K^+ Secretion across Frog Skin

Induction by Removal of Basolateral Cl^-

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ABSTRACT   We examined the development of K^+ secretion after removing Cl^- from the basolateral surface of isolated skins of *Rana temporaria* using noise analysis. K^+ secretion was defined by the appearance of a Lorentzian component in the power density spectrum (PDS) when Ba^{2+} was present in the apical bath (0.5 mM). No Lorentzians were observed when tissues were bathed in control, NaCl Ringer solution. Replacement of basolateral Cl^- by gluconate, nitrate, or SO_4^- (0-Cl_b) yielded Lorentzians with corner frequencies near 25 Hz, and plateau values (S_0) that were used to estimate the magnitude of K^+ secretion through channels in the apical cell membranes of the principal cells. The response was reversible and reproducible. In contrast, removing apical Cl^- did not alter the PDS. Reduction of basolateral Cl^- to 11.5 mM induced Lorentzians, but with lower values of S_0. Inhibition of Na^+ transport with amiloride or by omitting apical Na^+ depressed K^+ secretion but did not prevent its appearance in response to 0-Cl_b. Using microelectrodes, we observed depolarization of the intracellular voltage concomitant with increased resistance of the basolateral membrane after 0-Cl_b. Basolateral application of Ba^{2+} to depolarize cells also induced K^+ secretion. Because apical conductance and channel density are unchanged after 0-Cl_b, we conclude that K^+ secretion is "induced" simply by an increase of the electrical driving force for K^+ exit across this membrane. Repolarization of the apical membrane after 0-Cl_b eliminated K^+ secretion, while further depolarization increased the magnitude of the secretory current. The cell depolarization after 0-Cl_b is most likely caused directly by a decrease of the basolateral membrane K^+ conductance. Ba^{2+}-induced Lorentzians also were elicited by basolateral hypertonic solutions but with lower values of S_0, indicating that cell shrinkage per se could not entirely account for the response to 0-Cl_b and that the effects of 0-Cl_b may be partly related to a fall of intracellular Cl^-.

INTRODUCTION

A variety of high resistance epithelial tissues actively secrete K^+ from the basolateral to the apical cell surface. According to the model for steady-state transepithelial Na^+

absorption proposed by Koefoed-Johnsen and Ussing (1958), Na^+ enters cells passively across the apical membrane and is pumped out at the basolateral membrane in exchange for K^+, which then diffuses out of the cells across this membrane. Rather than simply cycling across the basolateral membrane, K^+ secretion could be achieved in parallel to Na^+ absorption if the apical membrane were permeable to K^+ as well as Na^+. The apical membrane of the principal cells of frog skin epithelia are selective for Na^+ in most species, but isolated skins of *Rana temporaria* also possess apical K^+ conductive channels (Nagel and Hirschmann, 1980; Van Driessche and Zeiske, 1980). It is therefore surprising that when skins of *Rana temporaria* are bathed symmetrically in NaCl Ringer solution, the short-circuit current, I_{sc}, is identical to the net, transepithelial flux of Na^+ (Ussing and Zerahn, 1951; Nielsen, 1984) despite high values of intracellular $[K^+]$ (Rick et al., 1978; Garcia-Diaz et al., 1985). This indicates that under these conditions no net secretion or absorpion of K^+ is observed. However, Nielsen (1984) found that eliminating Cl^- from the bathing solutions caused the net influx of Na^+ to exceed the I_{sc}. This was attributable to active K^+ secretion from the basolateral to the apical solution which is partly mediated by the principal cells (Nielsen, 1987).

The purpose of the present study was to examine this K^+ secretory process using current fluctuation (noise) analysis and microelectrodes to characterize the apical K^+ channels and gain some insight into the mechanism(s) underlying the dependence of K^+ secretion on external Cl^-. K^+ secretion by frog skin was easily detectable with noise analysis and was induced by maneuvers that depolarized the intracellular voltage, thus increasing the electrochemical driving force for K^+ exit through channels in the apical membrane. Indeed, modulation of this driving force may account for differences of the spontaneous K^+ secretory rate among high resistance epithelia such as the mammalian colon and renal distal tubule. The mechanism of cell depolarization subsequent to Cl^- removal was not resolved, but may be partly due to an influence of intracellular Cl^- directly on the basolateral membrane K^+ channels.

MATERIALS AND METHODS

Tissue Preparation

Frogs (*Rana temporaria*) were stored in cold rooms (17°C) with free access to tap water and were fed meal worms. It was previously found that apical K^+ permeability was augmented by storing frogs in dilute KCl solutions for several days before use (Van Driessche, 1984). Frogs were removed from the cold room and placed in small plastic tanks containing 30 mM KCl for 1–8 d before an experiment. Storage solutions were renewed daily. After decapitation, ventral skins were isolated, mounted in lucite chambers that were perfused with Ringer solution in both the apical and basolateral compartments, and short-circuited. Chambers for measurement of microscopic fluctuations of the short-circuit current, I_{sc}, were identical to those described previously (De Wolf and Van Driessche, 1986). Chamber perfusion rate was ~7 ml/min, the exposed tissue area was 0.5 cm^2, and the volume of each chamber half was 1.7 ml, resulting in exchange of bathing solutions within ~30 s. In some studies paired tissues were examined by cutting the ventral piece of skin longitudinally, yielding two tissues remarkably similar in electrical characteristics. Chambers were connected to a low-noise voltage clamp through 1 M KCl-agar (3%) bridges, 1 M KCl solution, and Ag-AgCl wires.

Electrical System

The low-noise voltage clamp was described previously (Van Driessche and Erlij, 1983). The filters, amplifiers, and data analysis system were as follows: Chambers and perfusion reservoirs were insulated from external noise sources by a solid metal Faraday cage and a vibration isolation table. After high-pass filtering (6 db/octave, cutoff frequency = 0.59 Hz) to remove the DC component of the I_{sc}, current fluctuations were amplified ($\times 1,000$) in the clamp. Outside the clamp the amplified signal was sent to two separate modules (designated high and low), each consisting of a pre-filter and an anti-aliasing filter.[1] The fundamental frequency was fixed in these studies at 0.5 Hz. The output of each stage was amplified ($\times 1$–20) to optimize the analog-to-digital conversion in a computer (RTI800 Multifunction A-D board [Analog Devices, Inc., Norwood, MA] in an IBM PC compatible computer). Data were digitized and displayed continuously on the computer's monitor. The required amplifier gain was a function of the noise level of each preparation as well as the experimental conditions and was adjusted before collecting and storing the data. After digitizing, data were converted from the time to the frequency domain using an FFT software routine (adapted from an algorithm as described by Van Driessche and Erlij, 1983, but written in "C" language for the PC) and displayed as a power density spectrum (PDS) on the computer's monitor.

An individual spectrum was obtained from a series of 30 periods of data acquisition (sweeps). Each sweep consisted of the sequential digitization of the low (1,024 points/2 s) and then the high (1,024 points/0.25 s) analog signals. The duration of a sweep was therefore 2.25 s, and ~ 3 min were required to obtain and process the 30 sweeps for a single spectrum, which was displayed immediately on the computer's monitor. The FFT output frequencies (512) for the low sweep ranged from 0.5 to 256 Hz at 0.5-Hz intervals and for the high sweep from 4 to 2,048 Hz. At frequencies > 8 Hz, overlapping frequencies were averaged resulting in eight values per octave, which yielded 74 spectral data points from 0.5 to 1,328 Hz in the PDS. Data were fit after the experiment over a frequency range that was selected for each PDS. In these studies 0.5 to ~ 100 Hz were usually chosen, which eliminated the influence of the amplifier noise at higher frequencies, allowing us to fit the data to $1/f$ and Lorentzian components as described previously (Van Driessche and Zeiske, 1980; Van Driessche and Erlij, 1983; cf. Eq. 1, below).

When paired tissues were studied, two chambers and two low-noise voltage clamps were enclosed in the Faraday cage, which permitted us to switch the input to the filter module between the two tissues. Thus, spectra for control and experimental pieces of paired tissues were obtained within ~ 5 min of each other.

Microelectrode studies were performed using procedures and criteria similar to those described previously (Nagel, 1976; Helman and Fisher, 1977). Tissues were short-circuited continuously and the intracellular voltage, V_a, was measured with respect to the outer solutions. The fractional resistance of the apical membrane, $fR_a = \Delta V_a/\Delta V_T$, was obtained with voltage pulses of 10 mV and 200 ms duration.

Transepithelial DC resistance (R_T) was measured with ~ 1-s voltage pulses of ± 10 or ± 20 mV. Potassium secretory current from the basolateral to the apical surface is expressed as a negative quantity.

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[1] Filter modules (Butterworth type) were built in the electronics section of the Labo voor Fysiologie, KUL. The fundamental frequency (f_i) was selectable (low signal: 0.2, 0.5, or 1 Hz). Pre-filter characteristics: stage 1 (high pass), 24 db/octave, cutoff frequency (f_{co}) = 75% of f_i; stage 2 (low pass), 24 db/octave, f_{co} = 120, 300, or 600 Hz for f_i of 0.2, 0.5, or 1 Hz, respectively. Anti-aliasing filter: 48 db/octave, f_{co} = 90, 225 or 450 Hz for the above f_i. The f_{co} and f_i for the high signal were eight times the low signal values for all filters.

Theoretical Considerations

Previously, fluctuating K^+ channels were observed when skins were bathed apically in high K^+ Ringer solution. The PDS for random, spontaneous channel fluctuations between a conducting (open) and a nonconducting (closed) state is characterized by low frequency $(1/f)$ noise summed with a Lorentzian component, $L(f)$ (Van Driessche and Zeiske, 1980):

$$L(f) = S_0/[1 + (f/f_c)^2] \qquad (1)$$

where $L(f)$ is the spectral density of the Lorentzian at frequency f, S_0 is the plateau value in the low frequency range, and f_c is the corner frequency $(f$ at $S_0/2)$. In the presence of micromolar quantities of Ba^{2+} in the apical bath, a second[2] Lorentzian component is observed which is the result of the two-state block of the K^+ channels by Ba^{2+} (Van Driessche and Zeiske, 1980). Accordingly, we hypothesized that induction of a K^+ secretory current through these channels should introduce either a spontaneous Lorentzian, a Ba^{2+}-induced Lorentzian, or both in the PDS.

Ba^{2+} blockage of open K^+ channels is a two-state process, and may be described by the following scheme:

$$\text{open} \underset{k_{off}}{\overset{Ba^{2+}}{\rightleftharpoons}} \text{blocked} \qquad (2)$$

k_{on} and k_{off} are the forward and reverse rate constants, respectively, for Ba^{2+} blockade. Thus,

$$2\pi f_c = k_{on}[Ba^{2+}] + k_{off} \qquad (3)$$

Given the rate constants, the open and blocked probabilities $(P_0$ and $P_B)$ are computed

$$P_0 = 1/(1 + [Ba^{2+}]/K_{Ba}) \qquad (4)$$

$$P_B = 1 - P_0 \qquad (5)$$

For a given $[Ba^{2+}]$,

$$S_0 = M_{Ba}\cdot(i_K)^2\cdot(2P_0P_B/\pi f_c) \qquad (6)$$

and

$$I_K = M_{Ba}\cdot i_K\cdot P_0 \qquad (7)$$

where M_{Ba} is K^+ channel density and i_K is the single-channel current. P_0, P_B, and f_c are functions of k_{on}, k_{off}, and the $[Ba^{2+}]$. If these parameters are constant (C), then S_0 is a function of M_{Ba} and i_K alone, and changes in S_0 may be taken to reflect changes in the total Ba^{2+}-inhibitable current, I_K^{Ba},

$$S_0 = I_K^{Ba}\cdot(i_K\cdot C) \qquad (8)$$

Although offsetting effects of M_{Ba} and i_K could lead to opposite changes of I_K^{Ba} and S_0, this seemed unlikely and appeared not to occur in these studies.

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[2] The Ba^{2+}-induced Lorentzian component also is described by Eq. 1 since it is an independent two-state process. The Ba^{2+}-induced parameters are not superscripted throughout the text for convenience.

Solutions

Control Ringer solution consisted of (in mM): 115 NaCl, 2.5 KHCO$_3$, and 1.0 CaCl$_2$. Four Cl$^-$-free solutions (0-Cl$_b$) were used: (a) NO$_3^-$ (115), 2.5 KHCO$_3$, and 1.0 Ca(NO$_3$)$_2$; (b) gluconate (115), 2.5 KHCO$_3$, and 4.0 CaSO$_4$; (c) SO$_4^=$ (57.5), 2.5 KHCO$_3$, and 2.0 CaSO$_4$; and (d) isotonic SO$_4^=$, which was identical to SO$_4^=$ Ringer solution with 61 mM mannitol added. Na$^+$-free Ringer solution contained N-methyl-D-glucamine as a substitute.

All values are given as mean ± SEM. Room temperature was 22–23°C. Tissues were incubated in control NaCl Ringer solution for 30–60 min until I_{sc} stabilized before beginning an experimental period.

GLOSSARY

Terms specific to this report:

0-Cl$_b$	Basolateral Cl$^-$-free solution
PDS	Power density spectrum
pdu	Power density unit = 10^{-20} A^2/s·cm^2
I_K^{Ba}	Transepithelial Ba^{2+}-inhibitable K$^+$ current
G_K; G_K^a	Transcellular K$^+$ conductance in the presence of apical amiloride; apical membrane conductance

Terms generally found in the literature and used below:

V_T; G_T	Transepithelial voltage; conductance
I_{sc}	Short-circuit current (V_T = 0)
V_a	Intracellular voltage measured across the apical membrane with respect to the bath under shorted conditions
fR_a	Fractional resistance of the apical membrane ($\Delta V_a/\Delta V_t$)
k_{on}; k_{off}	Microscopic forward and reverse rate constants for Ba^{2+} inhibition
K_{Ba}	Ba^{2+} inhibition constant (k_{off}/k_{on})
M_{Ba}	Channel density measured at a given inhibitor concentration (at the K_{Ba})
i_K	Single-channel current measured at the K_{Ba}
E_K	Nernst equilibrium potential for K$^+$
g_K	Single-channel conductance

RESULTS

Induction of K^+ Secretion

Previous studies by Nielsen indicated that bilateral Cl$^-$ removal from NaCl Ringer solution evokes K$^+$ secretion through the principal cells, which is inhibited by micromolar [Ba^{2+}] added to the apical bathing solution (Nielsen, 1987). Here we demonstrate that activation of K$^+$ secretion may be elicited by removing basolateral Cl$^-$ alone. A plot of I_{sc} vs. time is shown in Fig. 1. With control NaCl Ringer solution on both sides of the tissue, a pulse of 0.5 mM Ba^{2+} in the apical bath did not affect I_{sc}. After substituting either gluconate (Fig. 1, *top*) or NO$_3^-$ (Fig. 1, *bottom*) for Cl$^-$ in the basolateral solution, I_{sc} increased slightly and then declined. The response when gluconate was used as a Cl$^-$ substitute was considerably faster, and I_{sc} fell to lower

levels. However, regardless of the Cl^- substitute, changes of I_{sc} in response to the Ba^{2+} pulses developed with time. The response to Ba^{2+} was complex and was characterized by two phases: first, I_{sc} increased rapidly, consistent with inhibition of K^+ secretion, and then it decayed. The magnitude of initial, rapid change of I_{sc} increased gradually with time. When Ba^{2+} was removed, the mirror image was observed. Returning tissues to control NaCl Ringer solution caused I_{sc} to return near control values (Fig. 1, *top*; and Fig. 8, *top*, *open bars*) and the response to Ba^{2+} disappeared. Under control conditions amiloride (10^{-4} M) decreases I_{sc}, which represents active transepithelial Na^+ transport, to values near but greater than zero (Nagel et al., 1983; Fisher and Lockard, 1988). However, after $0\text{-}Cl_b$ amiloride caused I_{sc} to reverse polarity (Fig. 1,
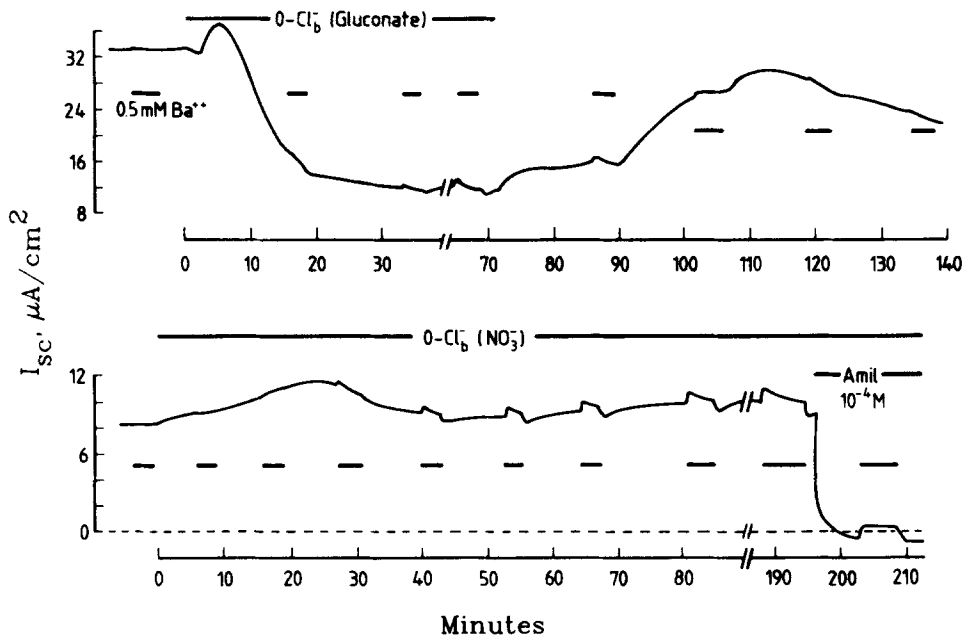

FIGURE 1. Response of I_{sc} to $0\text{-}Cl_b$. At $t = 0$ basolateral Cl^- was replaced by gluconate (*top*) or NO_3^- (*bottom*) in two separate experiments. Dark horizontal bars indicate presence of 0.5 mM Ba^{2+} in the apical solution. *Amil*, 10^{-4} M amiloride added to apical bath. Similar responses to Ba^{2+} after amiloride were observed when gluconate replaced Cl^- (not shown).

bottom right) consistent with K^+ secretion. Moreover, the secondary decline of I_{sc} during the Ba^{2+} pulse was absent after amiloride, indicating that this decline represents an effect of Ba^{2+} on the Na^+ pathway either directly or as a consequence of inhibiting K^+ secretion (cf. final pulse, Fig. 1, *bottom*).

We emphasize that the large decrease of I_{sc} and increase of R_T upon exposure to $0\text{-}Cl_b$ are primarily attributable to changes in Na^+ transport. Due to the complex nature of the I_{sc} response to Ba^{2+} and the comparatively low transport rate of K^+, we used noise analysis to examine the K^+ secretory process.

Identification of K^+ Secretion with Noise Analysis

We examined the effects of 0-Cl_b on the PDS before and during each of the Ba^{2+} pulses shown in Fig. 1. Typical spectra are shown in Fig. 2, A and B, which correspond to the current records depicted in Fig. 1, top and bottom, respectively. In the presence of serosal Cl^-, spectra (Fig. 2, A-1) were characterized by decreasing $1/f$ noise with increasing frequency. At frequencies greater than ~ 300 Hz amplifier noise increased significantly. Thus, detection of a Lorentzian component is dependent on the value of f_c. For example, at 25 Hz we could detect values of S_0 of 0.08 pdu (see Glossary). In general, we rarely observed spontaneous Lorentzians (see below), and addition of Ba^{2+} to the apical bath did not alter the PDS. However, after ~ 10–15 min of 0-Cl_b (i.e., during the decline of I_{sc}), a Lorentzian component appeared in response
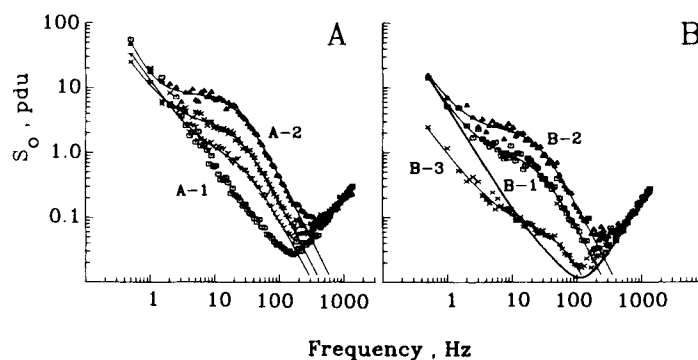

FIGURE 2.   Representative PDS before and after 0-Cl_b. Curves correspond to spectra measured during selected Ba^{2+} pulses shown in Fig. 1. (A) Gluconate. A-1, background spectrum observed under control conditions before 0-Cl_b exposure and 48 min after return to NaCl Ringer solution. A-2, 64 min in 0-Cl_b; $S_0 = 7.97$ pdu, $f_c = 23.7$ Hz. Internal curves (top): 16 min after return to control; $S_0 = 2.68$ pdu, $f_c = 25.1$ Hz. (bottom): 32 min after return to control; $S_0 = 0.81$ pdu, $f_c = 31.0$ Hz. (B) NO_3^-. Spectra at $t = 0$, 7, and 17 min in 0-Cl_b were identical and are represented by the curve drawn without data points for clarity. These spectra were indistinguishable from spectra measured in the absence of apical Ba^{2+}. B-1, 42 min in 0-Cl_b; $S_0 = 0.797$ pdu, $f_c = 28.5$ Hz. B-2, 84 min in 0-Cl_b; $S_0 = 2.18$ pdu, $f_c = 26.6$ Hz. B-3, 10^{-4} M amiloride in the apical bath in addition to 0.5 mM Ba^{2+}; $S_0 = 0.083$ pdu, $f_c = 52.3$ Hz.

to the Ba^{2+} pulse. For example, 64 min in gluconate yielded values of S_0 and f_c of 7.97 pdu and 23.7 Hz, respectively (Fig. 2, A-2). After returning Cl^- to the bath S_0 decreased slowly (similar to the return of I_{sc} toward control) to 2.68 and 0.81 pdu at 16 and 32 min, respectively (Fig. 2 A), and by 48 min the Lorentzian disappeared. Also, like the response of I_{sc}, the effects were reversible and reproducible, since removal of Cl^- a second time caused reappearance of the Lorentzian (although S_0 values were often lower). Similar to gluconate, Cl^- replacement by NO_3^- yielded time-dependent increases of S_0 without significant changes in f_c (Fig. 2, B-1 and B-2).

We also characterized the time course for development of the Lorentzian component after 0-Cl_b (NO_3^- substitute).Tissues were pulsed repeatedly at 15-min intervals
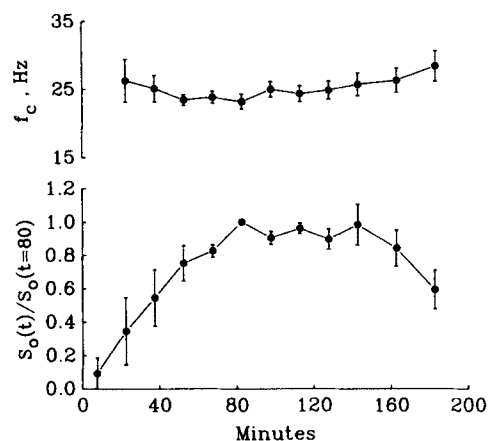
FIGURE 3. Time course of average changes of S_o and f_c after NO_3^- Ringer solution. At 80 min S_o averaged 3.74 ± 0.091 pdu ($n = 5$).

with 0.5 mM Ba^{2+} in the apical bath to determine S_o and f_c. As shown in Fig. 3, S_o increased to its maximum value after ~60–80 min and then remained fairly stable, while f_c averaged near 25 Hz throughout the 3-h experimental period. Thus, with time f_c was constant, S_o increased, and we observed a larger response of I_{sc} to apical Ba^{2+}. Since we used a single $[Ba^{2+}]$, we conclude that the changes of S_o reflect changes of the Ba^{2+}-inhibitable K^+ secretory current. Throughout this report, PDSs were obtained when tissues were pulsed apically with 0.5 mM Ba^{2+}, and the use of a this single $[Ba^{2+}]$ allowed us to examine changes in the K^+ secretory rate after various experimental maneuvers (see above).

We compared the effects of 11.5 mM Cl^- (11.5-Cl_b) with 0-Cl_b (Table I, top). In studies with paired tissues, the effect of Cl^- removal was dose dependent, since exposure to 11.5-Cl_b produced values of S_o that averaged 63% lower than values observed for 0-Cl_b. After 60 min of lowered basolateral Cl^- the skins were treated with amiloride (10^{-4} M) to block Na^+ transport. Then 10 mM $BaCl_2$ was added to the apical Ringer solution, thereby increasing I_{sc} (cf. Fig. 1). The change in I_{sc}, I_K^{Ba},

TABLE I

Comparison of Changes in I_k^{Ba} and S_o

	0-Cl_b	11.5-Cl_b	Paired ratio
I_K^{Ba}, $\mu A/cm^2$	−1.48 ± 0.27	−1.03 ± 0.20	0.66 ± 0.12 (5)
S_o, pdu	2.90 ± 0.61	1.69 ± 0.57	0.63 ± 0.12
	0-Cl_b	Hypertonic	
I_K^{Ba}, $\mu A/cm^2$	−1.42 ± 0.12	−0.46 ± 0.06	0.35 ± 0.06 (4)
S_o, pdu	6.06 ± 0.84	1.73 ± 0.93	0.32 ± 0.12

Ba^{2+}-induced Lorentzians were observed for all tissues exposed to 0-Cl_b. One tissue exposed to 11.5-Cl_b and two tissues treated with hypertonic solutions (9 or 21%) did not yield Lorentzians. The paired ratios were calculated only for tissues that yielded Ba^{2+}-induced Lorentzians. $n = 6$ for both groups of experiments.

represents the Ba^{2+}-inhibitable K^+ current. With 11.5-Cl_b, I_K^{Ba} was 66% of I_K^{Ba} in 0-Cl_b, which is similar to the difference observed in S_o.

In other studies we determined that exposure to hypertonic solutions in the basolateral chamber also caused the appearance of a small Ba^{2+}-induced Lorentzian component in the PDS relative to paired tissues exposed to 0-Cl_b (see below). In
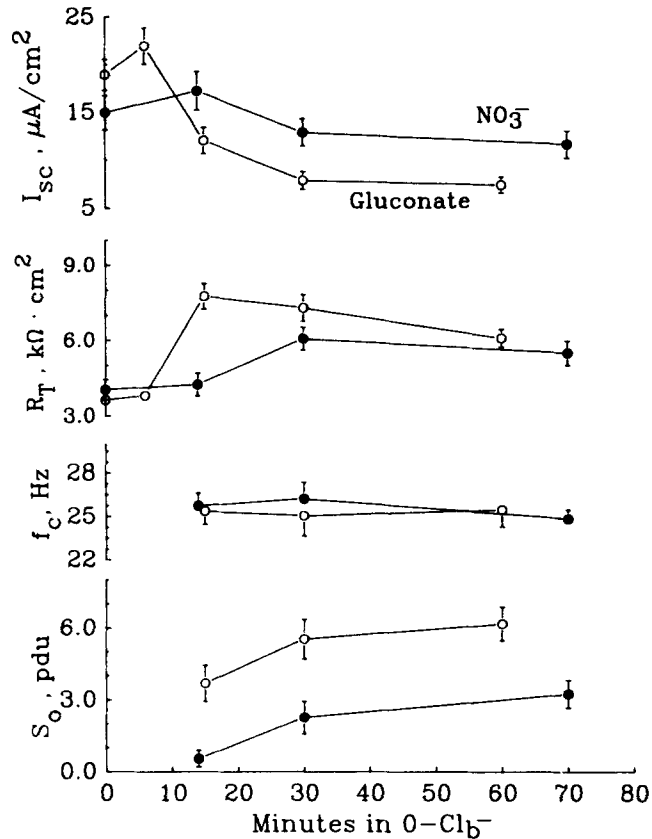

FIGURE 4.   Changes of electrical parameters after 0-Cl_b. At $t = 0$ tissues were exposed to basolateral solutions with either NO_3^- (*filled circles, n = 14*) or gluconate (*open circles, n = 14*) used as the Cl^- substitute (unpaired). At 15 min, 3 and 13 tissues yielded Ba^{2+}-induced Lorentzians in NO_3^- and gluconate, respectively. After this time all tissues yielded Ba^{2+}-induced Lorentzians. When no Lorentzian was observed, we assumed $S_o = 0$, and f_c was not included in the mean.

response to hyperosmotic solutions S_o averaged 32% less than the paired 0-Cl_b tissues, which was matched by a 35% decrease of I_K^{Ba} (Table I, *bottom*). Although the magnitude of I_K^{Ba} is certainly different when tissues are transporting Na^+ (see below), these data support the hypothesis that changes in S_o reflect changes in K^+ secretory current. Thus, "induction of K^+ secretion" is defined here by the appearance of a Ba^{2+}-induced Lorentzian component in the PDS.

K^+ secretion was observed when NO_3^-, gluconate, or isotonic $SO_4^=$ replaced basolateral Cl^- (cf. Fig. 4 and Table VIII, *left*), but no effect was observed upon removing apical Cl^-. In general, K^+ secretion was observed 10–15 min after removing Cl^- (i.e., when I_{sc} declined [Fig. 1]). Average time-dependent changes in the electrical parameters in response to basolateral NO_3^- and gluconate are shown in Fig. 4. The response to gluconate was more consistent and more rapid. After 6 min I_{sc} increased 16% above control, declined after 15 min to 63% of control, and then stabilized at ~40% of control after 30 min. As I_{sc} declined, R_T more than doubled, reaching a maximum at 15 min when a prominent Ba^{2+}-induced Lorentzian was observed with S_o averaging 3.7 pdu (Fig. 4, *open cirlces*). After 30 min in gluconate R_T did not change significantly, but S_o continued to increase. When NO_3^- was used as a Cl^-

TABLE II

Characteristics of Lorentzian Components

	S_o	f_c
	pdu	*Hz*
Active K^+ secretion		
Apical and basolateral NaCl Ringer solution		
Spontaneous (2)	0.1	112.4
Ba^{2+}-induced (9)	0.28 ± 0.083	38.6 ± 1.7
Apical NaCl Ringer solution and 0-Cl_b		
Spontaneous (13)	0.12 ± 0.037	114.6 ± 13.30
Ba^{2+}-induced (37)	2.39 ± 0.427	24.9 ± 0.78
K^+ Absorption		
Apical KCl Ringer solution; basolateral NaCl Ringer solution		
Spontaneous (4)	0.89 ± 0.106	122 ± 11.9
Ba^{2+}-induced (4)	33.6 ± 14.1	31.4 ± 2.2
Apical KCl Ringr solution and 0-Cl_b		
Spontaneous (1)	0.08	127
Ba^{2+}-induced (4)	6.6 ± 0.3	15.0 ± 0.78

0-Cl_b values were measured 60 min after removing Cl^-. Ba^{2+}-induced Lorentzians were obtained with 0.5 mM Ba^{2+} in the apical bath. For tissues with spontaneous Lorentzians, S_o and f_c averaged 3.94 ± 0.649 pdu and 25.8 ± 1.20 Hz, respectively, with apical Ba^{2+}. Number of tissues is given in parentheses.

substitute, the response was attenuated and slower, usually requiring 30 min for the Ba^{2+}-induced Lorentzian to appear (Fig. 4, *closed circles*).

In control NaCl Ringer solution only two tissues from a sample of 52 skins yielded spontaneous Lorentzians, and Ba^{2+}-induced Lorentzians with low values of S_o were observed in 17% of these skins (Table II). Thus, it is likely that for most tissues under control conditions the magnitude of K^+ secretion is near zero. Using NO_3^-, gluconate, or isotonic $SO_4^=$ as a Cl^- substitute, 25% of these tissues displayed spontaneous Lorentzians and virtually all of them (98%) yielded Ba^{2+}-induced Lorentzians. Spontaneous Lorentzians, i.e., a Lorentzian component in the PDS in the absence of apical Ba^{2+}, are indicative of random fluctuations of the apical K^+ channels. It is probable that for tissues demonstrating Ba^{2+}-induced Lorentzians but lacking spon-

taneous Lorentzians, the magnitude of the spontaneous K$^+$ current noise was below detectable levels. Conversely, when spontaneous Lorentzians were observed (Table II, *bottom*), the S_o for the Ba^{2+}-induced Lorentzian was greatest, which prevented us from simultaneously detecting the spontaneous component. Relative to the Ba^{2+}-induced fluctuations, the spontaneous noise was insignificant (cf. Table II), and the large Ba^{2+}-induced Lorentzian always masked the spontaneous component. This, coupled with the vastly different f_c (25 vs. 115 Hz), indicates that the Ba^{2+}-induced f_c was not significantly influenced by the spontaneous component, at least for low [Ba^{2+}] (i.e., 0.5 mM).

Finally, the values of f_c for the Ba^{2+}-induced Lorentzians were independent of the Cl$^-$ substitute and averaged near 25 Hz in all experiments (when tissues were transporting Na$^+$). The only deviation from this behavior occurred with very low values of S_o (which may be related to voltage-dependent effects; see below), again supporting the idea that S_o reflects the magnitude of the K$^+$ secretory current.

Determination of the Ba^{2+} Inhibition Constant (K$_{Ba}$)

Some tissues were subjected to sequential apical [Ba^{2+}] pulses of 0.1, 0.2, 0.5, 1.5, and 3.0 mM after 60 min incubation in 0-Cl$_b$ to determine the rate constants, k_{on} and k_{off}, and the Ba^{2+} inhibition constant, K_{Ba} ($=k_{off}/k_{on}$). For a two-state model (cf. Eq. 3), a plot of $2\pi f_c$ vs. [Ba^{2+}] is linear with slope k_{on} and intercept k_{off} (Van Driessche and Zeiske, 1980). However, the Ba^{2+}-induced noise is superimposed upon spontaneous K$^+$ channel fluctuations. Theoretically, these plots must deviate from linearity because the Ba^{2+}-induced and spontaneous Lorentzians are not independent, especially as the f_c for the Ba^{2+} block approaches the spontaneous f_c ($\sim$115Hz) (Van Driessche and Zeiske, 1980). As shown in Fig. 5 A, plots were generally nonlinear (at high [Ba^{2+}]), which must be partly attributed to this interaction, but may also reflect a voltage dependence of the blocking rate constants (see below). The linear portions of these plots (low [Ba^{2+}]) were used to calculate the rate constants, which on average yielded a K_{Ba} of $\sim$0.3 mM [Ba^{2+}] (Fig. 5 B, and Table III, row A).

Inhibition of Na$^+$ transport by amiloride hyperpolarizes the apical cell membranes (Nagel, 1976; Helman and Fisher, 1977). Tissues previously exposed to 0-Cl$_b$ were then subjected to another sequence of [Ba^{2+}]. These plots were also nonlinear above 0.2 mM Ba^{2+} (Fig. 5 A), but using the linear region of lower [Ba^{2+}] it was clear that amiloride increased k_{on} and decreased k_{off}, thereby causing a decrease of K_{Ba} to 0.061 mM (Fig. 5 B, and Table III, row B) consistent with voltage-sensitive inhibition by Ba^{2+}.

We assumed that the population of apical channels that mediate active K$^+$ secretion is identical to that which mediates gradient-driven K$^+$ absorption (cf. Van Driessche and Zeiske, 1980; Van Driessche, 1984; De Wolf and Van Driessche, 1986). Since secretion and absorption are studied under different conditions, the single-channel characteristics may differ substantially. Nevertheless, we examined the characteristics for the gradient-driven K$^+$ absorption, and these data are included in Tables II and III (rows C and D). Linear plots of $2\pi f_c$ vs. [Ba^{2+}] (0.05–1.0 mM) were observed. The computed rate constants (Table III, compare rows A and C) yielded a K_{Ba} (absorption) that averaged about half of the K_{Ba} (secretion). Pretreatment of these tissues with

0-Cl$_b$ decreased k_{on} and increased k_{off}; i.e., opposite effects compared with secreting tissues (see Discussion).

Changes of the Intracellular Voltage (V_a)

Response to 0-Cl$_b$. Previous studies have shown that 0-Cl$_b$ depolarizes the apical membrane during the same period that we observed induction of K$^+$ secretion (Biber et al., 1985; Duffey et al., 1986; Costa et al., 1987; Klemperer and Essig, 1988;
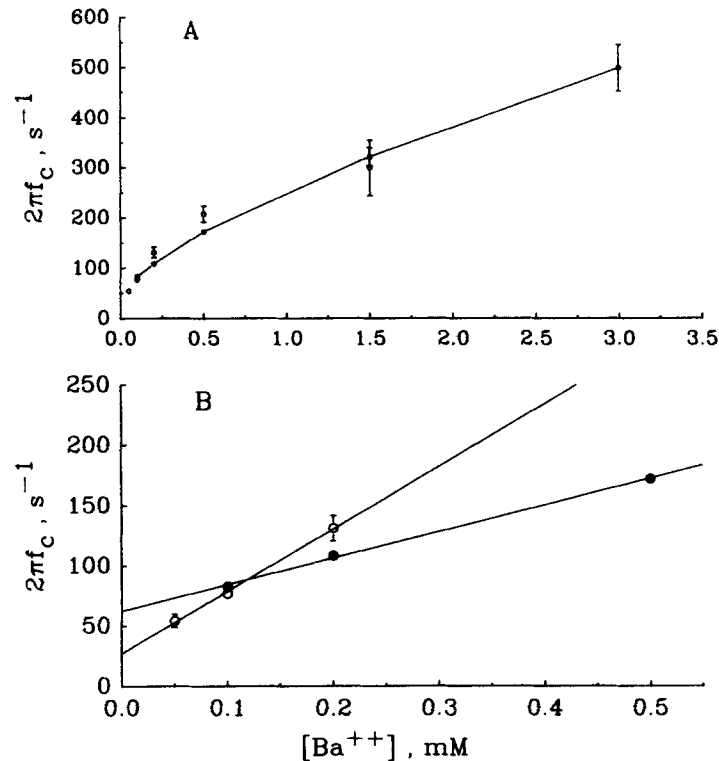

FIGURE 5. Comparison of $2\pi f_c$ vs. [Ba^{2+}] plots for control and amiloride-treated tissues (paired). (A) Full range of [Ba^{2+}] from 0.1 to 3.0 mM for control skins (*filled circles*) and 0.05 to 1.5 mM for amiloride-treated tissues (50 μM, *open circles*). (B) Expanded scale of linear regions of the curves shown in A.

Leibowich et al., 1988). Thus it seemed reasonable that under control conditions, with large negative intracellular voltages (Nagel, 1976; Helman and Fisher, 1977), K$^+$ secretory rates are extremely low because K$^+$ is near electrochemical equilibrium across the apical membrane, and exposure to 0-Cl$_b$ could "induce" K$^+$ secretion simply by depolarizing the cell, thereby favoring K$^+$ exit. Tissues were studied with microelectrodes to examine the changes of intracellular voltage after 0-Cl$_b$ (Table IV). Although punctures of frog skin epithelial cells are routinely accomplished, we encountered difficulty in maintaining cellular impalements after removing basolateral

TABLE III

Calculated Microscopic Parameters

Ringer solution		k_{on}	k_{off}	K_{Ba}	i_K	M_{Ba}
Apical	Basal					
		$s \cdot mM^{-1}$	s^{-1}	mM	pA	$\cdot 10^6 \cdot cm^{-2}$
Active K^+ secretion						
A NaCl	0-Cl$_b$	0.22 ± 0.02	62.3 ± 5.3	0.30 ± 0.05	—*	—*
B NaCl + Amil	0-Cl$_b$	0.52 ± 0.06	27.0 ± 3.4	0.06 ± 0.01	0.93 ± 0.10	3.5 ± 1.0
K^+ Absorption (apical K^+ Ringer solution, basolateral Na^+ Ringer solution)						
C KCl	NaCl	0.30 ± 0.01	41.5 ± 7.3	0.14 ± 0.03	3.22 ± 0.53	18.9 ± 10.1
D KCl	0-Cl$_b$	0.07 ± 0.01	62.4 ± 5.0	0.87 ± 0.10	2.06 ± 0.29	3.2 ± 1.4

A and *B*, Paired values obtained from the same tissues ($n = 7$). *C* and *D*, Values obtained from paired tissues shown in Fig. 7 ($n = 4$). Single-channel currents and densities were calculated according to Eqs. 6 and 7. Since these are defined for a given open probability (i.e., [Ba^{2+}]), average values for single-channel current (i_K) and channel density (M_{Ba}) were calculated for each tissue using three [Ba^{2+}] near the K_{Ba} (i.e., $P_o = 0.5$). *No Lorentzians were observed.

Cl^-. This probably reflects cell shrinkage after 0-Cl$_b$ (see below). However, we attempted to puncture cells before and within ~60 min of removing basolateral Cl^-. Cells depolarized dramatically concurrent with the appearance of the Ba^{2+}-induced Lorentzian. On average, V_a decreased 29.8 mV after 60 min (Table IV; $S_o = 8.11 ±$ 1.32 pdu, $f_c = 25.1 ± 1.1$), a value similar to the mean depolarization from the five other studies mentioned above (33.1 mV). The fR_a decreased to 0.42 from the control value of 0.66. Similar to previous observations by us and others, when cells were exposed to amiloride to inhibit Na$^+$ transport while bathed with control Ringer solution in the basolateral bath, V_a hyperpolarized and fR_a increased to values > 0.95 (not shown). Addition of amiloride after 0-Cl$_b$ hyperpolarized V_a by 36 mV, but only increased fR_a to 0.72, indicating that a significant conductance of the apical membrane remained relative to the basolateral membrane. Subsequent addition of 5–10 mM Ba^{2+} to the apical bath increased the fR_a to 0.96, indicating that the remaining apical conductance after exposure to 0-Cl$_b$ and apical amiloride was attributable to the apical K$^+$ channels.

Voltage clamp of V_a after 0-Cl$_b$. We examined the influence of apical membrane voltage on the rate of K$^+$ secretion. After exposure to 0-Cl$_b$ for at least 40 min, tissues were voltage clamped first to repolarize V_a near control values, and then, after a period of short-circuiting, to depolarize V_a ~30 mV below the short-circuited value.

TABLE IV

Effect of 0-Cl$_b$ on Intracellular Voltage, V_a, and Fractional Resistance, fR_a

	Control	0-Cl$_b$	Amiloride	Amiloride + Ba^{2+}
V_a, mV	−71.0 ± 3.6	−41.2 ± 3.1	−77.3 ± 3.2	−63.5 ± 4.6
fR_a	0.663 ± 0.051	0.429 ± 0.035	0.716 ± 0.036	0.958 ± 0.007

Values were measured 60–120 min after removal of Cl$^-$ (gluconate substitute). 10 mM Ba^{2+} was added to Ringer solution in apical bath containing 50 μM amiloride. Paired depolarization after addition of Ba^{2+} = 13.8 ± 3.38 mV ($n = 8$).

TABLE V

Influence of Transepithelial Voltage on V_a and S_o

$[Cl^-]_b$	100	0	0	0	0
V_T	0	0	Negative	0	Positive
V_a, mV	-65.9 ± 3.07	-39.7 ± 2.20	-60.3 ± 0.56	-46.7 ± 2.65	-18.5 ± 2.75
S_o, pdu	0	10.76 ± 1.36	0	5.89 ± 1.28	13.75 ± 3.30

V_T ranged about ± 100 mV and was chosen to either repolarize V_a near control, short-circuit values or depolarize it by ~ 30 mV; $n = 5$.

As shown in Table V, no Lorentzians were observed before exposure to 0-Cl$_b$ when V_a averaged -66 mV. After 0-Cl$_b$, V_a depolarized 26 mV, which was accompanied by the appearance of the Lorentzian. Repolarization of the apical membrane to -60 mV eliminated the Lorentzian component. After this repolarization period tissues were returned to short-circuit conditions, at which time they spontaneously hyperpolarized by 7 mV relative to the earlier short-circuit period. This was accompanied by a decrease of S_o to 5.9 pdu. Alternatively, depolarization of V_a to -18.5 mV led to an increase of S_o to 13.8 pdu, indicating that the K$^+$ secretory rate exceeded the level observed when tissues were short-circuited. Each tissue behaved qualitatively in the manner displayed by the mean values shown in Table V. We exploited the time-dependent changes of V_a and S_o, which varied from tissue to tissue (cf. Fig. 3), to generate a plot of all data points from eight tissues studied with microelectrodes regardless of clamp voltage or K$^+$ secretory rate (see Fig. 6). Indeed, S_o appears to increase as a function of V_a, supporting the idea that intracellular voltage directly influences the apical K$^+$ secretory rate.

Hyperpolarization by inhibiting Na^+ transport with apical amiloride or Na^+-free solutions. It is well documented that apical amiloride or Na$^+$-free solutions hyperpolarize cells, which should decrease the K$^+$ secretory rate. When amiloride was added
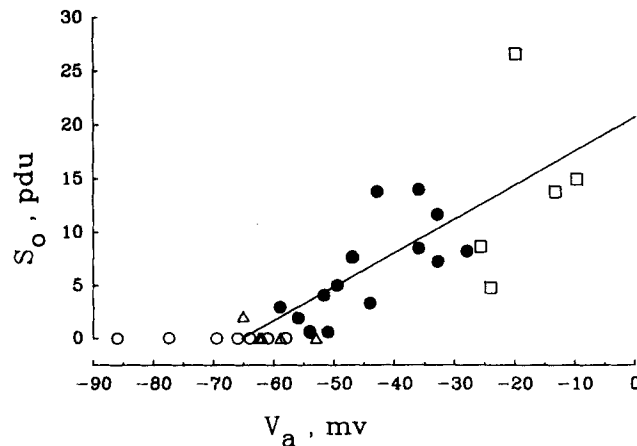

FIGURE 6.   K$^+$ secretory rate (S_o) as a function of intracellular voltage. *Open circles,* untreated tissues; *filled circles,* 40–60 min in 0-Cl$_b$; *open triangles,* repolarized near control values by transepithelial voltage clamp; *open squares,* depolarized from 0-Cl$_b$ state.

to the apical bath after induction of K^+ secretion, the rate constants for Ba^{2+} inhibition changed consistent with cell hyperpolarization (f_c increased from 25.9 ± 0.81 to 37.5 ± 2.9 Hz; n = 9). The total power density decreased dramatically after amiloride, as indicated by a decrease of the $1/f$ noise as well as S_o from 2.67 ± 0.34 to 0.3 ± 0.09 pdu (n = 9; cf. Fig. 2, *B-3*). In another series of experiments five skins were preincubated in apical Na^+-free solutions. After 1 h Cl^- was removed from the basolateral bath while tissues were maintained in apical 0-Na^+. K^+ secretion developed in the absence of Na^+ transport, but the appearance of the Ba^{2+}-induced Lorentzian required considerably more time (40–100 min). The values of S_o (0.249 ± 0.064) and f_c (36.8 ± 2.1) were similar to those measured when tissues were first exposed to 0-Cl_b and then treated with amiloride. Thus, K^+ secretion is a function of the voltage across the apical membrane, and in the absence of Na^+ transport is markedly reduced as expected for hyperpolarized cells.

Depolarization by serosal Ba^{2+}. We tested whether depolarization alone was sufficient to induce K^+ secretion by adding 1 mM Ba^{2+} to the basolateral NaCl Ringer solution. This maneuver depolarizes these cells by decreasing the basolateral membrane K^+ conductance (Nagel, 1979). Spectra were obtained 5 min after adding Ba^{2+} to the basolateral bath, i.e., when I_{sc} reached a minimum and R_T peaked (Table VI).

TABLE VI

Effect of 1.0 mM Ba^{2+} in the Basolateral Chamber

	0	Min after addition of Ba^{2+}		
		5	15	30
I_{sc}, $\mu A/cm^2$	18.2 ± 1.5	7.5 ± 0.7	8.8 ± 0.9	12.8 ± 1.7
R_T, $\Omega \cdot cm^2$	2,804 ± 216	4,544 ± 420	2,846 ± 218	1,813 ± 299
S_o, pdu	—*	2.83 ± 0.96	5.87 ± 1.76	4.95 ± 1.32
f_c, Hz	—*	25.2 ± 0.64	25.5 ± 1.0	28.9 ± 1.0

Spectra were measured with 0.5 mM Ba^{2+} in the apical solution. *No Lorentzian component was observed in spectrum. n = 6.

Lorentzians were observed for all tissues at this time. After 15 min S_o and I_{sc} increased, but R_T returned to control values. I_{sc} and R_T continued to change at 30 min, but S_o remained constant. The values of S_o observed after 5 min of basolateral Ba^{2+} treatment averaged 2.83 pdu, similar to values of S_o measured after 60 min in 0-Cl_b. We examined the response to 0-Cl_b in paired pieces of tissue for two of the skins summarized in Table VI and observed values of S_o at 60 min similar to the 5-min values observed in response to serosal Ba^{2+} for the paired piece of skin (5.6 vs. 5.8). We believe this is fortuitous, but it demonstrates that we can produce values of S_o identical to those observed after 0-Cl_b simply by depolarizing the cells. Thus, we conclude that induction of K^+ secretion is due to an increase of the electrical driving force for K^+ exit across the apical cell membrane.

Localization of the 0-Cl_b Effects

Since R_T increased and fR_a and V_a decreased with 0-Cl_b, it seemed likely that the K^+ secretion was attributable to intracellular depolarization brought about by a decrease

of the basolateral conductance. This was tested by examining the response of Na^+ transporting tissues bathed in NaCl Ringer solution to apical K^+ Ringer solution. Exchanging apical Na^+ for K^+ causes a decrease of I_{sc}, followed by a long-term increase lasting 1–3 h (cf. Fig. 7, control; also see Discussion). I_{sc}, under these conditions, represents net, transepithelial K^+ movement primarily through the Ba^{2+}-inhibitable apical K^+ channels of the principal cells (De Wolf and Van Driessche, 1986). Recall that after 0-Cl_b, inhibiting apical Na^+ channels with amiloride increases fR_a to 0.72. Thus, the apical membrane represents the high resistance barrier to K^+ movement under these conditions. If induction of K^+ secretion by 0-Cl_b is mediated by an increase of the apical membrane K^+ conductance, upon switching to apical K^+ Ringer solution initial values of I_{sc} should exceed those of paired tissues bathed with control [Cl^-] in the basolateral solution.

Paired tissues were incubated in NaCl Ringer solution for 60 min and I_{sc} averaged ~30 $\mu A/cm^2$. Next, the experimental piece was exposed to 0-Cl_b for the remainder of the experiment, which decreased I_{sc} to an average of 11.4 μA after 60 min (Fig. 7;
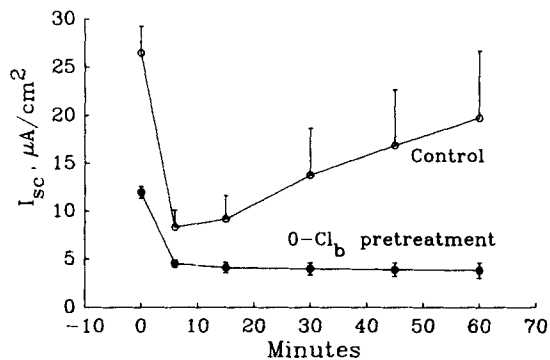

FIGURE 7. Effect of 0-Cl_b on I_{sc} after exchanging K^+ for Na^+ in the apical bath (paired). Before 0-Cl_b (i.e., $t = -60$ min; not shown), average I_{sc} and R_T for control tissues (*open circles*) were 26.5 ± 2.8 $\mu A/cm^2$ and 3,452 ± 1,056 $\Omega \cdot cm^2$, and for experimental tissues (*filled circles*) 29.4 ± 3.8 $\mu A/cm^2$ and 2,696 ± 187 $\Omega \cdot cm^2$. Thus, $t = 0$ min corresponds to 60 min of 0-Cl_b for the experimental piece. $n = 4$.

$t = 0$ min), by which time K^+ secretion was observed ($S_o = 5.97 \pm 1.8$ pdu, $f_c = 27.0 \pm 1.5$ Hz). Then Na^+ was replaced by K^+ in the apical bath for both pieces, which quickly decreased I_{sc} to 8.3 $\mu A/cm^2$ (control) vs. 4.5 $\mu A/cm^2$ (0-Cl_b; Fig. 6). Since 0-Cl_b diminished the absorptive K^+ current and increased R_T (cf. Fig. 4), it seems unlikely that the apical K^+ conductance is stimulated by 0-Cl_b. Alternatively, this is consistent with a decrease of basolateral K^+ conductance.

Additional support for the idea that depolarization of the apical membrane is a consequence of 0-Cl_b inhibiting the basolateral membrane conductance was obtained from studies of the transcellular K^+ conductance, G_K, as a function of [Cl^-]$_b$. We assumed that the electrical properties of the apical membrane are dominated by the Na^+ and K^+ channels, which are blocked by amiloride and Ba^{2+}, respectively. Thus, with amiloride (or 0-Na^+) and high [Ba^{2+}] in the apical bath, $1/R_T$ estimates the transepithelial shunt conductance (G_s). In the presence of amiloride alone (i.e., after removing Ba^{2+}) $G_K = 1/R_T - G_s$. Thus, G_K was determined for untreated, control tissues as well as for tissues secreting K^+ (Table VII). For these experiments, each

tissue was used as its own control. Two protocols were examined: (a) Skins were exposed to amiloride (10–30 min) and pulsed with 10 mM Ba^{2+} (1 min). After determining G_K, the inhibitors were washed out of the apical chamber. When I_{sc} returned to near control, 0-Cl_b or 11.5-Cl_b was introduced into the basolateral chamber and tissues were again exposed to the inhibitors (Table VII, A and B). (b) Skins were maintained in Na^+-free apical solutions for 1 h, pulsed with 5 mM Ba^{2+} to determine G_s, and then exposed to 0-Cl_b (100 min; Table VII C). Low Cl_b activated K^+ secretion for all groups as indicated by I_K^{Ba} values ranging from -0.94 to -1.7 μA/cm^2 (Table VII, A–C). Since the fractional resistance of the apical membrane is near unity under these conditions, we expected that an increase of G_K in response to 0-Cl_b should reflect an increase of the apical membrane K^+ conductance. Conversely, if the basolateral K^+ conductance decreased, we expected either a decrease of G_K or no change (since the basolateral resistance would have to increase to values near that of the apical membrane to be detectable electrically). The latter was observed (Table VII; compare paired G_K values on right). G_K did not change for group A, but

TABLE VII

Calculation of G_K

Ringer solution					
Apical	Basal	n	I_K^{Ba}	G_T	G_K
			μA/cm^2	mS/cm^2	mS/cm^2
A NaCl + amiloride	NaCl	(5)	-0.24 ± 0.09	0.1103 ± 0.0127	0.0406 ± 0.0051
	0-Cl_b		-1.70 ± 0.32	0.1619 ± 0.0317	0.0396 ± 0.0082
B NaCl + amiloride	NaCl	(5)	-0.11 ± 0.11	0.1513 ± 0.0182	0.0499 ± 0.0064 *
	11.5-Cl_b		-0.94 ± 0.19	0.1351 ± 0.0122	0.0251 ± 0.0091
C 0-Na^+	NaCl	(5)	-0.22 ± 0.08	0.1299 ± 0.0154	0.0678 ± 0.0153 ‡
	0-Cl_b		-1.14 ± 0.12	0.1435 ± 0.0209	0.0409 ± 0.0071

Paired tissues were used for each group. Paired ratios: *0.59 $\pm$ 0.25 ($P < 0.05$); ‡0.64 $\pm$ 0.07 ($P < 0.001$). Amiloride concentration, 50 μM.

decreased significantly for B and C (Table VII, right). Thus, low Cl_b probably serves to decrease basolateral membrane conductance, which in turn depolarizes cells.

Influence of Basolateral Osmolarity

Since basolateral KCl permeability is high and Cl^- is the principal anion in NaCl Ringer solution, 0-Cl_b must, in the absence of regulatory phenomena, cause a loss of cell Cl^- and a decrease of cell volume (Ussing, 1965; Ferreira and Ferreira, 1981; Stoddard et al., 1985). Thus, it was possible that in the present study cell shrinkage per se caused cell depolarization, which ultimately led to K^+ secretion. This was tested with a series of basolateral hyperosmotic challenges. Since estimates of cell [Cl^-] average near 35 mM, complete loss of cell Cl^- should result in a maximum shrinkage of ~30% (Ferreira and Ferreira, 1981). Paired tissues were exposed to either a hyperosmotic basolateral solution or to 0-Cl_b. Mannitol was added to the basolateral NaCl Ringer solution to increase its osmolarity either 9, 21, 33, or 46%

above control (Fig. 8). After 60 min in 0-Cl$_b$, I_{sc} decreased to ~30% of control values for all paired tissues (Fig. 8, left diagonal). I_{sc} was inversely proportional to basolateral osmolarity, decreasing monotonically to 10% of control values as the osmolarity was increased to a maximum of 46% above control. The decrease was rapid and similar to the declining phase of I_{sc} in response to 0-Cl$_b$. These changes were accompanied by increases of R_T at all hyperosmotic concentrations (Fig. 8, middle). Prominent Ba^{2+}-induced Lorentzians were observed for all paired 0-Cl$_b$ tissues, but not consistently for the hypertonic solutions. When Lorentzian components were seen, the response was always less than observed for the paired 0-Cl$_b$ tissue (cf. Fig. 8, lower plot). The K$^+$ secretory response was greatest in the 20–30% range of hyperosmotic concentrations, but was considerably reduced at 46%. Thus,
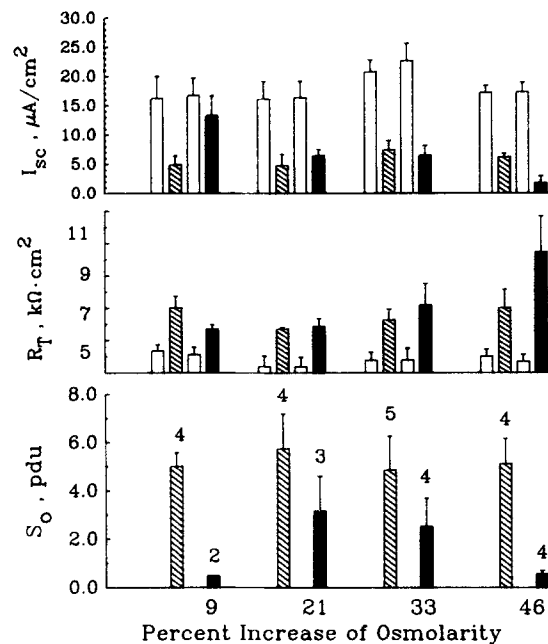

FIGURE 8. Changes of electrical parameters in response to hyperosmotic solutions and 0-Cl$_b$ in paired tissues. *Unshaded bars*, values measured when tissues were bathed with control NaCl Ringer solutions; *diagonal bars*, values measured after 60 min in 0-Cl$_b$; *solid bars*, values measured after 60 min in osmotic solutions indicated on abscissa. All tissues yielded Lorentzians after 0-Cl$_b$ (gluconate). The number of tissues examined at each osmolarity is indicated above the 0-Cl$_b$ bars in the graph of S_o. K$^+$ secretion was either lower or undetectable during the hyperosmotic challenge (number indicated above solid bars).

although Ba^{2+}-induced Lorentzians were often observed in response to hyperosmotic solutions, we could not mimic the response to 0-Cl$_b$ simply by increasing the osmotic strength of the basolateral bathing solution, and we conclude that cell shrinkage cannot account for the K$^+$ secretory response.

Since hypertonicity alone was insufficient to maximize the K$^+$ secretory response, we examined whether K$^+$ secretion could be induced by removing Cl$^-$ while minimizing or preventing cell shrinkage. To this end, we compared the effects of hypotonic to isotonic SO$_4^=$ Ringer solution. As stated above, I_{sc} decreased and R_T increased in response to isotonic SO$_4^=$ solutions (Table VIII), similar to the responses for other Cl$^-$ substitutes (cf. Fig. 4). Ba^{2+}-induced Lorentzians were observed with S_o averaging 6.2 pdu and f_c ~25 Hz. In contrast, after 60 min of hypotonic SO$_4^=$ Ringer solution I_{sc} was similar to control, R_T decreased significantly, and no Lorentzian

components appeared in the PDS (Table VIII). In accord with these observations, V_a does not depolarize when tissues are exposed to hypotonic SO_4^- Ringer solution (Nagel, 1977). Thus, 0-Cl$_b$ is insufficient to induce K^+ secretion in the face of a concurrent hypoosmotic shock (see Discussion).

DISCUSSION

The magnitude of K^+ secretion in frog skin is low relative to that of Na^+ absorption under control conditions. For this reason, we used noise analysis to characterize the induction of the K^+ secretory current under Na^+ transporting conditions. After removing basolateral Cl^-, distinct Lorentzians appeared in response to apically applied Ba^{2+}, consistent with the idea that K^+ is secreted from the basolateral to the apical surface of the epithelium. In the absence of apical Ba^{2+}, spontaneous Lorentzians, due to random fluctuations of the K^+ channels, were observed only for the tissues with the highest secretory rates. Thus, we defined the K^+ secretory current under control, Na^+-transporting conditions, by the appearance of a Ba^{2+}-induced Lorentzian component in the PDS. This effect appears to be specific for R. temporaria

TABLE VIII

Comparisons of Isotonic and Hypotonic SO_4^- Ringer Solution

	Control	Isotonic 0-Cl$_b$	Control	Hypotonic 0-Cl$_b$
I_{sc}, $\mu A/cm^2$	21.38 ± 2.25	7.32 ± 0.74	19.81 ± 2.54	16.22 ± 4.02
R_T, $\Omega \cdot cm^2$	2,735 ± 346	5,846 ± 823	2,703 ± 287	1,913 ± 327
S_o, pdu	—*	6.23 ± 0.87	—*	—*
f_c, Hz	—*	25.31 ± 1.31	—*	—*

Values in 0-Cl$_b$ were measured with 0.5 mM Ba^{2+} in the apical solution after 60 min exposure to 0-Cl$_b$. *No Lorentzian observed in PDS. $n = 4$.

since similar studies of R. ridibunda failed to produce changes in the PDS even after exposure to apical Ba^{2+} ($n = 4$, unpublished observations; Van Driessche and Zeiske, 1985).

Localization of the K^+ Secretory Pathway

Previously, Nielsen (1987) suggested that K^+ is secreted through two paths in frog skin (the glands and the principal cells), but only the principal cell pathway is inhibited by apical Ba^{2+}. We agree with his contention for the following reasons. (a) The rapid increase of I_{sc} in response to Ba^{2+} was concurrent with augmented current fluctuations (detected in < 1 s) which were maintained as long as Ba^{2+} was present in the outer bathing solution. Alternatively, if the channels were located basal to the outermost epithelial cell layer, such as the glands, we should have observed significant diffusion delays and possibly a time-dependent increase of the current fluctuations upon exposure of skins to apical Ba^{2+}. (b) Lorentzians were observed in

split skins which are, for the most part, gland-free ($n = 4$).[3] (c) Using microelectrodes, which certainly must access the principal cells ($>98\%$ of the apical surface area), we detected a Ba^{2+}-sensitive apical K^+ conductance consistent with previous observations by Nagel and Hirschmann (1980). We therefore conclude that the Lorentzians observed in the present studies reflect K^+ transport through apical K^+ channels of the principal cells.

We could not determine the secretory, macroscopic, Ba^{2+}-inhibitable current, I_K^{Ba}, under Na^+-transporting conditions because secondary effects of Ba^{2+} inhibition were also observed on the Na^+ pathway. After blocking Na^+ transport with amiloride, however, I_K^{Ba} averaged about -1.5 $\mu A/cm^2$ (cf. Tables I and VII). Nielsen (1984) measured net isotopic K^+ secretory fluxes averaging -1.6 $\mu A/cm^2$. In the absence of amiloride, Nielsen observed larger K^+ fluxes averaging -2.45 $\mu A/cm^2$. Given the voltage sensitivity of K^+ secretion, this difference was expected since inhibition of Na^+ transport with amiloride hyperpolarizes frog skin epithelial cells (Helman and Fisher, 1977; Nagel, 1978). For skins bathed in 0-Cl_b we observed a 36.1-mV hyperpolarization of the apical membrane in response to amiloride. Assuming $G_K = 0.04$ mS/cm^2 (Table VII), amiloride decreased the K^+ current across the apical membrane in the present studies by 1.4 μA, giving a total Ba^{2+}-inhibitable current of 2.9 $\mu A/cm^2$ under Na^+-transporting conditions, similar to the net flux observed by Nielsen (1984, 1987).

Unitary Characteristics of Apical K^+ Channels

It seems likely that the same K^+-selective channels in the apical membrane mediate active K^+ secretion and gradient-driven K^+ absorption. After hyperpolarization by amiloride, the K_{Ba} (secretion) was 20% of the preamiloride values, consistent with voltage-dependent block by Ba^{2+} described previously for absorptive tissues (Van Driessche and Zeiske, 1980; De Wolf and Van Driessche, 1986). Conversely, pretreatment of K^+ absorptive tissues with 0-Cl_b increased the absorptive K_{Ba} sixfold (cf. Table III, rows C and D). Although intracellular voltages were not measured under absorptive conditions, these data are consistent with the idea that cells exposed to 0-Cl_b remain depolarized after switching to apical K^+ Ringer's.

Calculation of single-channel parameters requires an estimate of I_K^{Ba}. This could be determined only after treating tissues with amiloride to eliminate the absorptive Na^+ current. Thus, after amiloride the single-channel secretory current (i_K) averaged about -0.9 pA (Table III, row B). While 0-Cl_b induces K^+ secretion, it inhibits gradient-driven K^+ absorption, consistent with a decrease of i_K (absorption) from 3.2 to 2.1 pA (Table III, rows C and D). These data also support the idea that relative to 100-Cl_b, K^+ absorbing cells were depolarized after 0-Cl_b.

Previous studies indicated that exposure to apical K^+ Ringer solution induces a long-term increase of I_{sc} over a period of 1–3 h (Van Driessche and Zeiske, 1980; Van Driessche, 1984). We inhibited this response by pretreating with 0-Cl_b, which served to lower values of i_K as well as channel density (cf. Table III, rows C and D). Despite

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[3] Isolated epithelia were prepared by the method of Fisher et al. (1980). Preparation of isolated epithelia is less successful for skins of R. temporaria than for R. pipiens, quite often causing deterioration of electrical parameters after splitting.

the different conditions, tissues, and protocols, the channel density (M_{Ba}) for absorptive tissues bathed in 0-Cl$_b$ averaged 3.2 million channels/cm^2, which is remarkably similar to the secretory value of ~3.5 million channels/cm^2 (cf. Table III, right column, rows B and D). These data are consistent with the following: (a) M_{Ba} of absorptive tissues pretreated with 0-Cl$_b$ represents the population of channels present in control tissues before the development of the long-term increase of I_{sc}. (b) Given the similarity of secretory and absorptive values of M_{Ba}, K$^+$ must pass through the same population of channels. (c) 0-Cl$_b$ does not increase the apical membrane conductance or the open probability of the K$^+$ channels. (d) Since the calculated values of M_{Ba} were similar to the values published previously by Van Driessche and Zeiske (1980), the density of apical K$^+$ channels may be relatively constant over a variety of populations and conditions.

The single-channel conductance (g_K) was estimated for tissues treated with amiloride according to the following equation:

$$g_K = i_K/(E_K - V_a) \tag{9}$$

where E_K is the Nernst potential for K$^+$ across the apical membrane. Using electron microprobe, intracellular [K$^+$] averaged 133 mM, which increased to 184 mM after 0-Cl$_b$ (Dörge et al., 1988). Given an i_K of -0.9 pA and a V_a of -77 mV (Table IV), $E_K = -112$ mV and $g_K = 26$ pS. Similarly, for absorptive K$^+$ flow, we assumed that $E_K = 0$, $i_K = 3.2$ pA (Table IV), $V_a = -60$ mV (unpublished observations, and Nagel and Hirschmann, 1980), and $g_K = 53$ pS. Given that absorptive channels are bathed bilaterally in high [K$^+$], it was not surprising that the g_K for absorption was greater than for secretion.

Estimates of i_K and g_K were also determined for 0-Cl$_b$ tissues not previously treated with amiloride. Analogous with Eq. 9,

$$G_K^a = I_K/(E_K - V_a) \tag{10}$$

and

$$G_K^a = G_T/fR_a \tag{11}$$

Combining Eqs. 6, 7, 10, and 11 and solving for i_K:

$$i_K = [S_o(G_K^a \pi f_c)]/[(E_K - V_a)(2P_B)] \tag{12}$$

We assumed that $E_K = -112$ mV and $P_B = 0.625$ for control tissues (Table III). For each tissue summarized in Table V, all the other parameters were determined independently and Eqs. 9 and 11 yielded values of i_K and g_K of 1.5 ± 0.4 pA and 22.4 ± 6.7 pS ($n = 4$). Thus, it is apparent that i_K increased as expected for depolarized cells with increased values of S_o. Moreover, g_K was similar to the values estimated for amiloride-treated tissues (22 vs. 26 pS), indicating that the channel behaves ohmically for intracellular voltages ranging between about -40 and -80 mV.

Mechanism of Induction of K^+ Secretion by 0-Cl_b

There are four known effects of 0-Cl$_b$ on principal cells: (a) intracellular voltage depolarizes (see above; Biber et al., 1985; Duffey et al., 1986; Costa et al., 1987; Klemperer and Essig, 1988; Liebowich et al., 1988); (b) intracellular [Cl$^-$] and [Na$^+$]

decrease (Ferreira and Ferreira, 1981; Giraldez and Ferreira, 1984; Dörge et al., 1985; Biber et al., 1985); (c) cell volume is reduced (Ussing, 1965; Ferreira and Ferreira, 1981); and (d) intracellular pH rises (Civan and Peterson-Yantorno, 1986). Any or all of these could play a role in the induction of K^+ secretion.

The activation of K^+ secretion appears to be a direct consequence of cell depolarization. Consistent with this idea, we observed a time course of cell depolarization in response to 0-Cl_b similar to the time course of activation of K^+ secretion. Other means of depolarizing cells, such as serosal application of Ba^{2+} or increases of basolateral [K^+] (unpublished observations), also yielded Ba^{2+}-induced Lorentzians in the PDS. Indeed, the intracellular voltage of frog skin epithelial cells is negative, often averaging near -80 to -90 mV, consistent with K^+ being near electrochemical equilibrium across the apical membrane when bathed in control NaCl Ringer solution containing 2.5 mM K^+. Similarly, the intracellular voltage of the principal cells of the rabbit cortical collecting duct averages near -60 mV (bath [K^+] = 5 mM). When treated with amiloride or luminal low [Na^+] the voltage hyperpolarizes to values approaching -90 mV, indicating that K^+ may be near equilibrium in this tissue as well (Koeppen et al., 1983; O'Neil and Sansom, 1984). In contrast, the apical membrane voltage of the rabbit colon is considerably reduced relative to the frog skin and cortical collecting duct, averaging -20 mV (bath [K^+] = 7 mM). However, this is paralleled by a substantially higher rate of K^+ secretion, which averaged ~ 10 $\mu A/cm^2$ (Wills and Biagi, 1982). Thus, the level of K^+ secretion in these tissues may be modulated simply by changes of the electrochemical gradient for K^+.

We determined the transcellular K^+ conductance, G_K, with amiloride in the apical bath to eliminate the influence of the Na^+ pathway. Since amiloride predominantly decreases the apical membrane Na^+ conductance, it is likely that under these conditions, before 0-Cl_b the basolateral membrane conductance greatly exceeded the apical conductance, and G_K approximated G_K^a. This was confirmed by measurements of fR_a averaging 0.75 under these conditions. Since G_K decreased or was unchanged after 0-Cl_b, we conclude that the primary effect of 0-Cl_b is a decrease of the basolateral K^+ conductance, which inhibits Na^+ absorption while stimulating K^+ secretion. A similar conclusion was drawn previously for toad urinary bladder (Lewis et al., 1985) as well as frog skin (Costa et al., 1987; Klemperer and Essig, 1988; Leibowich et al., 1988). Moreover, since the apical K^+ channel density was unchanged after 0-Cl_b, it appears that any direct effects on the apical K^+ channels were insignificant. Thus, a cellular maneuver that has drastic effects on the basolateral K^+ channels produced little or no effect on the apical channels, indicating that there is a fundamental difference in the two populations of channels.

At this juncture the cause of cell depolarization remains a mystery. Although we could duplicate the electrical effects of 0-Cl_b on I_{sc} and R_T with basolateral hypertonicity, K^+ secretion in response to 0-Cl_b exceeded osmotically induced K^+ secretion. Thus, we cannot ascribe the K^+ secretory response to cell shrinkage per se consequent to the lost intracellular Cl^-. Alternatively, tissues exposed to hypotonic $SO_4^=$ Ringer solution did not secrete K^+, which would seem to argue against a direct effect of intracellular Cl^-. However, since volume was not measured directly, it is possible that the hypotonic $SO_4^=$ solution caused cell swelling. This may open conductive channels to facilitate salt loss leading to regulatory volume decrease of swollen cells

(Grinstein et al., 1982; Larson and Spring, 1984; Dawson et al., 1988). Thus, it remains possible that the decrease of basolateral K^+ conductance is directly related to the loss of intracellular Cl^-, intracellular alkalinization, or a combination of several factors. We also cannot reject various schemes involving changes in cell volume. For example, cell shrinkage might decrease the conductance of both the apical and basolateral K^+ channels (as well as Na^+), but its influence on the apical K^+ channels may be blocked when intracellular $[Cl^-]$ is near control values. Elucidation of this requires studies of the effects of Cl^- and other factors directly on these channels.

Physiological Role of K^+ Secretion

In view of the relatively low K^+ conductance and transport rates measured by us and others when tissues are bathed in NaCl Ringer solution, it is worthwhile to consider the transport properties of tissues in vivo. The fact that apical K^+ permeability appears to be influenced by preincubation of animals in K^+-containing solutions led Van Driessche (1984) to suggest that these channels could be important in K^+ balance. Indeed, animals recently collected display higher apical K^+ permeability than those maintained in laboratory tanks. Furthermore, when skins are studied under "physiological" conditions (i.e., with low apical concentrations of Na^+ [<2 mM]), Na^+ transport rates are relatively low and are similar to the K^+ transport rates described above (Ehrenfeld and Garcia-Romeu, 1977). Although the magnitude of K^+ secretion was 2–4 $\mu A/cm^2$ under the conditions of our study (i.e., with 2.5 mM K^+ in the bathing solutions), physiologically the situation may be quite different, especially since this pathway is modulated by processes that appear to influence transepithelial Na^+ transport. Finally, we emphasize that any perturbation that depolarizes cells by increasing the transepithelial voltage (i.e., treatment with antidiuretic hormone) must stimulate K^+ secretion above that measured under the in vitro, short-circuit conditions of the present studies. Simply open-circuiting tissues (which depolarizes cells relative to the shorted state) increases the transepithelial driving force for K^+ secretion while decreasing the driving force for Na^+ absorption.

In conclusion, K^+ secretion in frog skin is "induced" by lowering the basolateral $[Cl^-]$ and is augmented by maneuvers that increase the electrochemical driving force for K^+ exit across the apical membranes. Loss of intracellular Cl^- eventually leads to inhibition of the basolateral membrane K^+ conductance, which in turn depolarizes cells. This emphasizes the fundamental regulatory role of the basolateral membrane conductance in transepithelial Na^+ and K^+ transport. The intermediate steps between the changes of intracellular $[Cl^-]$ and the basolateral K^+ conductance are unknown.

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