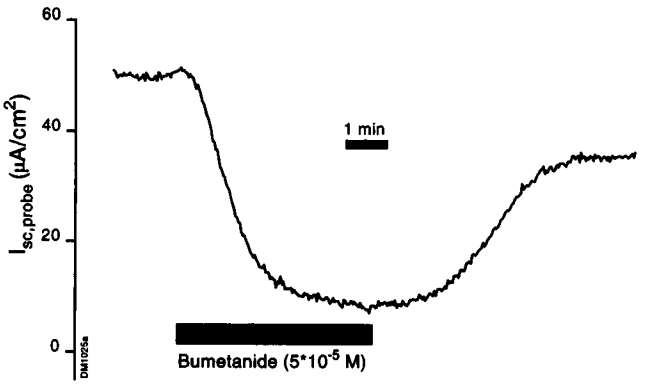

FIGURE 3 Representative record of the effect of basolateral bumetanide (50 μM) on $I_{sc,probe}$ at a fixed position over the apical surface of the dark cell epithelium.

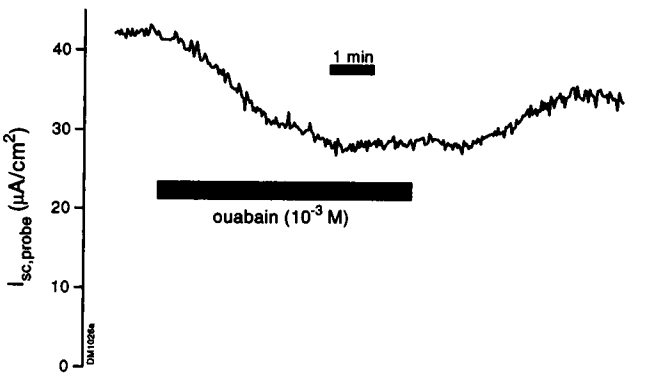

FIGURE 4 Representative record of the effect of basolateral ouabain (1 mM) on $I_{sc,probe}$ at a fixed position over the apical surface of the dark cell epithelium.

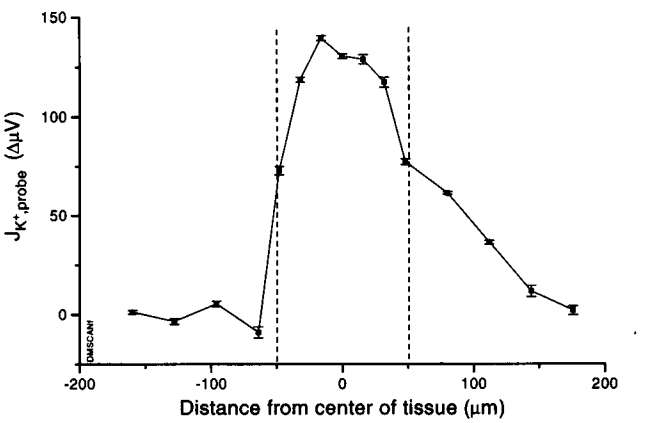

FIGURE 5 Representative spatial distribution of the magnitude of the voltage difference of the vibrating K^+-selective electrode ($J_{K+,probe}$), obtained by passing the tip of the electrode at a constant height over the VDC epithelium (as in Fig. 1). Dashed vertical lines indicate the edges of the dark cell epithelium, which was centered over the aperture of the chamber. Each point is the mean ± SE of 20 measurements at each position.

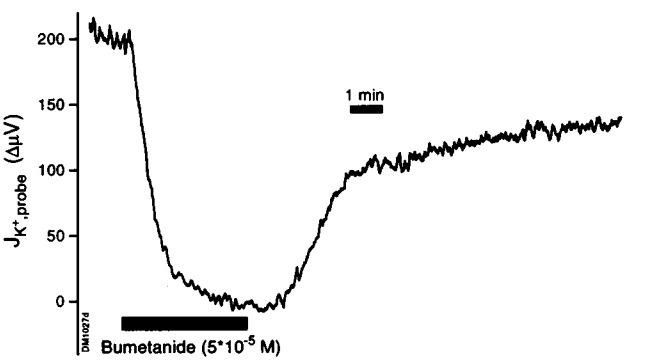

FIGURE 6 Representative record of the effect of basolateral bumetanide (50 μM) on $J_{K+,probe}$ at a fixed position over the apical surface of the dark cell epithelium.

parameters due to ouabain were not significantly different in unpaired samples.

DISCUSSION

We have shown directly for the first time that the VDC epithelium from the gerbil ampulla produces a luminally directed transepithelial K^+ flux. The current density and K^+ gradient scans over the tissue confirmed that the currents were from the tissue rather than being generated by spurious sources (e.g., K^+ gradients produced by convective flow induced by evaporation and/or thermal gradients) within the chamber. Inhibition of the current and flux by bumetanide and ouabain further strengthen this point.

Dark cells were previously thought to secrete K^+ based on a variety of observations. The presence of a high density of basolateral membrane infoldings, mitochondria, and $(Na^+ + K^+)$-ATPase activity were suggestive of a high solute transport rate by these cells (Kimura, 1969; Schulte and Adams, 1989). An oil-blocked tubular preparation of the nonsensory region of gerbil utricle (approximately 60% dark cells) secreted K^+ at a sufficient rate to account for the luminally directed transepithelial equivalent short circuit current (I_{sc} = transepithelial voltage/transepithelial resistance) (Marcus and Marcus, 1987). This electrogenic secretion was inhibited by ouabain and bumetanide. K^+ secretion was demonstrated in the entire isolated frog ampulla, and this was shown to be inhibited by ouabain and bumetanide (Bernard et al., 1986; Ferrary et al., 1989). The authors ascribed all changes in cation flux to the VDCs despite the presence of several other cell types.

The only in vitro preparation consisting almost solely of dark cells that has been used for transport studies is that from the ampulla (Marcus et al., 1994; Marcus et al., 1992; Wangemann and Marcus, 1990). With this preparation it was shown that dark cells produced an I_{sc} directed from the basolateral to the apical side under symmetrical conditions. This voltage was stimulated by raising the basolateral $[K^+]$ and was inhibited by basolateral perfusion of bumetanide or ouabain (Marcus et al., 1994). We have used those observations as a means to validate the new application of the

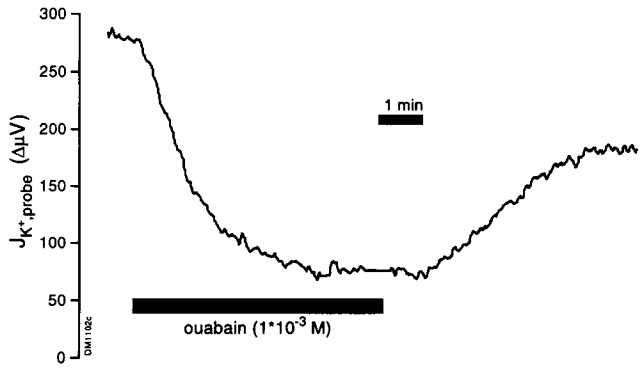

FIGURE 7 Representative record of the effect of basolateral ouabain (1 mM) on $J_{K+,probe}$ at a fixed position over the apical surface of the dark cell epithelium.

vibrating probe technique to this preparation. Indeed, we found the same response to these maneuvers of the current density magnitude over the apical membrane of the dark cells.

At present there are clearly quantitative limitations of both the voltage-sensitive and ion-selective vibrating probe techniques as applied to the inner ear tissue mounted in the micro-Ussing chamber. The basolateral application of elevated K^+, ouabain and bumetanide produced qualitatively the same effects on I_{sc} and $I_{sc,probe}$, although I_{sc} was stimulated or inhibited to a greater degree than $I_{sc,probe}$. The increase due to 25 mM K^+ was 164 ± 8% ($N = 43$) of I_{sc} and 35% of $I_{sc,probe}$, whereas the decrease due to bumetanide was 95 ± 1% ($N = 15$) of I_{sc} and 55% of $I_{sc,probe}$ and that due to ouabain was 94 ± 2% ($N = 14$) of I_{sc} and 39% of $I_{sc,probe}$ (Marcus et al., 1994). These discrepancies can be accounted for by the difference in the orientation of the epithelium in the chamber. For the present experiments it was necessary to mount the tissue with the basolateral side against the aperture. The reduced inhibition by bumetanide and ouabain was most likely due to dilution of the drug by a variable, unquantifiable leak from the apical bath laterally through and around the connective tissue into the perfused basolateral solution. By contrast, the apical membrane has no other structure to interfere with the seal to the edge of the aperture during measurements of I_{sc}.

The inhibition of $J_{K+,probe}$ by ouabain was indistinguishable from that of $I_{sc,probe}$. It is not clear why bumetanide inhibited $J_{K+,probe}$ more than $I_{sc,probe}$ (but to a similar extent as I_{sc}). It is most likely that this discrepancy is due to greater success in sealing the subpopulation of tissues used in that series of measurements with the K^+-selective vibrating probe.

The observations of potassium flux in the present experiments and its inhibition by bumetanide and ouabain are consistent with the earlier findings in the utricle (Marcus and Marcus, 1987). Although the absolute K^+ flux at the surface of the tissue in the present study could not be quantitatively evaluated, in several tissues it was possible to estimate the K^+ flux (J_{K+}), assuming an infinite flat sheet of epithelium. In three tissues J_{K+} was 27 ± 4 $\mu A/cm^2$ (at 48 ± 8 μm above the epithelium), which compares favorably with the average $I_{sc,probe}$ of 43 $\mu A/cm^2$ found for the vibrating voltage-sensitive probe. The calculated J_{K+} is expected to be lower than the K^+ flux at the surface of the epithelium due to the "efficiency factor" of the electrode (see Methods) and due to lateral diffusion of K^+ between the epithelial surface and the electrode. The present findings therefore substantiate the supposition that dark cells produce a K^+ flux and qualitatively support the correlation between this flux and the transepithelial current.

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