K⁺ Secretion across Frog Skin

Induction by Removal of Basolateral Cl⁻

RICHARD S. FISHER and WILLY VAN DRIESSCHE

From the Department of Nephrology, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100; and Laboratorium voor Fysiologie, KUL, Campus Gasthuisberg, B-3000 Leuven, Belgium

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 Ba²⁺ 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 SO₄²⁻ (0-Clb) yielded Lorentzians with corner frequencies near 25 Hz, and plateau values (S₀) 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 S₀. Inhibition of Na⁺ transport with amiloride or by omitting apical Na⁺ depressed K⁺ secretion but did not prevent its appearance in response to 0-Clb. Using microelectrodes, we observed depolarization of the intracellular voltage concomitant with increased resistance of the basolateral membrane after 0-Clb. Basolateral application of Ba²⁺ to depolarize cells also induced K⁺ secretion. Because apical conductance and channel density are unchanged after 0-Clb, 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-Clb eliminated K⁺ secretion, while further depolarization increased the magnitude of the secretory current. The cell depolarization after 0-Clb is most likely caused directly by a decrease of the basolateral membrane K⁺ conductance. Ba²⁺-induced Lorentzians also were elicited by basolateral hypertonic solutions but with lower values of S₀, indicating that cell shrinkage per se could not entirely account for the response to 0-Clb and that the effects of 0-Clb 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, Iₛ, 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 absorption of K⁺ is observed. However, Nielsen (1984) found that eliminating Cl⁻ from the bathing solutions caused the net influx of Na⁺ to exceed the Iₛ. 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, Iₛ, 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 cm², 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 \( I_{\text{Cl}} \) current, fluctuations were amplified (\( \times 1,000 \)) in the clamp. Outside the clamp, the amplified signal was sent to two separate modules (designed 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 (RT1800 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 about 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, \( V_i \), was measured with respect to the outer solutions. The fractional resistance of the apical membrane, \( fR_a = \Delta V_i / \Delta V_v \), 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.

\(^1\) Filter modules (Butterworth type) were built in the electronics section of the Labo voor Fysiologie, KUL. The fundamental frequency (\( f_0 \)) was selectable (low signal: 0.2, 0.5, or 1 Hz). Pre-filter characteristics: stage 1 (high pass), 24 db/octave, cutoff frequency (\( f_{\text{cutoff}} \)) = 75% of \( f_0 \); stage 2 (low pass), 24 db/octave, \( f_{\text{cutoff}} = 120, 300, \) or 600 Hz for \( f_0 \) of 0.2, 0.5, or 1 Hz, respectively. Anti-aliasing filter: 48 db/octave, \( f_{\text{cutoff}} = 90, 225 \) or 450 Hz for the above \( f_0 \). The \( f_{\text{cutoff}} \) and \( f_0 \) 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 (l/f) noise summed with a Lorentzian component, \( L(f) \) (Van Driessche and Zeiske, 1980):

\[
L(f) = S_o/[1 + (ff_c)^2]
\]

where \( L(f) \) is the spectral density of the Lorentzian at frequency \( f \), \( S_o \) is the plateau value in the low frequency range, and \( f_c \) is the corner frequency (at \( S_o/2 \)). In the presence of micromolar quantities of Ba²⁺ in the apical bath, a second Lorentzian component is observed which is the result of the two-state block of the K⁺ channels by Ba²⁺ (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 Ba²⁺-induced Lorentzian, or both in the PDS.

Ba²⁺ blockage of open K⁺ channels is a two-state process, and may be described by the following scheme:

\[
\text{Ba}^{2+} \quad \text{open} \xrightarrow{\text{Ba}^{2+}} \text{blocked}
\]

\( k_{on} \) and \( k_{of} \) are the forward and reverse rate constants, respectively, for Ba²⁺ blockade. Thus,

\[
2\pi f_c = k_{on}[\text{Ba}^{2+}] + k_{of}
\]

Given the rate constants, the open and blocked probabilities (\( P_o \) and \( P_b \)) are computed

\[
P_o = 1/(1 + [\text{Ba}^{2+}]/K_{ba})
\]

\[
P_b = 1 - P_o
\]

For a given [Ba²⁺],

\[
S_o = M_{Ba}(i_k)^2(2P_oP_b/\pi f_c)
\]

and

\[
i_k = M_{Ba}i_kP_o
\]

where \( M_{Ba} \) is K⁺ channel density and \( i_k \) is the single-channel current. \( P_o \), \( P_b \), and \( f_c \) are functions of \( k_{on}, k_{of}, \) and the [Ba²⁺]. If these parameters are constant \( C \), then \( S_o \) is a function of \( M_{Ba} \) and \( i_k \) alone, and changes in \( S_o \) may be taken to reflect changes in the total Ba²⁺-inhibitable current, \( I_{Ba}^o \)

\[
S_o = I_{Ba}^o(i_k/C)
\]

Although offsetting effects of \( M_{Ba} \) and \( i_k \) could lead to opposite changes of \( I_{Ba}^o \) and \( S_o \), this seemed unlikely and appeared not to occur in these studies.

---

\(^2\) The Ba²⁺-induced Lorentzian component also is described by Eq. 1 since it is an independent two-state process. The Ba²⁺-induced parameters are not superscripted throughout the text for convenience.
Control Ringer solution consisted of (in mM): 115 NaCl, 2.5 KHCO₃, and 1.0 CaCl₂. Four Cl⁻-free solutions (0-Cl⁻) were used: (a) NO₃ (115), 2.5 KHCO₃, and 1.0 Ca(NO₃)₂; (b) gluconate (115), 2.5 KHCO₃, and 4.0 CaSO₄; (c) SO₄ (57.5), 2.5 KHCO₃, and 2.0 CaSO₄; and (d) isotonic SO₄, which was identical to SO₄ 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 Iₓ stabilized before beginning an experimental period.

Terms specific to this report:
- 0-Cl⁻: Basolateral Cl⁻-free solution
- PDS: Power density spectrum
- pdu: Power density unit = 10⁻¹⁰ A²/s·cm²
- IₓBas: Transepithelial Ba²⁺-inhibitable K⁺ current
- Gtrx; Gk: Transcellular K⁺ conductance in the presence of apical amiloride; apical membrane conductance

Terms generally found in the literature and used below:
- Vt; Gt: Transepithelial voltage; conductance
- Iₛ: Short-circuit current (Vₛ = 0)
- Vₛ: Intracellular voltage measured across the apical membrane with respect to the bath under shorted conditions
- fRₛ: Fractional resistance of the apical membrane (ΔVₛ/ΔVₛ)
- kₘ₀; kₘₑ: Microscopic forward and reverse rate constants for Ba²⁺ inhibition
- Kₑ: Ba²⁺ inhibition constant (kₑ/kₘ₀)
- Mₑ: Channel density measured at a given inhibitor concentration (at the Kₑ)
- iₑ: Single-channel current measured at the Kₑ
- Eₑ: Nernst equilibrium potential for K⁺
- gₑ: 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 [Ba²⁺] 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 Iₓ vs. time is shown in Fig. 1. With control NaCl Ringer solution on both sides of the tissue, a pulse of 0.5 mM Ba²⁺ in the apical bath did not affect Iₓ. After substituting either gluconate (Fig. 1, top) or NO₃ (Fig. 1, bottom) for Cl⁻ in the basolateral solution, Iₓ increased slightly and then declined. The response when gluconate was used as a Cl⁻ substitute was considerably faster, and Iₓ fell to lower
levels. However, regardless of the Cl\(^–\) substitute, changes of \(I_\omega\) in response to the Ba\(^{2+}\) pulses developed with time. The response to Ba\(^{2+}\) was complex and was characterized by two phases: first, \(I_\omega\) increased rapidly, consistent with inhibition of K\(^+\) secretion, and then it decayed. The magnitude of initial, rapid change of \(I_\omega\) increased gradually with time. When Ba\(^{2+}\) was removed, the mirror image was observed. Returning tissues to control NaCl Ringer solution caused \(I_\omega\) 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 \(I_o\), which represents active transepithelial Na\(^+\) transport, to values near but greater than zero (Nagel et al., 1983; Fisher and Lockard, 1988). However, after 0-Cl\(_b\) amiloride caused Isc to reverse polarity (Fig. 1, bottom right) consistent with K\(^+\) secretion. Moreover, the secondary decline of \(I_\omega\) 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 \(I_\omega\) and increase of \(R_\tau\) upon exposure to 0-Cl\(_b\) are primarily attributable to changes in Na\(^+\) transport. Due to the complex nature of the \(I_\omega\) 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 $0-Cl^-$ 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-I) were characterized by decreasing $1/f$ noise with increasing frequency. At frequencies greater than $\sim 300$ 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 $S_o$ 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 $\sim 10-15$ min of $0-Cl^-$ (i.e., during the decline of $I_x$), a Lorentzian component appeared in response to the $Ba^{2+}$ pulse. For example, 64 min in gluconate yielded values of $S_o$ and $f_c$ of 7.97 pdu and 23.7 Hz, respectively (Fig. 2, A-2). After returning $Cl^-$ to the bath $S_o$ decreased slowly (similar to the return of $I_x$ toward control) to 2.68 and 0.81 pdu at 16 and 32 min, respectively (Fig. 2, A-4), and by 48 min the Lorentzian disappeared. Also, like the response of $I_x$, the effects were reversible and reproducible, since removal of $Cl^-$ a second time caused reappearance of the Lorentzian (although $S_o$ values were often lower). Similar to gluconate, $Cl^-$ replacement by $NO_3^-$ yielded time-dependent increases of $S_o$ without significant changes in $f_c$ (Fig. 2, B-I and B-2).

We also characterized the time course for development of the Lorentzian component after $0-Cl^-$ ($NO_3^-$ substitute). Tissues were pulsed repeatedly at 15-min intervals.

**Figure 2.** Representative PDS before and after $0-Cl^-$. Curves correspond to spectra measured during selected $Ba^{2+}$ pulses shown in Fig. 1. (A) Gluconate. A-I, background spectrum observed under control conditions before $0-Cl^-$ exposure and 48 min after return to NaCl Ringer solution. A-2, 64 min in $0-Cl^-$; $S_o = 7.97$ pdu, $f_c = 23.7$ Hz. Internal curves (top): 16 min after return to control; $S_o = 2.68$ pdu, $f_c = 25.1$ Hz. (bottom): 32 min after return to control; $S_o = 0.81$ pdu, $f_c = 51.0$ Hz. (B) $NO_3^-$. Spectra at $t = 0$, 7, and 17 min in $0-Cl^-$ 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-I, 42 min in $0-Cl^-$; $S_o = 0.797$ pdu, $f_c = 28.5$ Hz. B-2, 84 min in $0-Cl^-$; $S_o = 2.18$ pdu, $f_c = 26.6$ Hz. B-3, $10^{-4}$ M amiloride in the apical bath in addition to $0.5$ mM Ba$^{2+}$; $S_o = 0.083$ pdu, $f_c = 52.3$ Hz.
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 \(\sim 60-80\) 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 \(I_x\) 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 \(I_x\) (cf. Fig. 1). The change in \(I_x\), \(I_x^{Ba}\),

<table>
<thead>
<tr>
<th>Table I</th>
<th>Comparison of Changes in (I_x^{Ba}) and (S_o)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paired ratio</td>
<td></td>
</tr>
<tr>
<td>(I_x^{Ba}, \mu A/cm^2)</td>
<td>(0-Cl_b)</td>
</tr>
<tr>
<td>(S_o, pdu)</td>
<td>2.90 ± 0.61</td>
</tr>
<tr>
<td>(I_x^{Ba}, \mu A/cm^2)</td>
<td>(0-Cl_b)</td>
</tr>
<tr>
<td>(S_o, pdu)</td>
<td>6.06 ± 0.84</td>
</tr>
</tbody>
</table>

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\(_b\), \(I_{K}^{a}\) was 66% of \(I_{K}^{b}\) 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 response to hyperosmotic solutions \(S_o\) averaged 32% less than the paired 0-Cl\(_b\) tissues, which was matched by a 35% decrease of \(I_{K}^{a}\) (Table I, bottom). Although the magnitude of \(I_{K}^{a}\) 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.

**FIGURE 4.** Changes of electrical parameters after 0-Cl\(_b\). At \(t = 0\) tissues were exposed to basolateral solutions with either NO\(_3\) \((\text{filled circles, } n = 14)\) or gluconate \((\text{open circles, } n = 14)\) 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 \(f\) was not included in the mean.
K⁺ secretion was observed when NO₃⁻, gluconate, or isotonic SO₄²⁻ 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 $I_w$ declined [Fig. 1]). Average time-dependent changes in the electrical parameters in response to basolateral NO₃⁻ and gluconate are shown in Fig. 4. The response to gluconate was more consistent and more rapid. After 6 min $I_w$ increased 16% above control, declined after 15 min to 63% of control, and then stabilized at ~40% of control after 30 min. As $I_w$ declined, $R_t$ more than doubled, reaching a maximum at 15 min when a prominent Ba²⁺-induced Lorentzian was observed with $S_o$ averaging 3.7 pdu (Fig. 4, open circles). After 30 min in gluconate $R_t$ did not change significantly, but $S_o$ continued to increase. When NO₃⁻ was used as a Cl⁻ substitute, the response was attenuated and slower, usually requiring 30 min for the Ba²⁺-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 Ba²⁺-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 NO₃⁻, gluconate, or isotonic SO₄²⁻ as a Cl⁻ substitute, 25% of these tissues displayed spontaneous Lorentzians and virtually all of them (98%) yielded Ba²⁺-induced Lorentzians. Spontaneous Lorentzians, i.e., a Lorentzian component in the PDS in the absence of apical Ba²⁺, are indicative of random fluctuations of the apical K⁺ channels. It is probable that for tissues demonstrating Ba²⁺-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 Ba²⁺-induced Lorentzian was greatest, which prevented us from simultaneously detecting the spontaneous component. Relative to the Ba²⁺-induced fluctuations, the spontaneous noise was insignificant (cf. Table II), and the large Ba²⁺-induced Lorentzian always masked the spontaneous component. This, coupled with the vastly different $f_c$ (25 vs. 115 Hz), indicates that the Ba²⁺-induced $f_c$ was not significantly influenced by the spontaneous component, at least for low [Ba²⁺] (i.e., 0.5 mM).

Finally, the values of $f_c$ for the Ba²⁺-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 Ba²⁺ Inhibition Constant ($K_{ba}$)**

Some tissues were subjected to sequential apical [Ba²⁺] pulses of 0.1, 0.2, 0.5, 1.5, and 3.0 mM after 60 min incubation in 0-Cl⁻ to determine the rate constants, $k_{on}$ and $k_{off}$, and the Ba²⁺ inhibition constant, $K_{ba}$ ($=k_{off}/k_{on}$). For a two-state model (cf. Eq. 3), a plot of $2\pi f_c$ vs. [Ba²⁺] is linear with slope $k_{on}$ and intercept $k_{off}$ (Van Driessche and Zeiske, 1980). However, the Ba²⁺-induced noise is superimposed upon spontaneous K⁺ channel fluctuations. Theoretically, these plots must deviate from linearity because the Ba²⁺-induced and spontaneous Lorentzians are not independent, especially as the $f_c$ for the Ba²⁺ block approaches the spontaneous $f_c$ (~115 Hz) (Van Driessche and Zeiske, 1980). As shown in Fig. 5 A, plots were generally nonlinear (at high [Ba²⁺]), 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 [Ba²⁺]) were used to calculate the rate constants, which on average yielded a $K_{ba}$ of ~0.3 mM [Ba²⁺] (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⁻ were then subjected to another sequence of [Ba²⁺]. These plots were also nonlinear above 0.2 mM Ba²⁺ (Fig. 5 A), but using the linear region of lower [Ba²⁺] 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 Ba²⁺.

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. [Ba²⁺] (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-Clb decreased $k_{on}$ and increased $k_{off}$; i.e., opposite effects compared with secreting tissues (see Discussion).

**Changes of the Intracellular Voltage ($V_i$)**

*Response to 0-Clb.* Previous studies have shown that 0-Clb 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; 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-Clb 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-Clb (Table IV). Although punctures of frog skin epithelial cells are routinely accomplished, we encountered difficulty in maintaining cellular impalements after removing basolateral...
**K⁺ Secretion across Frog Skin after Cl⁻ Removal**

### Table IV

<table>
<thead>
<tr>
<th>Ringer solution</th>
<th>Apical</th>
<th>Basal</th>
<th>$k_m$ s⁻¹ M⁻¹</th>
<th>$k_m$ s⁻¹ M⁻¹</th>
<th>$K_m$ mM⁻¹</th>
<th>$i_N$ pA</th>
<th>$M_{ik}$ μM⁻¹ cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active K⁺ secretion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A NaCl</td>
<td>0-Cl⁻</td>
<td>0.22 ± 0.02</td>
<td>62.3 ± 5.3</td>
<td>0.30 ± 0.05</td>
<td>—*</td>
<td>—*</td>
<td></td>
</tr>
<tr>
<td>B NaCl + Amil</td>
<td>0-Cl⁻</td>
<td>0.52 ± 0.06</td>
<td>27.0 ± 3.4</td>
<td>0.06 ± 0.01</td>
<td>0.93 ± 0.10</td>
<td>3.5 ± 1.0</td>
<td></td>
</tr>
<tr>
<td><strong>K⁺ Absorption (apical K⁺ Ringer solution, basolateral Na⁺ Ringer solution)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C KCl NaCl</td>
<td>0.30 ± 0.01</td>
<td>41.5 ± 7.3</td>
<td>0.14 ± 0.03</td>
<td>3.22 ± 0.53</td>
<td>18.9 ± 10.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D KCl NaCl</td>
<td>0.07 ± 0.01</td>
<td>62.4 ± 5.0</td>
<td>0.87 ± 0.10</td>
<td>2.06 ± 0.29</td>
<td>3.2 ± 1.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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., $P_o = 0.5$), average values for single-channel current ($i_N$) and channel density ($M_{ik}$) were calculated for each tissue using three $[Ba^{2+}]$ near the $K_m$ (i.e., $P_o = 0.5$). *No Lorentzians were observed.

Cl⁻. This probably reflects cell shrinkage after 0-Cl⁻ (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 Ba²⁺-induced Lorentzian. On average, $V_s$ decreased 29.8 mV after 60 min (Table IV; $S_o = 8.11 ± 1.32$ pA, $f_s = 25.1 ± 1.1$), a value similar to the mean depolarization from the five other studies mentioned above (33.1 mV). The $fR_s$ 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, $V_s$ hyperpolarized and $fR_s$ increased to values > 0.95 (not shown). Addition of amiloride after 0-Cl⁻ hyperpolarized $V_s$ by 36 mV, but only increased $fR_s$ to 0.72, indicating that a significant conductance of the apical membrane remained relative to the basolateral membrane. Subsequent addition of 5–10 mM Ba²⁺ to the apical bath increased the $fR_s$ to 0.96, indicating that the remaining apical conductance after exposure to 0-Cl⁻ and apical amiloride was attributable to the apical K⁺ channels.

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

### Table IV

| Effect of 0-Cl⁻ on Intracellular Voltage, $V_s$, and Fractional Resistance, $fR_s$ |
|-----------------|--------------|--------------|----------------|
| Control | 0-Cl⁻ | Amiloride | Amiloride + Ba²⁺ |
| $V_s$, mV | -71.0 ± 3.6 | -41.2 ± 3.1 | -77.3 ± 3.2 | -63.5 ± 4.6 |
| $fR_s$ | 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 Ba²⁺ was added to Ringer solution in apical bath containing 50 μM amiloride. Paired depolarization after addition of Ba²⁺ = 13.8 ± 3.38 mV (n = 8).
TABLE V

<table>
<thead>
<tr>
<th>[Cl−]₀</th>
<th>100</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vₐ, mV</td>
<td>-65.9 ± 3.07</td>
<td>-39.7 ± 2.20</td>
<td>-60.3 ± 0.56</td>
<td>-46.7 ± 2.65</td>
<td>-18.5 ± 2.75</td>
<td></td>
</tr>
<tr>
<td>S₀, pmol</td>
<td>0</td>
<td>10.76 ± 1.36</td>
<td>0</td>
<td>5.89 ± 1.28</td>
<td>13.75 ± 3.30</td>
<td></td>
</tr>
</tbody>
</table>

Vₐ ranged about ±100 mV and was chosen to either repolarize Vₐ 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-Cl₀ when Vₐ averaged -66 mV. After 0-Cl₀, Vₐ 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 S₀ to 5.9 pmol. Alternatively, depolarization of Vₐ to -18.5 mV led to an increase of S₀ to 13.8 pmol, 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 Vₐ and S₀, 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, S₀ appears to increase as a function of Vₐ, 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 (S₀) as a function of intracellular voltage. Open circles, untreated tissues; filled circles, 40-60 min in 0-Cl₀; open triangles, repolarized near control values by transepithelial voltage clamp; open squares, depolarized from 0-Cl₀ state.](image-url)
to the apical bath after induction of K$^+$ secretion, the rate constants for Ba$^{2+}$
inhibition changed consistent with cell hyperpolarization ($f_r$ 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 $f_r$ (36.8 ± 2.1) were similar to those measured when tissues were
first exposed to 0-Cl$, and then treated with amiloride. Thus, K$^+$ 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 mem-
brane K$^+$ conductance (Nagel, 1979). Spectra were obtained 5 min after adding Ba$^{2+}$
to the basolateral bath, i.e., when $I_\infty$ reached a minimum and $R_T$ peaked (Table VI).

| TABLE VI |
|-----------------|-----------------|-----------------|-----------------|
| **Effect of 1.0 mM Ba$^{2+}$ in the Basolateral Chamber** |
| Min after addition of Ba$^{2+}$ |
| 0 | 5 | 15 | 30 |
| $I_\infty$, $\mu A/cm^2$ | 18.2 ± 1.5 | 7.5 ± 0.7 | 8.8 ± 0.9 | 12.8 ± 1.7 |
| $R_T$, $\Omega/cm^2$ | 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 |
| $f_r$, 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 $I_\infty$
increased, but $R_T$ returned to control values. $I_\infty$ 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$. We examined the response to 0-Cl$^-$ 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$^-$ 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$^-$ Effects

Since $R_T$ increased and $I R_A$ and $V_e$ decreased with 0-Cl$, it seemed likely that the K$^+$
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 \(I_{ac}\) followed by a long-term increase lasting 1–3 h (cf. Fig. 7, control; also see Discussion). \(I_{ac}\) 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 \(I_{ac}\) 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 \(I_{ac}\) 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 \(I_{ac}\) averaged \(\sim 30\) μA/cm\(^2\). Next, the experimental piece was exposed to 0-Cl\(_b\) for the remainder of the experiment, which decreased \(I_{ac}\) to an average of 11.4 μA after 60 min (Fig. 7; \(t = 0\) min), by which time K\(^+\) secretion was observed (\(S_0 = 5.97 \pm 1.8\) pdm, \(f_0 = 27.0 \pm 1.5\) Hz). Then Na\(^+\) was replaced by K\(^+\) in the apical bath for both pieces, which quickly decreased \(I_{ac}\) to 8.3 μA/cm\(^2\) (control) vs. 4.5 μA/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 Ba²⁺ (1 min). After determining G_K, the inhibitors were washed out of the apical chamber. When Iₜ returned to near control, 0-Cl₅ or 11.5-Cl₅ 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 Ba²⁺ to determine Gᵢ, and then exposed to 0-Cl₅ (100 min; Table VII C). Low Cl₅ activated K⁺ secretion for all groups as indicated by Iₜ values ranging from -0.94 to -1.7 µA/cm² (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-Cl₅ 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 decreased significantly for B and C (Table VII, right). Thus, low Cl₅ probably serves to decrease basolateral membrane conductance, which in turn depolarizes cells.

### Table VII

<table>
<thead>
<tr>
<th>Ringer solution</th>
<th>Apical</th>
<th>Basal</th>
<th>n</th>
<th>Iₜ⁰</th>
<th>Gᵢ</th>
<th>Gₚ</th>
</tr>
</thead>
<tbody>
<tr>
<td>A NaCl + amiloride</td>
<td>NaCl</td>
<td>(5)</td>
<td>-0.24 ± 0.09</td>
<td>0.1103 ± 0.0127</td>
<td>0.0406 ± 0.0051</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0-Cl₅</td>
<td></td>
<td>-1.70 ± 0.32</td>
<td>0.1619 ± 0.0317</td>
<td>0.0396 ± 0.0082</td>
<td></td>
</tr>
<tr>
<td>B NaCl + amiloride</td>
<td>NaCl</td>
<td>(5)</td>
<td>-0.11 ± 0.11</td>
<td>0.1515 ± 0.0182</td>
<td>0.0499 ± 0.0064</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.5-Cl₅</td>
<td></td>
<td>-0.94 ± 0.19</td>
<td>0.1351 ± 0.0122</td>
<td>0.0251 ± 0.0091</td>
<td></td>
</tr>
<tr>
<td>C 0-Na⁺</td>
<td>NaCl</td>
<td>(5)</td>
<td>-0.22 ± 0.08</td>
<td>0.1299 ± 0.0154</td>
<td>0.0678 ± 0.0153</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0-Cl₅</td>
<td></td>
<td>-1.14 ± 0.12</td>
<td>0.1435 ± 0.0209</td>
<td>0.0469 ± 0.0071</td>
<td></td>
</tr>
</tbody>
</table>

Paired tissues were used for each group. Paired ratios: *0.59 ± 0.25 (P < 0.05); *0.64 ± 0.07 (P < 0.001).

Amiloride concentration, 50 µM.

Influence of Basolateral Osmolarity

Since basolateral KCl permeability is high and Cl⁻ is the principal anion in NaCl Ringer solution, 0-Cl₅ 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-Cl₅. 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-Clb, $I_w$ decreased to ~30% of control values for all paired tissues (Fig. 8, left diagonal). $I_w$ 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 $I_w$ in response to 0-Clb. 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-Clb tissues, but not consistently for the hypertonic solutions. When Lorentzian components were seen, the response was always less than observed for the paired 0-Clb 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, although Ba$^{2+}$-induced Lorentzians were often observed in response to hyperosmotic solutions, we could not mimic the response to 0-Clb 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, $I_w$ 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 pdm and $f_c$ ~ 25 Hz. In contrast, after 60 min of hypotonic SO$_4^-$ Ringer solution $I_w$ 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 $\text{SO}_4^-$ Ringer solution (Nagel, 1977). Thus, 0-$\text{Cl}_b$ 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 $\text{Cl}^-$, distinct Lorentzians appeared in response to apically applied $\text{Ba}^{2+}$, consistent with the idea that $K^+$ is secreted from the basolateral to the apical surface of the epithelium. In the absence of apical $\text{Ba}^{2+}$, 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 $\text{Ba}^{2+}$-induced Lorentzian component in the PDS. This effect appears to be specific for *R. temporaria*

<table>
<thead>
<tr>
<th>Table VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comparisons of Isotonic and Hypotonic $\text{SO}_4^-$ Ringer Solution</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Isotonic 0-$\text{Cl}_b$</th>
<th>Control</th>
<th>Hypotonic 0-$\text{Cl}_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_o$, $\mu A/cm^2$</td>
<td>21.38 ± 2.25</td>
<td>7.32 ± 0.74</td>
<td>19.81 ± 2.54</td>
<td>16.22 ± 4.02</td>
</tr>
<tr>
<td>$R_o$, $\Omega/cm^2$</td>
<td>2.735 ± 346</td>
<td>5.846 ± 823</td>
<td>2.705 ± 287</td>
<td>1.913 ± 327</td>
</tr>
<tr>
<td>$S_o$, $\text{pS/cm}$</td>
<td>—*</td>
<td>6.29 ± 0.87</td>
<td>—*</td>
<td>—*</td>
</tr>
<tr>
<td>$f$, Hz</td>
<td>23.31 ± 1.31</td>
<td>—*</td>
<td>—*</td>
<td>—*</td>
</tr>
</tbody>
</table>

Values in 0-$\text{Cl}_b$ were measured with 0.5 mM $\text{Ba}^{2+}$ in the apical solution after 60 min exposure to 0-$\text{Cl}_b$. *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 $\text{Ba}^{2+}$ (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 $\text{Ba}^{2+}$. We agree with his contention for the following reasons. (a) The rapid increase of $I_o$ in response to $\text{Ba}^{2+}$ was concurrent with augmented current fluctuations (detected in < 1 s) which were maintained as long as $\text{Ba}^{2+}$ 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 $\text{Ba}^{2+}$. (b) Lorentzians were observed in
split skins which are, for the most part, gland-free \((n = 4)\).\(^{3}\) Using microelectrodes, which certainly must access the principal cells (> 98% of the apical surface area), we detected a \(\text{Ba}^{2+}\)-sensitive apical \(\text{K}^{+}\) conductance consistent with previous observations by Nagel and Hirschmann (1980). We therefore conclude that the Lorentzians observed in the present studies reflect \(\text{K}^{+}\) transport through apical \(\text{K}^{+}\) channels of the principal cells.

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

**Unitary Characteristics of Apical \(\text{K}^{+}\) Channels**

It seems likely that the same \(\text{K}^{+}\)-selective channels in the apical membrane mediate active \(\text{K}^{+}\) secretion and gradient-driven \(\text{K}^{+}\) absorption. After hyperpolarization by amiloride, the \(K_{\text{Na}}\) (secretion) was 20% of the preamiloride values, consistent with voltage-dependent block by \(\text{Ba}^{2+}\) described previously for absorptive tissues (Van Driessche and Zeiske, 1980; De Wolf and Van Driessche, 1986). Conversely, pretreatment of \(\text{K}^{+}\) absorptive tissues with 0-\(\text{Cl}^{-}\) increased the absorptive \(K_{\text{Na}}\) 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-\(\text{Cl}^{-}\) remain depolarized after switching to apical \(\text{K}^{+}\) Ringer's.

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

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

---

\(^{3}\) 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$^-$ 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$^-$ 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$^-$ 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 ($g_\kappa$) was estimated for tissues treated with amiloride according to the following equation:

$$g_\kappa = i_\kappa/(E_\kappa - V_a)$$

(9)

where $E_\kappa$ 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$^-$ (Dörge et al., 1988). Given an $i_\kappa$ of $-0.9$ pA and a $V_a$ of $-77$ mV (Table IV), $E_\kappa = -112$ mV and $g_\kappa = 26$ pS. Similarly, for absorptive K$^+$ flow, we assumed that $E_\kappa = 0$, $i_\kappa = 3.2$ pA (Table IV), $V_a = -60$ mV (unpublished observations, and Nagel and Hirschmann, 1980), and $g_\kappa = 53$ pS. Given that absorptive channels are bathed bilaterally in high [K$^+$], it was not surprising that the $g_\kappa$ for absorption was greater than for secretion.

Estimates of $i_\kappa$ and $g_\kappa$ were also determined for 0-Cl$^-$ tissues not previously treated with amiloride. Analogous with Eq. 9,

$$G_\kappa = I_\kappa/(E_\kappa - V_a)$$

(10)

and

$$G_{\kappa} = G_t/\Omega_{R_a}$$

(11)

Combining Eqs. 6, 7, 10, and 11 and solving for $i_\kappa$:

$$i_\kappa = [S_c(G_{\kappa} \pi f_\kappa)]/[\{(E_\kappa - V_a)(2P_b)\}]$$

(12)

We assumed that $E_\kappa = -112$ 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 $i_\kappa$ and $g_\kappa$ of $1.5 \pm 0.4$ pA and $22.4 \pm 6.7$ pS ($n = 4$). Thus, it is apparent that $i_\kappa$ increased as expected for depolarized cells with increased values of $S_c$. Moreover, $g_\kappa$ 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$^-$**

There are four known effects of 0-Cl$^-$ 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 O-Clb similar to the time course of activation of K⁺ secretion. Other means of depolarizing cells, such as serosal application of Ba²⁺ or increases of basolateral [K⁺] (unpublished observations), also yielded Ba²⁺-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 μA/cm² (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, Gₖ, 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 O-Clb, the basolateral membrane conductance greatly exceeded the apical conductance, and Gₖ approximated Gᵦ. This was confirmed by measurements of ERaverage 0.75 under these conditions. Since Gₖ decreased or was unchanged after O-Clb, we conclude that the primary effect of O-Clb 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; Leibovich et al., 1988). Moreover, since the apical K⁺ channel density was unchanged after O-Clb, 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 O-Clb on Iᵦ and Rᵦ with basolateral hypertonicity, K⁺ secretion in response to O-Clb 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 SO₄⁻ 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 SO₄⁻ solution caused cell swelling. This may open conductive channels to facilitate salt loss leading to regulatory volume decrease of swollen cells.
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 μA/cm² 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.

We thank Mrs. J. De Beir-Simaels, Mr. M. Decré, and Mr. T. Hendrickx for their excellent technical assistance, and Dr. W. Nagel and Dr. S. Sarhan-Sohraby for their helpful discussions and comments on the manuscript.

Original version received 11 November 1988 and accepted version received 13 August 1990.

REFERENCES


FISHER AND VAN DRIESSCHE  

K⁺ Secretion across Frog Skin after Cl⁻ Removal  


