Chloride transport in rabbit esophageal epithelial cells

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Abstract

We investigated Cl\textsuperscript{−} transport pathways in the apical and basolateral membranes of rabbit esophageal epithelial cells (EEC) using conventional and ion-selective microelectrodes. Intact sections of esophageal epithelium were mounted serosal or luminal side up in a modified Ussing chamber, where transepithelial potential difference and transepithelial resistance could be determined. Microelectrodes were used to measure intracellular Cl\textsuperscript{−} activity (a\textsubscript{Cl})\textsubscript{m}, basolateral or apical membrane potentials (V\textsubscript{mBL} or V\textsubscript{mC}), and the voltage divider ratio. When a basal cell was impaled, V\textsubscript{mBL} was −73 ± 4.3 mM and a\textsubscript{Cl} was 16.4 ± 2.1 mM, which were similar in presence or absence of bicarbonate. Removal of serosal Cl\textsuperscript{−} caused a transient depolarization of V\textsubscript{mBL} and a decrease in a\textsubscript{Cl} of 6.5 ± 0.9 mM. The depolarization and the rate of decrease of a\textsubscript{Cl} were inhibited by ~60% in the presence of the Cl\textsuperscript{−}-channel blocker flufenamate. Serosal bumetanide significantly decreased the rate of change of a\textsubscript{Cl} on removal and readdition of serosal Cl\textsuperscript{−}. When a luminal cell was impaled, V\textsubscript{mC} was −65 ± 3.6 mM and a\textsubscript{Cl} was 16.3 ± 2.2 mM. Removal of luminal Cl\textsuperscript{−} depolarized V\textsubscript{mC} and decreased a\textsubscript{Cl} by only 2.5 ± 0.9 mM. Subsequent removal of Cl\textsuperscript{−} from the serosal bath decreased a\textsubscript{Cl} in the luminal cell by an additional 6.4 ± 1.0 mM. A plot of V\textsubscript{mBL} measurements vs. log a\textsubscript{Cl}/log a\textsubscript{Cl} was linear (slope (S) = 67.8 mV/decade of change in a\textsubscript{Cl}/a\textsubscript{Cl}). In contrast, V\textsubscript{mC} correlated very poorly with log a\textsubscript{Cl}/a\textsubscript{Cl} (S = 18.9 mV/decade of change in a\textsubscript{Cl}/a\textsubscript{Cl}). These results indicate that 1) in rabbit EEC, a\textsubscript{Cl} is higher than equilibrium across apical and basolateral membranes, and this process is independent of bicarbonate; 2) the basolateral cell membrane possesses a conductive Cl\textsuperscript{−} pathway sensitive to flufenamate; and 3) the apical membrane has limited permeability to Cl\textsuperscript{−}, which is consistent with the limited capacity for transepithelial Cl\textsuperscript{−} transport. Transport of Cl\textsuperscript{−} at the basolateral membrane is likely the dominant pathway for regulation of intracellular Cl\textsuperscript{−}.

The esophagus is lined by a moist stratified squamous epithelium, which actively transports Na\textsuperscript{+} from lumen to serosa. Active Na\textsuperscript{+} transport accounts for the main portion (85%) of the short-circuit current developed across this tissue (~10 μAmp/cm\textsuperscript{2}), whereas the majority of the remaining current is due to the transport of Cl\textsuperscript{−} from serosa to mucosa (20). Although the contribution of Cl\textsuperscript{−} to the short-circuit current is very small, cellular transport of Cl\textsuperscript{−}, similar to other epithelia, is fundamental for the maintenance of the intracellular milieu. Regulation of cellular Cl\textsuperscript{−} transport is particularly important in the esophageal epithelium, a tissue regularly exposed to wide fluctuations in luminal content including high levels of NaCl (salt) in food and HCl (gastric acid) due to the reflux of gastric content (14, 16). Because very little information is available about Cl\textsuperscript{−} transport in esophageal epithelial cells, the purpose of this study was to examine its transport pathways across both the basolateral and the apical cell membranes of this epithelium. This was done using microelectrodes to measure intracellular Cl\textsuperscript{−} activity and basolateral and apical membrane potential of esophageal cells within the intact epithelium. Our study indicates that the a\textsubscript{Cl} in this tissue is maintained above electrochemical equilibrium independently of the presence of bicarbonate. We have also identified a Ca\textsuperscript{2+}-dependant conductive pathway for Cl\textsuperscript{−} transport across the basolateral membrane that is sensitive to the nonsteroidal anti-inflammatory aromatic Cl\textsuperscript{−}-channel blocker flufenamate. A component of Cl\textsuperscript{−} transport in the serosal cells is sensitive to bumetanide. Moreover, there is little capacity for the transport of Cl\textsuperscript{−} across the apical membrane, which likely accounts for the minimal contribution of Cl\textsuperscript{−} to net active transport of ions across this epithelium.

METHODS

Animal and Tissue Preparation

New Zealand rabbits were killed by administration of an intravenous overdose of pentobarbital sodium (60 mg/ml). The esophagus was excised, opened longitudinally, and pinned mucosal side down in a paraffin tray containing ice-cold oxygenated Ringer. The muscle layers were lifted up.
with forceps, and the underlying mucosa were dissected free with a scalpel. The sheet of mucosa thus obtained was cut, and a section was mounted horizontally serosal side up (for basal cells) or mucosal side up (for luminal cell impalements) in a modified Ussing chamber with an aperture of 1.13 cm². The chamber allows independent and continuous perfusion of the apical and the serosal side of the tissue. The fluid for the perfusion of the tissue is delivered by gravity. The perfusion solutions can be switched quickly and with minimal dead space by means of a combination of rotary and slider valves (Rainin, Emeryville, CA) that allow one of six experimental solutions to flow to each side of the chamber. The volume of fluid in the upper and in the lower sides of the chamber was ~0.25 ml. The rate of flow of the solution to the upper chamber was 2 ml/min, which yielded a turnover rate (i.e., time for a solution to be washed out completely from the chamber) of ~8 s. Perfusion fluid to the lower part of the chamber was delivered at 2 ml/min; however, only part of this fluid (~1 ml/min) entered the chamber, the rest leaching through a drain that was kept at a fixed level. The turnover rate in the lower side of the chamber was ~15 s. All solutions were placed at the same height from the chamber. The solutions were prewarmed and delivered to the chamber at 37°C.

**Electrodes**

Transepithelial potential ($V_{TE}$) was measured as the voltage difference between a free-flowing KCl (tip ~10 μm) electrode placed in the bath fluid of the luminal side and a similar electrode placed in the bath fluid of the serosal side (luminal electrode potential = serosal electrode potential). Both electrodes were fitted with an Ag-AgCl wire, and the leads were connected to the amplifier of a voltage clamp (model VCC 600 Physiologic Instruments, San Diego, CA). The voltage clamp allowed automatic compensation for voltage errors due to the resistance of the fluid and was also used to deliver a direct-current (DC) pulse (I) of 5–15 μA via platinum wires located in each side of the chamber. This allowed us to determine the transepithelial resistance ($R_{TE}$) from the voltage deflection [$\Delta V_{TE}$] as follows:

$$R_{TE} = \Delta V_{TE}/I$$ (1)

The esophagus possesses relatively large basal cells (20–40 μm) that allow stable impalements with double-barreled microelectrodes. Double-barreled microelectrodes were used in basal cells to measure simultaneously and in the same cell the cell membrane potential difference (basolateral membrane potential ($V_{mBL}$)) and the intracellular activity of Cl⁻ ($a_{Cl}^{−}$). Luminal cells are exceptionally long (100–250 μm) and slender (5–10 μm) so that single-barreled microelectrode impalements proved more stable. Thus, for apical membrane studies, two adjacent luminal cells were impaled with two single-barreled microelectrodes, one a Ling-Gerard microelectrode for cell membrane potential measurement and the other a Cl⁻-sensitive microelectrode for intracellular Cl⁻ measurements.

The following is a brief description of microelectrode preparation: double-barreled microelectrodes were prepared from two 1.2-mm OD borosilicate glass fiber capillaries (A-M Systems, Everett, WA) that were held together with shrinkable tubing and twisted 360° over an open flame. The electrodes were then pulled on a vertical microelectrode puller (David Kopf, Tujunga, CA) to a tip smaller than 0.2 μm. One barrel was exposed to hexamethyldisilazane (Sigma, St. Louis, MO) vapor for 30 min, after which the electrodes were baked at 100°C for 2 h. The Cl⁻ exchanger (WI, Sarasota, FL) was then introduced into the tip of the silanized barrel by means of very fine glass capillaries. The silanized barrel was further backfilled with 0.5 M KCl, and the reference barrel was filled with 1 M KCl. Each barrel was connected to an Ag-AgCl half cell and connected to one of the probes of a high-input impedance electrometer (WPI). The resistance of the reference barrel ranged from 50 to 100 MΩ, and the tip potential was <5 mV. Single-barreled Cl⁻-selective microelectrodes were pulled from 1.2-mm-OD borosilicate glass capillaries dried in the oven at 200°C for 2 h. Ten microliters of tri-n-butyl-chlorosilane were then introduced in a closed vessel (300 ml) that contained the microelectrodes, after which the silane fumes were vented and the electrodes were left in the oven for an additional 30 min. The electrodes were then filled and backfilled as described for the ion-sensitive barrel above. Ling-Gerard microelectrodes were pulled from 1.2-mm-OD borosilicate glass fiber capillaries and filled with 3 M KCl. Their resistance ranged from 20 to 30 MΩ, and the tip potentials were <5 mV.

The slope ($S$) of the Cl⁻ microelectrode (or ion-sensitive) barrel was determined from the equation

$$S = \frac{V_{100NaCl} - V_{10NaCl}}{0.94}$$ (2)

where $V_{100NaCl}$ and $V_{10NaCl}$ denote the electrode potential in the solutions as noted, 0.94 equals the logarithm (base 10) of the Cl⁻ activity ratio of pure 100 mM to pure 10 mM NaCl. The $S$ of the electrodes averaged 55 mV/decade of change in activity of Cl⁻. The selectivity coefficient $k_{Cl⁻-HCO₃}$ for Cl⁻ over HCO₃⁻ was calculated from the equation

$$k_{Cl⁻-HCO₃} = \frac{10^{V_{100HCO₃}/V_{100Cl⁻}}}{10^{V_{100Cl⁻}/V_{100HCO₃}}}$$ (3)

where $V_{100HCO₃}$ is the voltage of the Cl⁻ electrode in 100 mM NaHCO₃ and $V_{100Cl⁻}$ is the voltage of the Cl⁻ electrode in 100 mM NaCl.

The intracellular ionic activities ($a_{Cl}^{−}$) were calculated from the potential readings of cellular impalements of the Cl⁻ sensitive barrel of the double-barreled microelectrode or of the single-barreled Cl⁻ sensitive microelectrode. The total potential ($V^{Cl⁻}$) of the ion-sensitive electrode was measured as the voltage difference between the ion-sensitive microelectrode and the free flowing reference electrode in the bath. The pure ionic potential was obtained by subtracting electronically $V_{mBL}$ or apical membrane potential ($V_{mC}$) from the total potential of the ion-sensitive electrode.

The intracellular ionic activity was calculated according to the equation

$$a_{Cl}^{−} = a_{Cl}^{−} \times 10^{\frac{V_{cell} - V_{em}}{K_{Cl-CO₂}} - K_{Cl-CO₂}}$$ (4)

where $a_{Cl}^{−}$ indicates the activity of Cl⁻ in the bathing solution, the activity coefficient being 0.76 at 37°C, $V_{cell}$ is the cell membrane potential difference ($V_{mBL}$ or $V_{mC}$), and $V_{em}$ is the reading of the electrode in the bathing solution. In 50 Cl⁻ electrodes used for the study, the mean Cl⁻-to-HCO₃⁻ selectivity ratio was 10:1. Because of the low estimated intracellular activity of bicarbonate in the cell and the good selectivity of our electrodes, the correction factor ($K_{Cl-CO₂}$) $a_{HCO₃}^{−}$ for the selectivity was dropped from the calculations of $a_{Cl}^{−}$ (2).

When a basal cell was impaled, the basolateral membrane potential ($V_{mBL}$) was read as the voltage difference between the reference barrel of the double-barreled microelectrode and the reference electrode in the serosal bath. When a luminal cell was impaled, the $V_{mC}$ was read as the voltage...
difference between the single-barreled microelectrode (Ling- 
Gard) and the reference electrode in the luminal bath.

The apparent ratio (23) of the apical to the basolateral, 
membrane resistance (Ra/Rb) was determined from the 
ratio of the voltage deflections produced by the transepithelial DC 
pulse across the apical and the basolateral membranes 
(change in VmB, (ΔVmB) and VmBL, (ΔVmBL), respectively) 
according to the equation

\[ \frac{Ra}{Rb} = \frac{\Delta V_{mB}}{\Delta V_{mBL}} \] (5)

when a serosal cell was impaled, ΔVmBL produced by the 
transepithelial current pulse was measured directly and 
ΔVmB was calculated as ΔVTE - ΔVmBL. When a luminal cell 
was impaled, ΔVmC was measured directly and ΔVmBL was 
calculated as ΔVTE - ΔVmC. This calculation relies on the 
assumption that VTE = VmBL + VmC, which was verified only 
in simple tissues. To validate that in this multilayered tissue 
this calculation is a reasonable approximation we did the 
following. We used the signal from the bath voltage electrode 
at either the serosal or the luminal side of the tissue to drive 
the circuit ground of the intracellular measuring electrometer, 
thus allowing the intracellular potential (from the volt-
eter microelectrode) to be referenced to either the serosal or 
the mucosal side of the tissue. We used this approach to 
compare during serosal impalements a calculated VmC (from 
VmBL - VTE) to a presumed VmC measured as cell potential 
referred to the luminal electrode. On the other hand, 
during luminal impalements, we compared a calculated 
VmBL (from VmC - VTE) with a presumed VmBL measured as 
cell potential referenced to the serosal electrode. The differences 
between the calculated and measured values of VmBL 
or of VmC were within an acceptable range close to the 
standard error of the measurement.

Readings were recorded on a three-channel strip chart 
recorder (Kipp & Zonen, Bohemia, NY).

The Nernst equilibrium potential for Cl\(^-\) was calculated 
from the equation

\[ E_{Cl} = \frac{RT}{2F} \ln \left( \frac{a_{Cl}^i}{a_{Cl}} \right) \] (6)

where R is the gas constant, T the absolute temperature, z is 
the valence, F is the Faraday constant, \( a_{Cl}^i \) is the intracellular 
Cl\(^-\) activity, and \( a_{Cl} \) indicates the activity of Cl\(^-\) in the 
luminal or serosal bath.

**Solutions**

The composition of Ringer solutions is given in Table 1. We 
have measured ionized Ca\(^{2+}\) in gluconate-containing 
solutions and found that ionized Ca\(^{2+}\) concentration was reduced by 
almost 50% compared with control Ringer. Consequently, 
we have doubled the concentration of Ca\(^{2+}\) in all solutions 
containing gluconate. Chelation of Ca\(^{2+}\) in 0 Cl\(^-\) solution 
was corrected in a similar manner by Boron and Boulpaep (3). 
Indanyloxyacetic acid 94 (R(+)-IAA-94) and R(+)-butylindola-
zone (R(+)-DIOA) were purchased from RBI-Sigma (St Louis, 
MO), diphenylamine-2-carboxylate (DPC) was from Fluka 
(Milwaukee, WI), 5-nitro-2-(3-phenylpropylamino) benzoic 
acid (NPPB) was from Calbiochem (La Jolla, CA), and DIDS, 
STIS, and all other chemicals were from Sigma. Inhibitors 
insoluble in aqueous solutions were dissolved in a small 
volume of dimethyl sulfoxide and added to the solution. The 
concentrations used were based on the concentrations re-
The results are presented as means ± SE. Data were 
analyzed using the two-tailed paired Student’s t-test unless 
otherwise indicated. \( n \) is the number of observations.

**RESULTS**

**Basolateral Membrane Studies in Basal Cells**

**Measurements in control bicarbonate Ringer.** Intracellular 
measurements of basal cells obtained in tissues from 10 rabbits, 
bathed bilaterally with control HCO\(_3\)/HCO\(_2\) Ringer solution (Table 1, solution 1) yielded the following values: VmB = -70 ± 4.2 mV, VTE = -12.9 ± 0.7 mV, a\(_i\)Cl = 16.2 ± 2.0 mM, R\(_{TE}\) = 2,807 ± 
147 Ω·cm\(^2\) and Ra/Rb = 4.74 ± 0.6 (\( n = 16 \)).

On the basis of the measured a\(_i\)Cl, the electrochemical 
equilibrium for Cl\(^-\) (ECl), as calculated from Eq. 6, is 
-46 mV, a value significantly smaller than the electrical 
potential difference of -70 mV observed across the 
cell basolateral membrane. Thus, in the basal cells, a\(_i\)Cl 
is higher than the a\(_i\)Cl predicted by electrochemical 
equilibrium across the basolateral membrane.

**Effect of removal of serosal Cl\(^-\) in the presence of 
CO\(_2\)/HCO\(_3\).** When the serosal bath solution was 
switched to a Cl\(^-\)-free solution (solution 2, Table 1), a\(_i\) 
decreased from 14.3 ± 1.9 to 5.7 ± 1.1 mM (\( n = 12, P < 0.001 \)), 
and this decrease was accompanied by a rapid 
(<1 min) initial depolarization of VmB, as shown in 
Fig. 1, segment ab, from -66 ± 3.0 to -61 ± 3.5 mV 
(\( n = 21, P < 0.001 \)). This initial depolarization was 
then followed by a slower (5–8 min) repolarization of 
VmB (segment bc) until the membrane potential 
reached -67 ± 3.3 mV, a value not significantly differ-
ent from control. The switch to Cl\(^-\)-free solution also 
resulted in the rapid depolarization of VTE from 
-12.8 ± 0.6 to -4.9 ± 0.6 mV and then partial 
repolarization to -8.9 ± 0.8 mV. R\(_{TE}\) increased from 
2,530 ± 162 Ω·cm\(^2\) to 2,787 ± 170 Ω·cm\(^2\), whereas 
Ra/Rb decreased from 4.32 ± 0.85 to 3.53 ± 0.73 (\( n = 10, P < 0.001 \)). Data from this and similar experiments 
are summarized in Fig. 2. When Cl\(^-\) was returned to 
the serosal solution, all changes were completely re-

**Table 1. Composition of solutions**

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Composition given in mM unless otherwise indicated.

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versed. \(V_{\text{mBL}}\) and \(V_{\text{TE}}\) hyperpolarized rapidly (<1 min.) and transiently (Fig. 1, segment cd) to \(-74 \pm 5.8\) and \(-16.1 \pm 0.9\) mV, respectively, before going back to their control values. \(R_{\text{TE}}\) and \(R_{\text{a/Rb}}\) returned to normal, and \(a_i^{\text{Cl}}\) monotonically increased to its control value over the course of 5–8 min.

**Effect of SITS or DIDS on removal of serosal Cl\(^-\) in CO\(_2\)/HCO\(_3\)\) Ringer.** Cl\(^-\)/HCO\(_3\) exchange and other bicarbonate-dependent transporters are inhibited by the stilbene derivatives SITS and DIDS in a variety of tissues. To check the contribution of such a mechanism to Cl\(^-\) exit on removal of serosal Cl\(^-\), we treated tissues from four different animals with 0.5 mM SITS for 15 min before the removal of extracellular Cl\(^-\). SITS did not have an effect on the steady-state parameters in the treated tissues. Moreover, when Cl\(^-\) was removed in the presence of SITS, we observed a transient depolarization of \(V_{\text{mBL}}\) of \(8.6 \pm 0.6\) mV, after which \(V_{\text{mBL}}\) hyperpolarized to a value not significantly different from the control value in the presence of SITS. Also, \(a_i^{\text{Cl}}\) decreased from \(20.8 \pm 5.0\) to \(9.7 \pm 2.0\) mM. The changes in \(V_{\text{mBL}}\) observed on removal of serosal Cl\(^-\) in

![Fig. 1. Tracing from an experiment showing the effect of serosal bath removal of Cl\(^-\) in a basal cell.](image)

In control bicarbonate Ringer, removal of Cl\(^-\) from the serosal bath caused an initial transient depolarization (ab) of the basolateral membrane potential (\(V_{\text{mBL}}\)) that was followed by a quick recovery (bc) to a value not significantly different from control. The transepithelial potential (\(V_{\text{TE}}\)) also depolarized partially recovered, whereas intracellular Cl\(^-\) activity (\(a_i^{\text{Cl}}\)) decreased substantially. The changes were reversible on restoring bath Cl\(^-\).

![Fig. 2. Effect of Cl\(^-\) removal from the serosal bath in HCO\(_3\) Ringer in a basal cell, on \(V_{\text{mBL}}\) (A), \(V_{\text{TE}}\) (B), \(a_i^{\text{Cl}}\) (C), transepithelial resistance \((R_{\text{TE}}); D)\), and apical-to-basolateral membrane resistance ratio (\(R_{\text{a/Rb}}\); E). Removal of serosal bath Cl\(^-\) caused an initial depolarization in \(V_{\text{mBL}}\) of \(5.2 \pm 0.8\) mV, which then recovered to a value not significantly different from control \((n = 21; P < 0.001)\). \(V_{\text{TE}}\) depolarized initially by \(7.9 \pm 0.5\) mV and recovered partially to a value \(4.3 \pm 0.5\) mV more positive than control. \(a_i^{\text{Cl}}\) decreased by \(8.6 \pm 1.4\) mM \((n = 12; P < 0.001)\). \(R_{\text{TE}}\) increased by \(257 \pm 35\ \Omega\cdot\text{cm}^2 \ (n = 21; P < 0.001)\), and \(R_{\text{a/Rb}}\) decreased by \(0.79 \pm 0.14 \ (n = 10; P < 0.001)\). *Significantly different from control, paired data \((P < 0.05)\).](image)
the presence of SITS are not significantly different ($n = 4, P > 0.05$) from those observed in its absence (see above). The rate of change in $a_i^{\text{Cl}}$ on removal and readadition of serosal Cl$^-$ were not different in the absence and presence of SITS (Table 2).

In a different set of experiments, the esophageal tissue from 4 different rabbits were exposed to DIDS (0.1 mM) for 15 min before the removal of serosal Cl$^-$. DIDS had no significant effect on the steady-state parameters. The initial depolarization of $V_{\text{mBL}}$ on removal of serosal Cl$^-$ was $4.3 \pm 1.9$ and $3.3 \pm 1.9$ mV in the absence and presence of DIDS, respectively. This initial depolarization was followed by a hyperpolarization of $V_{\text{mBL}}$ and a reduction in the $a_i^{\text{Cl}}$ by $7.4 \pm 1.2$ mM in the absence and $6.2 \pm 3.05$ mM ($n = 4, P > 0.05$) in the presence of DIDS. All the observed changes in cell membrane potential difference, resistance, and transepithelial parameters were not statistically different in the absence or presence of DIDS ($n = 4, P > 0.05$). The rates of change in $a_i^{\text{Cl}}$ on removal and readadition of serosal Cl$^-$ were not different in the absence and presence of DIDS. (Table 2).

**Measurements of intracellular Cl$^-$ in the nominal absence of bicarbonate.** The lack of inhibition by SITS or DIDS of the changes in $a_i^{\text{Cl}}$ and the depolarization of $V_{\text{mBL}}$ associated with Cl$^-$ removal suggest that a conductive pathway for Cl$^-$ exists across the basolateral membrane of basal cells. If this is the case, then the cellular changes induced by removal of serosal Cl$^-$ are expected to persist in the absence of CO$_2$/HCO$_3$\(^-\). Tissues from 11 rabbits were therefore perfused with a bicarbonate-free HEPES Ringer solution (solution 3, Table 1), and the following values were obtained: $V_{\text{mBL}}$ was $-73 \pm 4.3$ mV, $V_{\text{TE}}$ was $-12.6 \pm 1.3$ mV, and $a_i^{\text{Cl}}$ was $16.4 \pm 2.1$ mM. $R_{\text{TE}}$ was $2,357 \pm 263$ $\Omega$ cm$^2$, and $R_{\text{Cl/Rb}}$ was $5.47 \pm 1.1$ ($n = 13$). From these data, the $E_{\text{Cl}}$, as calculated from the Nernst equation (Eq. 6), was $-48$ mV, which is significantly smaller than the electrical potential difference of $-73$ mV observed across the basolateral cell membrane. Thus the $a_i^{\text{Cl}}$

remains higher than the activity of Cl$^-$ predicted by electrochemical equilibrium across the basolateral membrane even in the absence of bicarbonate. This indicates that the mechanism for maintenance of Cl$^-$ above electrochemical equilibrium is essentially bicarbonate independent.

**Effect of removal of serosal Cl$^-$ in the absence of bicarbonate.** When the serosal bath HEPES solution was switched to a Cl$^-$free HEPES solution (solution 4, Table 1) the observed changes in cellular and transepithelial parameters were very similar to the changes observed on removal of Cl$^-$ in the presence of HCO$_3$\(^-\). On removal of bath Cl$^-$, $a_i^{\text{Cl}}$ decreased significantly from $16.4 \pm 2.1$ to $9.9 \pm 1.5$ mM ($n = 13, P < 0.001$). This was accompanied by a very rapid (<1 min) initial depolarization of $V_{\text{mBL}}$ (Fig. 3, segment ab) from $-68 \pm 2.5$ to $-63 \pm 2.8$ mV ($n = 30, P < 0.001$) followed by a slow (~5–8 min) repolarization (segment bc) until the membrane potential reached $-66 \pm 2.5$ mV, a value not significantly different from control. $V_{\text{TE}}$ depolarized rapidly from $-11.5 \pm 1.0$ to $-2.9 \pm 1.1$ mV and then repolarized to $-5.5 \pm 1.2$ mV ($n = 32, P < 0.001$). $R_{\text{TE}}$ increased from $1,995 \pm 144$ to $2,278 \pm 158$ $\Omega$ cm$^2$ ($n = 26, P < 0.001$), whereas $R_{\text{Cl/Rb}}$ decreased from $5.47 \pm 1.1$ to $3.56 \pm 0.6$ (n = 13, P < 0.001). Data from this and similar experiments are summarized in Fig. 4.

All changes were fully reversed when Cl$^-$ was restored to the bath. There was a rapid (<1 min) and transient hyperpolarization of $V_{\text{mBL}}$ and $V_{\text{TE}}$ (Fig. 3, segment cd) by $6.1 \pm 0.5$ and $-8.9 \pm 0.3$ mV, respectively, before going back to their control values (segment de). $R_{\text{TE}}$ and $R_{\text{Cl/Rb}}$ returned to normal values, whereas $a_i^{\text{Cl}}$ monotonically increased to its control value over the course of 5–8 min. The rates of change of $a_i^{\text{Cl}}$ on removal and restoration of serosal Cl$^-$ were not different in the presence and absence of bicarbonate (Table 2).

**Removal of serosal Cl$^-$ in the presence of anion transport inhibitors.** To further investigate the ion transport processes involved in the cellular responses to removal of serosal Cl$^-$, we tested the effect of two known inhibitors of Cl$^-$ cotransport. The experiments were run in the absence of bicarbonate to minimize the effect of putative bicarbonate transporting mechanisms. Bumetanide blocks Na-K-2Cl cotransport in a large variety of tissues, and DIOA blocks K-Cl cotransport (6). In these experiments, esophageal tissues were exposed to the inhibitors for ~15 min, after which the solution was switched to a chloride-free solution also containing the inhibitor. Application of bumetanide (0.1 mM) or DIOA (0.1 mM), each in three different rabbits, had no effect on steady-state $V_{\text{mBL}}$ or $V_{\text{TE}}$. The change in $a_i^{\text{Cl}}$ ($\Delta a_i^{\text{Cl}}$) on removal of serosal Cl$^-$ was $-4.6 \pm 2.6$ mM in the presence of bumetanide, which is slightly but not significantly different from $\Delta a_i^{\text{Cl}}$ of $-7.4 \pm 1.7$ mM in the absence of bumetanide ($n = 5, P > 0.05$). However, the rate of decrease in $a_i^{\text{Cl}}$ on removal of serosal Cl$^-$ was $-0.96 \pm 0.3$ in the presence of bumetanide, a value significantly smaller than $-2.67 \pm 0.79$ mM/min in its absence. Moreover, the rate of increase in $a_i^{\text{Cl}}$ on restoration of serosal Cl$^-$ was $0.97 \pm 0.3$ mM/min in the presence of bumetanide, a value significantly smaller

### Table 2. Rates of change in $a_i^{\text{Cl}}$ of basal cells caused by removal and readadition of bath Cl$^-$ under different conditions

<table>
<thead>
<tr>
<th></th>
<th>$\text{Rate of decrease in } a_i^{\text{Cl}}$ on Cl$^-$ removal, mM/min</th>
<th>$\text{Rate of increase in } a_i^{\text{Cl}}$ on Cl$^-$ readdition, mM/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$-1.32 \pm 0.25$</td>
<td>$1.55 \pm 0.39$</td>
</tr>
<tr>
<td>HEPES</td>
<td>$-2.14 \pm 0.24$ (NS)</td>
<td>$1.29 \pm 0.18$ (NS)</td>
</tr>
<tr>
<td>Control</td>
<td>$-1.50 \pm 0.44$</td>
<td>$2.28 \pm 0.46$</td>
</tr>
<tr>
<td>SITS (0.5 mM)</td>
<td>$-2.01 \pm 0.66$ (NS)</td>
<td>$2.33 \pm 1.33$ (NS)</td>
</tr>
<tr>
<td>Control</td>
<td>$-4.44 \pm 1.31$</td>
<td>$2.65 \pm 0.12$</td>
</tr>
<tr>
<td>DIDS (0.1 mM)</td>
<td>$-5.6 \pm 1.40$ (NS)</td>
<td>$5.24 \pm 1.82$ (NS)</td>
</tr>
<tr>
<td>HEPES</td>
<td>$-2.67 \pm 0.79$</td>
<td>$1.93 \pm 0.63$</td>
</tr>
<tr>
<td>Bumetanide ($0.1$ mM)</td>
<td>$-0.97 \pm 0.30$</td>
<td>$0.97 \pm 0.31$</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.05$, (5)</td>
<td>$P &lt; 0.05$, (5)</td>
</tr>
<tr>
<td>HEPES</td>
<td>$-3.28 \pm 0.28$</td>
<td>$2.02 \pm 0.30$</td>
</tr>
<tr>
<td>R(+)-DIOA</td>
<td>$-2.83 \pm 0.44$ (NS)</td>
<td>$1.09 \pm 0.36$ (NS)</td>
</tr>
<tr>
<td>Control</td>
<td>$-2.19 \pm 0.20$</td>
<td>$2.51 \pm 0.05$</td>
</tr>
<tr>
<td>8-Bromo-cAMP</td>
<td>$-2.42 \pm 0.43$ (NS)</td>
<td>$2.08 \pm 0.32$ (NS)</td>
</tr>
</tbody>
</table>

Values are means ± SE; the number between parenthesis is the number of observations. *Significantly different from control, paired data, $P < 0.05$. R(+)butylisodamine [R(+)-DIOA]
than $1.93 \pm 0.63$ mM/min in its absence ($n = 5, P < 0.05$; Table 2). In the presence of DIOA, $\Delta a_{i Cl}^{\text{Cl}}$ was $-9.0 \pm 2.3$ mM, a value not significantly different from $\Delta a_{i Cl}^{\text{Cl}}$ of $-9.1 \pm 1.7$ mM in the absence of DIOA ($n = 5, P > 0.05$). Also, the $\Delta V_{mBL}$ and $\Delta V_{TE}$ were similar in the absence and presence of bumetanide or DIOA ($P > 0.05$). The rate of change in $a_{i Cl}^{\text{Cl}}$ on removal and addition of serosal $\text{Cl}^-$ was $-3.3 \pm 0.28$ and $2.02 \pm 0.3$ mM/min, respectively, in the absence and $-2.83 \pm 0.44$ and $1.09 \pm 0.36$ mM/min, respectively, in the presence of DIOA. Although the rates were slightly reduced in the presence of the inhibitor, the values were not statistically different. These data indicate that although under steady-state conditions, bumetanide or DIOA do not alter $a_{i Cl}^{\text{Cl}}$, bumetanide decreased the rate of change of $a_{i Cl}^{\text{Cl}}$ on removal and addition of serosal $\text{Cl}^-$ without affecting the accompanying changes in cell membrane potential difference. This finding is consistent with the

Fig. 3. Tracing from an experiment showing the effect of removal of serosal Cl$^-$ in a basal cell, on $a_{i Cl}^{\text{Cl}}$, $V_{mBL}$, and $V_{TE}$ in the absence of CO$_2$/HCO$_3$ (HEPES Ringer). Removal of Cl$^-$ from the serosal bath caused an initial transient depolarization (ab) of $V_{mBL}$ that was followed by a quick hyperpolarization and recovery (bc) to a value slightly lower but not significantly different from control. $V_{mBL}$ also quickly depolarized and partially recovered, whereas $a_{i Cl}^{\text{Cl}}$ decreased substantially. The changes were reversible on restoring bath Cl$^-$.

Fig. 4. Effect of removal of Cl$^-$ from the serosal bath in the absence of CO$_2$/HCO$_3$ in a basal cell, on $V_{mBL}$ (A), $V_{TE}$ (B), $a_{i Cl}^{\text{Cl}}$ (C), $R_{TE}$ (D), and $Ra/Rb$ (E). Removal of serosal Cl$^-$ caused an initial depolarization of $V_{mBL}$ of $5.0 \pm 0.8$ mV, which then recovered to a value not significantly different from control ($n = 30; P < 0.001$). $V_{TE}$ depolarized initially by $8.6 \pm 0.6$ mV and recovered partially to a value $6.3 \pm 0.7$ mV more positive than control ($n = 32; P < 0.001$). $a_{i Cl}^{\text{Cl}}$ decreased by $6.5 \pm 0.94$ mM ($n = 13; P < 0.001$). $R_{TE}$ increased by $283 \pm 29 \Omega \cdot \text{cm}^2$ ($n = 26; P < 0.001$), and $Ra/Rb$ decreased by $1.91 \pm 0.54$ ($n = 13; P < 0.001$). *Significantly different from control, paired data ($P < 0.05$).
presence of an electrogenic transport mechanism for Cl⁻ transport at the basolateral membrane, likely Na-K-2Cl.

Removal of serosal Cl⁻ in the presence of Cl⁻-channel blockers. Because the depolarization of $V_{mBL}$ on removal of serosal Cl⁻ indicates that a conductive pathway for Cl⁻ exists at the basolateral membrane of basal cells, the following four compounds reported to inhibit different types of Cl⁻ channels in other preparations were investigated: anthracene-9-carboxylate (A-9-COOH), DPC, NPPB, and R(+)-IAA94 (7, 12, 13). Application of each of these compounds, A-9-COOH (1 mM), DPC (0.1 mM), NPPB (0.1 mM), or R(+)-IAA94 (20 μM) in at least three tissues from different animals had no effect on steady-state $V_{mBL}$ or $a_i^{Cl}$ in either bicarbonate- or HEPES-containing solutions. $\Delta V_{mBL}$, $\Delta a_i^{Cl}$, and the rate of decrease in $a_i^{Cl}$ in the removal of serosal Cl⁻ were not significantly different in the absence and presence of each of these four inhibitors (Table 3).

Flufenamate, at concentrations ranging from 0.1 to 0.5 mM, is an inhibitor of Cl⁻ conductance, particularly the Ca²⁺-activated Cl⁻ channel (27, 29). Similar to A-9-COOH, DPC, NPPB, and R(+)-IAA94, serosal application of the Cl⁻-channel inhibitor flufenamate (0.5 mM) did not cause significant changes in steady-state values of $V_{mBL}$ or $a_i^{Cl}$. Moreover, in the presence of flufenamate, removal of serosal Cl⁻ decreased $a_i^{Cl}$ by 5.9 ± 0.74 mM, a value not significantly different from the decrease in $a_i^{Cl}$ (6.8 ± 1.8 mM) in the absence of the inhibitor. However, in the presence of flufenamate, $V_{mBL}$ depolarized by only 2.9 ± 0.5 mV (Fig. 5, segment $a'b'$) compared with 7.4 ± 1 mV in control (Fig. 5, segment $ab$), and the repolarization was only partial (Fig. 5, segment $b'c'$). Similarly, the initial depolarization of $V_{TE}$ was only 5.8 ± 0.6 mV compared with 9.0 ± 0.7 mV in control, thus the peak depolarizations of $V_{mBL}$ and $V_{TE}$ caused by Cl⁻ removal in the presence of flufenamate were significantly smaller than those in its absence ($P < 0.01, n = 14$). The rate of decrease of $a_i^{Cl}$ was markedly reduced from $-2.9 ± 0.8$ to $-0.9 ± 0.2$ mM/min in the presence of flufenamate ($n = 5, P < 0.05$). These results are summarized in Table 3.

Effect of removal of serosal Cl⁻ in the absence of Ca²⁺. The effect of flufenamate is indicative of the presence of Ca²⁺-activated Cl⁻ channels in the basolateral membrane (5, 27). Prolonged exposure of the tissue to a Ca²⁺-free solution in the presence of a Ca²⁺ chelator is likely to result in reduction of intracellular Ca²⁺. Moreover, studies in other epithelial cells have indicated that Cl⁻ channels can be regulated by external and internal Ca²⁺ (24, 28). We, therefore, investigated the effect of Cl⁻ removal in the absence of Ca²⁺ from the bathing solutions. In seven esophageal tissues from six different rabbits, Ca²⁺ removal was achieved by perfusing the esophagus bilaterally for ~20 min with a Ca²⁺-free HEPES solution to which 3 mM EDTA was added (solution 5). Figure 6 is a representative tracing showing the results of those experiments. The removal of Ca²⁺ caused a slow depolarization of $V_{mBL}$ from $-57 ± 3.5$ to $-42 ± 1.9$ mV and of $V_{TE}$ from $-11.9 ± 0.6$ to $-8.6 ± 0.5$ mV ($n = 12, P < 0.001$), whereas $a_i^{Cl}$, $R_{TE}$, and $Ra/Rb$ did not change significantly. The removal of Cl⁻ in the absence of Ca²⁺ (solution 6) caused an initial small depolarization of $V_{mBL}$, which was followed by a repolarization (segment $a'b'c'$). In the absence of Ca²⁺, the initial depolarization caused by Cl⁻ removal (segment $a'b'$) averaged 2.9 ± 0.8 mV, which was significantly smaller than the depolarization of 6.1 ± 0.6 mV in control (segment $ab; n = 10, P < 0.006$). Subsequent to this depolarization, in the absence of Ca²⁺, $V_{mBL}$ repolarized to a value of 9.3 ± 1.0 mV ($n = 10, P < 0.001$), more negative than the initial value (segment $b'c'$) whereas in the presence of Ca²⁺, it repolarized to a value not significantly different from control (segment $bc$). The transient hyperpolarization of $V_{mBL}$ (segment $cd; -5.8 ± 0.9$ mV) on the return of Cl⁻ to the bath was also inhibited (~2.0 ± 0.4 mV) in the absence of Ca²⁺ (segment $c'd'$). $V_{TE}$ depolarized initially by 8.6 ± 0.6 mV on removal of Cl⁻ in the

Table 3. Effect of Cl⁻ channel inhibitors on changes in $V_{mBL}$, $a_i^{Cl}$, and $V_{TE}$ upon removal of Cl⁻ from the serosal bath

<table>
<thead>
<tr>
<th></th>
<th>$\Delta V_{mBL}$ initial, mV</th>
<th>$\Delta V_{mBL}$ final, mV</th>
<th>$\Delta a_i^{Cl}$, mM</th>
<th>Rates of decrease in $a_i^{Cl}$, mM/min</th>
<th>$\Delta V_{TE}$ initial, mV</th>
<th>$\Delta V_{TE}$ final, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.0 ± 0.9</td>
<td>2.4 ± 4.3</td>
<td>-10.9 ± 3.4</td>
<td>-3.4 ± 0.6</td>
<td>9.6 ± 1.7</td>
<td>7.8 ± 2.2</td>
</tr>
<tr>
<td>A-9-COOH (1 mM)</td>
<td>5.4 ± 0.9 NS (3)</td>
<td>-0.2 ± 3.1 NS (3)</td>
<td>-9.2 ± 4.0 NS (3)</td>
<td>-3.7 ± 0.9 NS (3)</td>
<td>5.9 ± 1.2 NS (3)</td>
<td>4.2 ± 0.9 NS (3)</td>
</tr>
<tr>
<td>Control</td>
<td>8.0 ± 1.0</td>
<td>1.0 ± 3.0</td>
<td>-6.4 ± 3.1</td>
<td>-2.1 ± 0.4</td>
<td>9.1 ± 0.1</td>
<td>4.1 ± 2.1</td>
</tr>
<tr>
<td>DPC (0.1 mM)</td>
<td>8.5 ± 0.5 NS (4)</td>
<td>-0.5 ± 2.5 NS (4)</td>
<td>-3.7 ± 1.5 NS (4)</td>
<td>-2.5 ± 0.9 NS (4)</td>
<td>9.0 ± 1.0 NS (4)</td>
<td>4.1 ± 2.9 NS (4)</td>
</tr>
<tr>
<td>Control</td>
<td>6.8 ± 1.3</td>
<td>1.3 ± 1.8</td>
<td>-4.0 ± 1.3</td>
<td>-3.4 ± 0.9</td>
<td>7.4 ± 0.6</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>NPPB (0.1 mM)</td>
<td>5.5 ± 1.2 NS (4)</td>
<td>0.8 ± 2.8 NS (4)</td>
<td>-7.9 ± 2.8 NS (3)</td>
<td>-5.9 ± 2.1 NS (3)</td>
<td>6.2 ± 0.5 NS (4)</td>
<td>-1.0 ± 3.2 NS (4)</td>
</tr>
<tr>
<td>Control</td>
<td>5.7 ± 0.3</td>
<td>3.3 ± 2.7</td>
<td>-8.1 ± 1.9</td>
<td>-2.5 ± 0.7</td>
<td>8.9 ± 0.9</td>
<td>8.9 ± 0.9</td>
</tr>
<tr>
<td>IAA-94 (20 μM)</td>
<td>4.7 ± 0.9 NS (3)</td>
<td>-3 ± 4.7 NS (3)</td>
<td>-13.1 ± 1.8 NS (3)</td>
<td>-2.7 ± 0.9 NS (3)</td>
<td>5.3 ± 1.8 NS (3)</td>
<td>4.9 ± 1.5 NS (3)</td>
</tr>
<tr>
<td>Control</td>
<td>7.4 ± 1.0</td>
<td>-2.3 ± 1.5</td>
<td>-6.8 ± 1.8</td>
<td>-2.9 ± 0.8</td>
<td>9.0 ± 0.7</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>Flufenamate</td>
<td>2.9 ± 0.5</td>
<td>0.2 ± 1.3</td>
<td>-5.9 ± 1.0</td>
<td>-0.9 ± 0.2</td>
<td>5.8 ± 0.6</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td>(0.5 mM)</td>
<td>*P &lt; 0.001 (14)</td>
<td>NS (14)</td>
<td>NS (7)</td>
<td>*P &lt; 0.05 (5)</td>
<td>*P &lt; 0.01 (14)</td>
<td>NS (14)</td>
</tr>
</tbody>
</table>

Changes ($\Delta$) are of basolateral membrane potential ($V_{mBL}$), intracellular Cl⁻ activity ($a_i^{Cl}$), and transepithelial potential ($V_{TE}$), respectively, observed on removal of Cl⁻ from the bath in the absence (control) and presence of the inhibitor. The initial change in $V_{mBL}$ is the rapid transient depolarization observed on removal of Cl⁻ from the bath. $\Delta V_{mBL}$ final is the value reached after $V_{mBL}$ has reached a steady state in 0 Cl⁻ (Na-glucosinate). Positive changes in $V_{mBL}$ and $V_{TE}$ indicate a depolarization. Values are means ± SE; the number in parenthesis is the number of observations. *Significantly different from control, paired data ($P < 0.05$). NS, not significant; DPC, diphenylamine-2-carboxylate; NPPB, 5-nitro-2-(3-phenylpropyl-amino)benzoic acid.
absence of Ca\textsuperscript{2+}, a value slightly smaller than in control (10.1 \pm 0.6). The changes in \(R_{TE}\) and \(Ra/Rb\) were similar in the absence and presence of Ca\textsuperscript{2+}. \(a_i^{Cl}\) decreased by 4.6 \pm 1.8 mM in control, a value not significantly different from 7.8 \pm 0.7 mM in the absence of Ca\textsuperscript{2+} (\(n = 3, P > 0.05\)). The inhibition of the depolarization on removal of Cl\textsuperscript{-} both in the presence of flufenamate and in the absence of Ca\textsuperscript{2+} strongly supports the presence of Ca\textsuperscript{2+}-sensitive Cl\textsuperscript{-} channels on the basolateral membrane.

**Cyclic AMP.** To examine the effect of a Cl\textsuperscript{-}-channel activator on Cl\textsuperscript{-} transport in the basal esophageal cell, we studied the effect of cAMP on the changes induced by the removal of Cl\textsuperscript{-}. We exposed the serosal side of the tissue to a Ringer solution containing the permeable analog of cAMP, 8-bromo-cAMP (10\textsuperscript{-4} M), for several minutes. 8-bromocyclic AMP did not have an effect on the steady-state values of \(V_{mBL}\), \(V_{TE,0}\) or \(ai^{Cl}\). When Cl\textsuperscript{-} was removed from the serosal bath, \(V_{mBL}\) depolarized transiently by 4.8 \pm 3.0 mV, after which it repolarized to a value not significantly different from control, whereas \(a_i^{Cl}\) decreased by 10.2 \pm 1.5 mM, with a rate of change in \(a_i^{Cl}\) that was not different from control (Table 2). \(V_{TE}\) depolarized transiently by 4.0 \pm 2.3 mV and then repolarized to a value 2.4 \pm 0.4 mV more positive than control. Those values were not significantly different from the values observed in the absence of 8-bromo-cAMP in the same experiments (\(n = 4, P > 0.05\)).

**Apical Membrane Studies in Luminal Cells**

For apical membrane studies, two adjacent luminal cells were impaled with two single-barreled micro-electrodes, one a Ling-Gerard microelectrode for cell membrane potential measurement and the other a Cl\textsuperscript{-}-sensitive microelectrode for intracellular Cl\textsuperscript{-} measurements. For these measurements to be valid, the membrane potentials recorded by the two electrodes should be identical and ionic substitutions in the luminal bath should cause equal changes in the two cell membrane potential differences. To validate the measurements thus obtained, experiments were performed in which two different luminal cells in the same tissue were impaled using a Ling-Gerard microelectrode and a dummy microelectrode identical to the ion-selective
microelectrode but filled with 3 M KCl. As shown in Fig. 7, cells 1 and 2 have similar membrane potential differences, and the potential changes produced by the removal of Na⁺ or Cl⁻ from the luminal bath are the same in the two cells. This experiment was repeated in tissues from three different rabbits, and the luminal cell membrane potential difference was −62 ± 1.9 and −61 ± 2.1 mV in the simultaneously impaled cells, cells 1 and 2 (n = 9, P > 0.05). In the absence of Cl⁻ from the luminal bath those values were −60 ± 3.3 and −59 ± 2.3, mV respectively (n = 4, P > 0.05).

Measurements of intracellular Cl⁻ in the presence of HCO₃⁻ Ringer. In tissues from six rabbits perfused with control HCO₃⁻ Ringer (Table 1, solution 1), VₘC was −53 ± 5.1 mV, Vₜₑ was −11.1 ± 2.5 mV, and aᵥCl was 26.7 ± 3.3 mM. Rₜₑ was 2,237 Ω·cm², and Rₐ/Rb was 1.75 ± 0.37. On the basis of the measured value of aᵥCl, the calculated electrochemical equilibrium potential in luminal cells for Cl⁻ (EₜₑCl) was −34 mV, which is significantly smaller than the luminal cell potential difference VₘC of −53 mV observed in these cells. Thus, in the presence of CO₂/HCO₃⁻, intracellular Cl⁻ is higher than predicted from electrochemical equilibrium.

Measurements of intracellular Cl⁻ in the nominal absence of bicarbonate. In tissues from five rabbits perfused with HEPES Ringer (solution 3, Table 1), VₘC was −65 ± 3.6 mV, Vₜₑ was −14.5 ± 1.5 mV, and aᵥCl was 16.3 ± 2.2 mM. Rₜₑ was 2,413 ± 177 Ω·cm², and Rₐ/Rb was 2.27 ± 0.2 (n = 12). EₜₑCl, calculated from Eq. 6, was −49 mV in the absence of CO₂/HCO₃⁻, a value significantly smaller than the electrical potential difference of −64 mV observed across the apical cell membrane. These experiments indicate that aᵥCl is higher than the activity of Cl⁻ predicted by electrochemical equilibrium across both the apical and the basolateral membranes both in the presence or absence of CO₂/HCO₃⁻.

Effect of removal of luminal and basolateral Cl⁻ in the absence of CO₂/HCO₃⁻. To investigate Cl⁻ transport at the apical membrane of luminal cells of EE, we conducted the experiment depicted in Fig. 8. When the luminal bath solution was switched to a Cl⁻-free solution in the nominal absence of CO₂/HCO₃⁻, VₘC depolarized from −65 ± 3.0 to −57 ± 2.7 mV (segment abc). Vₜₑ hyperpolarized from −14.5 ± 1.5 to −21.8 ± 1.1 mV, and aᵥCl decreased from 16.3 ± 2.2 to 13.8 ± 1.9 mM. As shown in Fig. 8, the changes in VₘC and Vₜₑ were not transient, which is different from the changes in VₘBL and Vₜₑ when Cl⁻ was removed from the basolateral bath. Rₜₑ increased from 2,413 ± 177 to 3,100 ± 247 Ω·cm², and Rₐ/Rb increased from 2.27 ± 0.2 to 2.63 ± 0.2 (n = 12, P < 0.02). The hyperpolarization of Vₜₑ on luminal Cl⁻ removal could be explained by a diffusion potential for Cl⁻ across the paracellular shunt, a fact also supported by the observed increase in Rₜₑ.

When Cl⁻ was subsequently removed from the basolateral solution in the continuous absence of luminal Cl⁻, there was an initial transient hyperpolarization of VₘC from −55 ± 2.2 to −63 ± 2.4 mV after which VₘC depolarized to −51 ± 3.6 mV and Vₜₑ depolarized transiently from −23.6 ± 1.4 to −13.2 ± 1.4 mV to recover to −18.5 ± 2.2 mV. Rₜₑ increased from 3,292 ± 264 to 3,675 ± 323 Ω·cm². The aᵥCl in the luminal cell decreased from 15.2 ± 3.4 to 8.8 ± 2.4 mM (n = 6, P < 0.001) in response to basolateral Cl⁻ removal. Thus the decrease in aᵥCl in the apical cell is significantly larger (6.3 ± 1.0 mM) when Cl⁻ is removed from the basolateral side than when removed from the luminal side (2.6 ± 0.9 mM; unpaired t-test, n = 15, P < 0.02). These data are summarized in Fig. 9.

Fig. 7. Tracing from an experiment in which 2 different luminal cells in the same tissue were impaled using a Ling-Gerard microelectrode and a dummy microelectrode identical to the ion-selective microelectrode but filled with 3 M KCl. Cells 1 and 2 indicate 2 adjacent cells a few millimeters apart. The 2 cells have similar membrane potential differences (VₘC), and the potential changes produced by the removal of Na⁺ or Cl⁻ from the luminal bath are the same in the 2 cells.
Effect of cyclic AMP. To investigate the possibility that a cystic fibrosis transmembrane conductance regulator-like channel is expressed at the luminal cell membrane, we examined the effect of removal of luminal Cl⁻/H¹¹⁰₀²⁻ in the presence of the permeable analog of cAMP, 8-bromo-cAMP. We exposed the luminal side of the tissue to a control HEPES solution containing 8-bromo-cAMP (10⁻⁴ M) for several minutes. 8-bromo-cAMP did not have an effect on the steady-state values of $V_{mc}$, $V_{TE,a}$, $i_{Cl}$, $R_{a/b}$, or $R_{TE}$. The removal of Cl⁻ from the luminal solution in the continuous presence of 8-bromo-cAMP caused $V_{mc}$ to depolarize by 6.3 ± 2.2 mV, and $V_{TE}$ hyperpolarized by 7.8 ± 0.4 mV, $Ra/Rb$ remained unchanged, and $R_{TE}$ increased by 282 ± 3 Ω·cm². Those changes are not significantly different from the changes observed in the absence of cAMP ($n = 4$, $P > 0.05$).

The above experiment was repeated in the presence of bicarbonate to examine the possible presence of a...
cAMP-stimulated apical Cl⁻/HCO₃⁻ exchange. In the presence of bicarbonate Ringer, we exposed the luminal side of the tissue to 8-bromo-cAMP (10⁻⁴ M) for several minutes. Similar to the experiment in the absence of bicarbonate, 8-bromo-cAMP did not have an effect on the steady-state values of \(V_m\), \(V_{TE}\), \(a^{Cl}\), \(R_a/\), or \(R_{TE}\). The removal of Cl⁻ from the luminal solution in the continuous presence of 8-bromo-cAMP caused \(V_{TE}\) to depolarize by 5.5 ± 2.5 mV, and \(a^{Cl}\) decreased by 2.7 ± 1.9 mM at a rate of 0.2 ± 0.12 mM/min. \(V_{TE}\) hyperpolarized by 7.45 ± 0.9 mV, \(R_a/\) increased slightly but not significantly, and \(R_{TE}\) increased by 353 ± 41Ω·cm². These changes are not significantly different from the changes observed in the absence of cAMP (n = 4, P > 0.05). It should be noted that the rate of decrease in \(a^{Cl}\) (−0.2 ± 0.12 mM/min) in the apical cell on removal of luminal Cl⁻ was about 10-fold smaller than the rate of decrease in \(a^{Cl}\) in the basal cell on removal of serosal Cl⁻, a fact consistent with the lower permeability of the luminal cell membrane to Cl⁻.

**DISCUSSION**

*Steady-State Measurements*

The aim of this study was to investigate the mechanisms for Cl⁻ transport across the apical and basolateral membranes of esophageal epithelial cells. Intracellular measurements were obtained either in luminal cells (across the apical membrane) or basal cells (across the basolateral membrane) of intact sections of rabbit esophageal epithelium using conventional and ion-selective microelectrodes. The results of these investigations establish for the first time the level of intracellular Cl⁻ activity in the luminal and basal cells of this stratified squamous epithelium. Intracellular activity of Cl⁻ was higher than equilibrium in both cell types even when the esophageal tissue was exposed to a nominally bicarbonate-free solution, indicating that active transport mechanism(s) for Cl⁻ entry exists in these cells and that these mechanism(s) are not dependent on the presence of bicarbonate. Moreover, in the presence of CO₂/HCO₃⁻ (and, therefore, HCO₃⁻-dependent Cl⁻ transports could be active), neither SITS nor DIDS changed the steady-state value of \(a^{Cl}\) nor the changes observed on removal of serosal Cl⁻. This finding is consistent with the fact that removal of bicarbonate from the perfusing solutions did not alter \(a^{Cl}\) appreciably.

Among the possible bicarbonate-independent mechanisms that could be responsible for Cl⁻ entry are Na-K-2Cl and/or K-Cl cotransport. In our experiments, the rate of change in \(a^{Cl}\) on removal and addition of serosal Cl⁻ was significantly reduced in the presence of bumetanide, which indicates that Na-K-2Cl is contributing to the movement of Cl⁻ across the basolateral membrane at least when Cl⁻ gradient is perturbed across this membrane. The possibility exists that under physiological conditions, one or both transporters are present but only activated in the face of an osmotic challenge to the cell (15, 25). Other transporters likely to drive Cl⁻ into the cells in the absence of bicarbonate are Cl⁻/OH⁻, or other Cl⁻-base exchangers similar to those reported in the kidney (10, 26).

Although the mechanism by which \(a^{Cl}\) is maintained above the value predicted by equilibrium potential in esophageal cells, undoubtedly, results from the action of several transporters including Na⁺/K/2Cl⁻, a leak pathway for Cl⁻ must exist to counterbalance active Cl⁻ loading to maintain a steady-state \(a^{Cl}\). A likely mechanism for such a leak is through a conductive pathway for Cl⁻.

**Basolateral Cl⁻ Channels**

Our experiments support the presence of a conductive pathway for Cl⁻, which is more prominent on the basolateral than on the apical membrane of the cell. This finding is supported by several pieces of evidence. First, the removal of external Cl⁻ causes a fast depolarization of the basolateral membrane potential difference in the basal cell. Although \(V_{BL}\) gradually recovers to its original value, this recovery is likely due to compensatory secondary mechanisms. This behavior of the cell membrane is consistent with the presence in the basolateral membrane of two conductive pathways, one for K⁺ and the other for Cl⁻. The rapid depolarization due to Cl⁻ exit causes K⁺ to leave the cell (9), which brings back the cell membrane PD to its original value. In fact, the presence of a conductive pathway for K⁺ in the basolateral membrane of esophageal basal cells has already been established (11). Second, a linear relationship between \(V_{BL}\) and \(a^{Cl}\) exists. When \(V_{BL}\) is plotted vs. the ratio of \(a^{Cl}\) in tissues bathed in control HEPES Ringer, a straight line is generated (Fig. 10A), the S of which is 67.8 mV/decade of change in \(a^{Cl}\) (R² = 0.83, n = 13, P < 0.001). Third, serosal furosemate, a Cl⁻-channel blocker blocked the depolarization induced by the removal of Cl⁻ by 60%. Fourth, the rate of decrease in \(a^{Cl}\) on removal of serosal Cl⁻ was significantly inhibited in the presence of furosemate. It is also of importance to note that this conductive pathway for Cl⁻ was less sensitive to treatment with other Cl⁻-channel inhibitors, such as SITS, DIDS, DPC, A-9-COOH, NPPB, or IAA-94, and that 8-bromocyclic-AMP did not stimulate this Cl⁻ conductance.

The removal of serosal Cl⁻ caused apparent \(R_a/\) to decrease substantially. Although we cannot rule out a change in the shunt resistance contributing to the observed change in the divider ratio, the observed decrease in \(R_a/\) on removal of serosal Cl⁻ reflects a possible increase in basolateral membrane resistance, indicating that Cl⁻ may contribute significantly to the conductance across this membrane. It should be noted that the calculation of \(R_a/\) (Eq. 5) from the voltage divider ratio is an approximation that holds true only if \(V_{TE} = V_{BL} + V_m\). This assumption was validated in relatively simple epithelia (21, 22). Given the complexity of the esophageal epithelium (~25 layers of cells (17)), we relied on the following observations as an
**Fig. 10.** Plot of the logarithm of the ratio of intracellular to extracellular activity of Cl⁻ ($a_i^{Cl}$/$a_o^{Cl}$) against $V_{mBL}$ (A) and $V_{mc}$ (B) when the tissues were perfused with control HEPES Ringer. The regression equation for $V_{mc}$ against $a_i^{Cl}$/$a_o^{Cl}$ is $y = 18.9x - 51.9, R^2 = 0.24$ ($n = 14; P > 0.05$), whereas the regression equation for $V_{mBL}$ against $a_i^{Cl}$/$a_o^{Cl}$ is $y = 67.8x - 17.0, R^2 = 0.83$ ($n = 13; P < 0.001$).

indication that $V_{TE}$ approximately equals $V_{mBL} + V_{mc}$. First, our pooled data of measured $V_{mBL}$ and $V_{TE}$ during basolateral impalements indicate that the average calculated value of $V_{mc}$ in these experiments ($-57 \pm 4.1$ mV in HCO$_3^-$ and $-61 \pm 4.1$ mV in HEPES Ringer) was not statistically different from the measured values of $V_{mc}$ ($-50 \pm 4.8$ and $-66 \pm 3.6$ mV, respectively) obtained from experiments in which luminal impalements were performed. Similarly, the data of measured $V_{mc}$ and $V_{TE}$ during luminal impalements yielded an average calculated value of $V_{mBL}$ in these experiments of $-64 \pm 5.5$ in HCO$_3^-$ and $-78 \pm 2.6$ mV, respectively, in HEPES, which was not statistically different from the average measured $V_{mBL}$ ($-70 \pm 4.2$ mV in HCO$_3^-$ and $-73 \pm 4.3$ mV in HEPES Ringer) obtained from basolateral impalements. Second, as described in METHODS, the calculated value of $V_{mc}$ (as $V_{TE} - V_{mBL}$; $-56 \pm 4.8$ mV) obtained from serosal impalements and the measured value of a presumed $V_{mc}$ ($-57 \pm 5.0$ mV) obtained from readings of the intracellular voltage microelectrode against the reference electrode in the luminal bath (rather than the serosal bath) were also similar.

The inhibition by flufenamate suggests the presence of a Ca$^{2+}$-sensitive Cl⁻ channel at the basolateral membrane of the esophageal cell. This is supported by the fact that in the absence of extracellular Ca$^{2+}$, the initial depolarization induced by Cl⁻ removal was reduced by ~50%. Moreover, in the absence of Ca$^{2+}$, the transient hyperpolarization on readdition of basolateral Cl⁻ was significantly inhibited. It remains to be determined whether this Cl⁻ channel shares common characteristics with the Ca$^{2+}$-activated Cl⁻ channel family, which play an important role in anion transport and cell volume regulation and may have a role as cell adhesion molecules (4, 8, 19).

**Apical Membrane Cl⁻ Transport**

The conductance of the apical membrane of luminal cells to Cl⁻ is very limited and could not be stimulated by cAMP. Transport of Cl⁻ is more prominent on the basolateral side. This is supported by several observations. First, the apical membrane potential is not linearly proportional to changes in $a_i^{Cl}$. For example, when $V_{mc}$ is plotted vs. the ratio of $a_i^{Cl}$ to $a_o^{Cl}$ in tissues bathed in control HEPES Ringer, a straight line (Fig. 10B) with poor correlation is generated. The $S$ of this line is 18.9 mV/decade of change in $a_i^{Cl}$/a_o^{Cl} ($R^2 = 0.24, n = 14, P = 0.07$). Second, whereas removal of basolateral Cl⁻ reduced $a_i^{Cl}$ in the basal cell by 40%, luminal Cl⁻ removal reduced $a_i^{Cl}$ in the luminal cell by only 15%. In fact, basolateral Cl⁻ deletion caused an even larger drop in luminal cell $a_i^{Cl}$ than did luminal Cl⁻ deletion (see RESULTS). Third, the rate of decrease in $a_i^{Cl}$ in the apical cell on removal of luminal Cl⁻ is severalfold smaller than the rate of decrease in $a_i^{Cl}$ in the serosal cell on removal of serosal Cl⁻. It is also to be noted that the removal of basolateral Cl⁻ caused Ra/Rb to decrease by 35%, whereas luminal Cl⁻ removal caused an increase in Ra/Rb of only 16%. These findings indicate that Cl⁻ transport at the basolateral membrane is the major determinant of $a_i^{Cl}$ and that there is little transport of Cl⁻ across the apical membrane. Such findings are also in agreement with the observation that Cl⁻ transport from serosa to mucosa contributes only a small (~10%) component of net active ion transport (as reflected in the short-circuit current) across rabbit esophageal epithelium. Such limited conductance of the apical membrane of luminal cells, however, is likely a reflection of its barrier properties, protecting the surface cells from hydrochloric acid refluxing from the stomach and from osmotic and concentration gradients in various ingested solutes.

In summary, our study indicates the presence of a conductive pathway for Cl⁻ transport at the basolateral membrane of the cell, which is sensitive to flufenamate and to the absence of Ca$^{2+}$. The fact that $a_i^{Cl}$ in the esophageal epithelium is maintained above electrochemical equilibrium is indicative of additional bicarbonate-independent secondary active pathways for Cl⁻ influx into the cell. Our results also indicate that there is little capacity for the transport of Cl⁻ across the apical membrane, and this likely accounts for the minimal contribution of Cl⁻ to net active transport of ions across this epithelium.

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