Voltage-gated proton channels help regulate pH_ı in rat alveolar epithelium

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Murphy, Ricardo, Vladimir V. Cherny, Deri Morgan, and Thomas E. DeCoursey. Voltage-gated proton channels help regulate pH_ı in rat alveolar epithelium. Am J Physiol Lung Cell Mol Physiol 288: L398–L408, 2005. First published October 29, 2004; doi: 10.1152/ajplung.00299.2004.—Voltage-gated proton channels are expressed highly in rat alveolar epithelial cells. Here we investigated whether these channels contribute to pH regulation. The intracellular pH (pH_ı) was monitored using BCECF in cultured alveolar epithelial cell monolayers and found to be 7.13 in nominally HCO_3⁻-free solutions [at external pH (pH_e) 7.4]. Cells were acid-loaded by the inhibition of all known pH regulators, slow pH_ı recovery persisted, suggesting the existence of a yet-undefined acid extrusion mechanism in these cells.

proton conductance; pH regulation; hydrogen ion; acid load; 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; intracellular pH

ALVEOLAR TYPE II EPITHELIAL CELLS exhibit an impressive panoply of pH-regulating mechanisms, perhaps reflecting their role in extruding enormous quantities of acid in the form of CO₂ during respiration as well as their exposure to an asymmetrical pH environment. Whereas the basolateral membranes face typical interstitial fluid, the apical membranes face the alveolar subphase, the fluid at the interface between air and tissues in the alveolus. This fluid is highly acidic compared with plasma or interstitial fluid, with estimates at pH 6.69 in dog lung (17), pH 6.27 in fetal lamb lung (1), and pH 6.92 in rabbit lung (31). The properties and localization of several transporters that influence pH_ı have been studied previously. Sodium-proton exchange, Na⁺/H⁺-antiport, is active at pH_ı <7.0 during recovery from an acid load (32) and appears to be localized in basolateral membranes (22, 26). Sodium-independent Cl⁻/HCO_3⁻ exchange contributes to recovery from an alkaline load (33), and the alveolar type II epithelial isoform is restricted to the basolateral surface of alveolar epithelial monolayers (27). A Cl⁻-independent Na⁺-HCO_3⁻ symporter is detected in basolateral membranes (27). Evidence for a K⁺-H⁺-ATPase exists in guinea pig but not rat type II pneumocytes (24). A plasma membrane V-type H⁺-ATPase reportedly is active at physiological pH and may keep pH_ı near 7.5 (28), although Brown et al. (6) found no evidence for a V-type H⁺-ATPase in rat type II cells and concluded that ATP modulates Na⁺/H⁺-antiport. The possibility that Cl⁻/OH⁻ exchange (38) might occur in rat alveolar epithelial cells was suggested by highly indirect evidence (13).

Finally, voltage-gated proton channels comprise a major Conductance in rat alveolar epithelial type II cells (10), are demonstrably present in the apical membranes of cultured cells (12), and might be present in all membranes. Proton channels are opened by membrane depolarization, decreased pH_ı, increased extracellular pH (pH_e), or a combination of these factors (9, 11). Their regulation by pH and voltage ensures that these channels open only when there is an outward electrochemical gradient for protons (11). Because this gradient is normally inward (10), we predict that H⁺ current inhibition with Zn²⁺ should have no effect on pH_ı in unchallenged alveolar epithelial cells. In several other cells, proton channels mediate pH_ı recovery after an acid load. However, until now, no direct evidence for this or any other specific function for proton channels had been demonstrated in alveolar epithelial cells. Effects of Zn²⁺ reported here indicate that voltage-gated proton channels are closed at normal pH_ı but contribute to H⁺ extrusion after acid loading rat alveolar epithelial type II cells.

MATERIALS AND METHODS

Rat alveolar epithelial cells. Type II alveolar epithelial cells were isolated from adult male Sprague-Dawley rats by enzyme digestion, lectin agglutination, and differential adherence, as described in detail elsewhere (15), with three exceptions. First, the solution used to perfuse the lungs is 40 ml of HBSS (catalog no. 14170-112; GIBCO Laboratories, Grand Island, NY). Second, we use 0.2 mg/ml elastase without trypsin to dissociate the cells. Third, the filtrate is centrifuged at 2,000 rpm (instead of 1,500 rpm). Before invasive procedures were initiated, the rats were anesthetized deeply with pentobarbital sodium. The rats were treated humanely in compliance with local law, our Institutional Animal Care and Use Committee, and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The lungs were lavaged to remove macrophages, elastase was instilled, and then the tissue was minced and forced through fine gauze. Lectin agglutination and differential adherence further removed contaminating cell types. The preparation at first included mainly type II alveolar epithelial cells, but after several days in culture, the properties of the cells are more like type I cells. Studies were done on monolayers of cells grown on cover glass chips for 5–25 days. Plotting the parameters defined in Fig. 2 against time in culture did not reveal any trends (data not shown).

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Chemicals. SCH-28080 and some of the 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM) used here were purchased from Calbiochem (La Jolla, CA). All of the remaining chemicals, including nigericin, 5-(N,N-dimethyl)amiloride hydrochloride (DMA), bafilomycin A1, and some BCECF-AM, were obtained from Sigma Chemical (St. Louis, MO).

Measurement of pH. Cells were loaded for 20–60 min with 10–20 μg/ml BCECF-AM, the nonfluorescent, membrane-permeant acetoxyethyl ester of BCECF dissolved in 1 ml of Ringer solution (in mM: 160 NaCl, 4.5 KCl, 1 MgCl2, 5 HEPES; pH 7.4) or culture medium. Intracellular esterases cleave the three AM ester groups to form the charged, membrane-impermeant, pH-sensitive, fluorescent dye BCECF (30). pH was monitored ratiometrically with a model LS50B luminescence spectrometer (Perkin-Elmer, Norwalk, CT) at excitation wavelengths of 440 and 495 nm and an emission wavelength of 525 nm. Excitation and emission slit widths were 5 and 3 nm, respectively. We took background readings before loading the dye and subtracted them from the fluorescence intensities (F_x, where λ is the excitation wavelength) before calculating the fluorescence ratio (R = F_440/F_495 measured at 525 nm). Because of a progressive detachment of cells from the cover glass (especially during solution changes), fluorescence intensities frequently declined over the course of the experiment. Data for which F_440 was less than twice background were discarded.

At the end of some experiments we performed a calibration (Fig. 1A) using the nigericin technique (30, 39). Specifically, the tissue was transferred to solutions containing 80–100 mM KCl, 3–10 mM NaCl, 4.5 KCl, 2 CaCl_, 1 MgCl_, 5 HEPES; pH 7.4) or 160 NaCl, 4.5 KCl, 2 CaCl_, 1 MgCl_, 5 HEPES; pH 7.4) or to Ringer solution at time (t) = 0, pH440 falls rapidly (acid load as NH4 leaves the cells (C). This is followed by a slower recovery, presumably due to proton (or proton equivalent) efflux (D). The bold curve for t > 0 is a fit of Eq. 3. For t < 0 the data were fitted with an exponential decay. This component was not used for analysis, except to establish pH(0) (Eq. 3). pHmin, limiting value to which the pH apparently recovers as t → ∞, tmin, the time to reach the pH minimum at the end of phase C; pHmax, pH at that minimum; τ, time constant of pH recovery.

Fig. 2. An example of an acid-load/recovery cycle. The tissue was initially in Ringer. Addition of 30 mM NHCl results in a transient increase in pH(A) associated with a rapid influx of NH3, followed by a slower fall in pH as NH3 enters the cells (B). On transferring the tissue to NHCl-free potassium Ringer (K-Ringer) solution at time (t) = 0, pH440 falls rapidly (acid load) as NH4 leaves the cells (C). This is followed by a slower recovery, presumably due to proton (or proton equivalent) efflux (D). The bold curve for t > 0 is a fit of Eq. 3. For t < 0 the data were fitted with an exponential decay. This component was not used for analysis, except to establish pH(0) (Eq. 3).

Experimental protocol. After dye loading, cells were initially placed in 1 ml of Ringer solution and were then acid-loaded by the NH4Cl prepulse technique (35). Specifically, 250 μl of 150 mM NH4Cl solution in water was added to the 1 ml of Ringer to give a final NH4Cl concentration close to 30 mM. As shown in Fig. 2, this resulted in an abrupt rise in pH, followed by a slower decline (phases A and B). The sharp rise in pH is believed to reflect the rapid influx

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Fig. 1. A: calibration of the absolute pH at the end of one experiment. Intracellular pH (pH) values of 5.5–8.0 (indicated by the numbers) were obtained by incubating the tissue in “high” (80–100 mM) KCl solutions containing 10 μM nigericin, 2 mM CaCl_, and 100 mM appropriate buffers (pH 5.5 and 6.0, MES; 6.5, bis-Tris; 7.0, BES; 7.55, HEPES; 8.0, Tricine). Any glassware or other apparatus that came into contact with nigericin was soaked overnight in ethanol (3). Failure to observe this precaution resulted in rapid recovery from an acid load [time constant (τ) ~ 4 min]. In later experiments the use of disposable cuvettes obviated the need to wash cuvettes, but tissue holders were still soaked overnight in ethanol, even when nigericin was not used (as a precaution against contamination). Calibration was not possible in all experiments because of cell detachment and the consequent fall in F_x. Accordingly, a mean calibration curve was used to calculate pH from the fluorescence-ratio data. We obtained this curve by fitting the following equation to data pooled from six experiments (Fig. 1B)

\[
R = \frac{R_{max} + R_{min}\log(pK^+ - pH)}{1 + 10^{pK^+ - pH}}
\]

where R_{max} is the value of R as [H^+] → 0, R_{min} is the value of R as [H^+] → ∞ and pK^+ is the negative log of acidic dissociation constant (pK_A) of BCECF by

\[
pK_A = pK^+ + \log_{10}[F_{440}/F_{640}]
\]

where F_440 and F_640 are the values of F_440 as [H^+] → ∞ and [H^+] → 0, respectively. Because 440 nm is close to the isosbestic point of BCECF, the log term in Eq. 2 should be close to zero so that pK^+_A = pK_A (provided external [K^+] is chosen correctly, see Refs. 4, 5). Equations 1 and 2 follow from the treatment of fluorescent calcium probes by Grynkiewicz et al. (21). The estimated parameter values were pK^+_A = 7.337 ± 0.071, R_{min} = 1.329 ± 0.066, and R_{max} = 8.83 ± 0.52.

Experimental protocol. After dye loading, cells were initially placed in 1 ml of Ringer solution and were then acid-loaded by the NH4Cl prepulse technique (35). Specifically, 250 μl of 150 mM NH4Cl solution in water was added to the 1 ml of Ringer to give a final NH4Cl concentration close to 30 mM. As shown in Fig. 2, this resulted in an abrupt rise in pH, followed by a slower decline (phases A and B). The sharp rise in pH is believed to reflect the rapid influx
and protonation of NH₃, whereas the slower decay is thought to be associated with the entry of NH₄⁺, which then releases protons to the cytosol. When the pH had fallen to ~7, the cells were transferred to an NH₄Cl-free “recovery” solution. The ensuing efflux of NH₃ then resulted in a rapid fall in pHi (acid load, phase D) followed by a slower recovery (phase D), presumably due to proton equivalent efflux. The recovery solution was either Ringer plus 100 μM DMA (an inhibitor of the Na⁺/H⁺ exchanger, Refs. 26, 28) or potassium Ringer (K-Ringer), in which NaCl is replaced by KCl (which should also prevent Na⁺/H⁺ antiport). Both solutions contained 100 nM bafilomycin A₁ to inhibit any H⁺-/ATPase activity (28), 100 μM SCH-28080 to inhibit any H⁺/K⁺-ATPase activity (24, 37), and 1 mg/ml glucose. The K-Ringer also contained 1.5 μM of the K⁺ ionophore valinomycin as a precaution to ensure adequate charge compensation of electrogenic H⁺ efflux. pHₒ was 7.4 in Ringer and either 7.4 or 8.0 in K-Ringer; HEPES buffer was used in all solutions.

To test for Zn²⁺ sensitivity (an indication of the involvement of voltage-gated H⁺ channels) the recovery solution also contained either 10 μM ZnCl₂ or 1 mM of the divalent cation chelator EGTA (plus an extra 1 mM CaCl₂ to maintain normal free Ca²⁺). A ZnCl₂ concentration of 10 μM should effectively abolish any voltage-gated H⁺ flux, even in cells depolarized to 0 mV (8).

During experiments, a cover glass with attached cell monolayer was held in a cover glass holder inside a spectrometer cuvette containing ~1 ml of solution, with constant stirring. We changed solutions by transferring the holder to one or two successive beakers containing 16–18 ml of the next solution in the series and then to a cuvette containing 1 ml of that solution. When not in use, rinsing solutions were stored in an incubator at 37°C; cuvette solutions were maintained at 37°C in the spectrometer with a circulating water bath. The order of acid-load/recovery cycles with or without Zn²⁺ was varied between experiments (e.g., Fig. 3). In ~50% of experiments two acid-load/recovery cycles were achieved, and in ~10% three cycles were obtained. In the remaining experiments only a single acid-load/recovery cycle was possible because of cell loss.

**Data analysis.** To quantify the changes in pHi following the removal of NH₄Cl (i.e., on transferring the cells to the recovery solution), we fitted the data for phases C and D in Fig. 2 with the following equation by nonlinear least squares (bold curve in Fig. 2)

$$pHi(t) = pHi(0) + \Delta pHi(1 - e^{-\alpha t}) + \Delta pHi(1 - e^{-\beta t})$$

(3)

where, having removed the artifact associated with the solution change, we took time \( t = 0 \) as midway between the end of phase B and the start of phase C. Referring to Fig. 2, data are reported in terms of \( t_{min} \) (the time to reach the pH minimum at the end of phase C), \( pHi_{min} \) (the pH at that minimum), \( \tau = t_2 \) in Eq. 3 (effectively the time constant of the recovery phase, D), and \( pH_{min} \) (the limiting value to which the pH apparently recovers as \( t \to \infty \)). \( pH_{fin} = [pHi(0) + \Delta pH_{1} + \Delta pH_{2}] \) and \( \tau \) (related to \( \tau_2 \)) are obtained directly from the fits of Eq. 3. We determined \( t_{min} \) by setting the derivative of Eq. 3 to zero and solving for \( t_{min} \), which was then calculated by setting \( t = t_{min} \) in Eq. 3.

In some cases the minimum was obscured by the solution-change artifact, and so the term in \( \Delta pHi \) in Eq. 3 was omitted. \( pH_{min} \) and \( t_{min} \) were then estimated as pHi and \( t \) for the first reliable data point following the solution change (i.e., after removing the artifact). Although this is somewhat arbitrary, it was done to avoid biasing the mean value of \( t_{min} \) toward larger values. In other cases there was insufficient curvature in phase D for a fit of the second exponential and so it was replaced with a linear term

$$pHi(t) = pHi(0) + \Delta pHi(1 - e^{-\alpha t}) + bt$$

(4)

where \( b \) is a constant. We again determined \( pH_{min} \) and \( t_{min} \) by setting the derivative of Eq. 4 to zero or from the first point in phase D if a minimum was absent (in which case the term in \( \Delta pHi \) was omitted); estimation of \( \tau \) and \( pH_{min} \) was not possible.

From the point of view of data analysis, the ideal experiment is like those shown in Fig. 3, in which Zn²⁺ and control (EGTA) data are available in the same experiment. If the data from control and Zn²⁺-exposed cells are correlated, the use of such paired data will improve the precision of parameter-ratio estimates and hence increase the power of statistical tests. Paired data were analyzed as described in RESULTS. However, to limit the analysis to paired data would be to discard about one-third of the experiments. Hence, if paired data are not correlated it is better to use all the data (paired and unpaired) and so increase the sample size. For recovery in Ringer at a single pHₒ (7.4) this is easily achieved with one-way analysis of variance (ANOVA). For recovery in Ringer at two different pHₒ (7.4 and 8.0), the following model was fitted to all the data by least squares

$$x = \mu (pHi = 7.4, [Zn⁺²] = 0)$$

(5a)

$$x = \alpha \mu (pHi = 7.4, [Zn⁺²] > 0)$$

(5b)

$$x = \beta \mu (pHi = 8.0, [Zn⁺²] = 0)$$

(5c)

$$x = \alpha \beta \gamma \mu (pHi = 8.0, [Zn⁺²] > 0)$$

(5d)

where \( x \) represents \( t_{min}, pH_{min}, \tau, \) or \( pH_{min}; \mu \) is the value of \( x \) when \( pH_{0} = 7.4 \) and [Zn⁺²] = 0; \( \alpha \) is the factor by which \( x \) is changed by Zn⁺² at \( pH_{0} = 7.4 \); \( \beta \) is the factor by which \( x \) is changed by a change in pHₒ (7.4 → 8.0) when [Zn⁺²] = 0; and \( \gamma \) allows for the possibility that the effect of Zn⁺² is different at different pHₒ and vice versa (if there is no such interaction between the effects of Zn⁺² and pHₒ, then \( x = \alpha \beta \mu \) when \( pH_{0} = 8.0 \) and [Zn⁺²] > 0). Various reduced models were then fitted by setting \( \alpha, \beta, \) or \( \gamma \) to unity. A minimum-parameter model was chosen by using \( F \) tests at the 5% significance level as described by Walpole and Myers (40). Specifically, for two models
with $k$ and $k - 1$ parameters (e.g., for $k = 4$, models with parameters $\mu, \alpha, \beta, \gamma$ and $\mu, \alpha, \beta$) an $F$ value with $(1, n - k)$ degrees of freedom (where $n$ is the number of $x$ values) was calculated as

$$F = \frac{RSS_{n-1} - RSS_k}{RSS_k/(n - k)}$$

where $RSS$ is the residual sum of squares. If the $F$ value was significant the $k$-parameter model was retained, otherwise it was rejected in favor of the reduced model with $k - 1$ parameters. When nonsignificant $F$ values were obtained with more than one reduced model, the one with the smallest $RSS_{n-1}$ was chosen for the next round of $F$ tests (i.e., against models with $k - 2$ parameters).

As judged by $t$-tests at the 5% level and visual inspection of the data, there was no evidence that the order of the applied treatments (i.e., $Zn^{2+} \rightarrow$ EGTA or EGTA $\rightarrow Zn^{2+}$) affected $x$, and so order does not appear as a factor in Eq. 5. Rather, for all types of analysis (paired, ANOVA, and Eq. 5, $a-d$), control data obtained before and after the $Zn^{2+}$ treatment were pooled, as were the $Zn^{2+}$ data obtained before and after the EGTA treatments. (In this case the ANOVA is equivalent to a two-sample $t$-test; Ref. 40) For $t_{min}$ and $\tau$, a log transformation was applied to both sides of Eq. 5, $a-d$, to stabilize the variance and reduce positive skew; this was not necessary for $pH_{min}$ and $pH_{fin}$ (presumably because these variables are already log-transformed). Mean values are given with standard errors and, where appropriate, the number of observations in parenthesis. A significance level of 0.05 was used for all statistical tests.

Boyarsky et al. (4) concluded that in a variety of cells at around $pH_{i}$ 7, the high $K^+/nigerinicin$ calibration technique led to estimates of $pHi$ ($pHi_{true}$) that were 0.08–0.26 $pH$ units above the true $pHi$ ($pHi_{true}$). At least part of the error probably arose because $[K^+]_i$ in the calibration solutions was too low; this was likely true in the present study also. In a subsequent paper on smooth muscle cells, Boyarsky et al. (5) found that the error varied from $0$ to $0.3$ $pH$ units over the $pHi_{nig}$ range 6–8. Furthermore, the error increased approximately linearly with $pHi_{nig}$ such that

$$pHi_{nig} - pHi_{true} = A + BpHi_{nig}$$

where $A$ and $B$ are constants. As pointed out by Boyarsky et al. (5), such a linear relationship will mean that a linear relationship will mean that $pHi$ values in the $pHi_{min}$, $pHi_{fin}$, and $pHi_{fin}$, as illustrated in Fig. 2. Often it was possible to estimate these parameters in the presence and absence of $Zn^{2+}$ within a single experiment (Fig. 3), although sometimes cell detachment meant that only a single acid-load/recovery cycle was obtained (i.e., either $Zn^{2+}$ or EGTA, but not both in the same experiment).

Mean values of $t_{min}$, $\tau$, $pH_{min}$, and $pH_{fin}$ before, during, and after the $Zn^{2+}$ treatment are summarized in Fig. 5. (The “Before” and “After” solutions contained 1 mM EGTA, and so $Zn^{2+}$ should have been essentially absent.) In considering these mean values, we find the overall impression to be that $Zn^{2+}$ slows recovery (i.e., increases $t_{min}$ and $\tau$; Fig. 5, A and B) but does not reduce the final level of recovery ($pHi_{fin}$; Fig. 5D). Also the acid load itself is more extreme (i.e., $pHi_{min}$ is lower;
Fig. 5. Mean values of $t_{\text{min}}$, $\tau$, $pH_{\text{min}}$, and $pH_{\text{fin}}$ for pH recovery in K-Ringer in the presence and absence of Zn$^{2+}$ (see Fig. 2 for the meaning of these parameters). Statistical analyses (Table 1 and Fig. 6) showed that Zn$^{2+}$ significantly increased $t_{\text{min}}$ and $\tau$ (A and B) and significantly reduced $pH_{\text{min}}$ (C). Zn$^{2+}$ had no significant effect on $pH_{\text{min}}$ (D). Zn$^{2+}$ solutions contained 10 μM ZnCl$_2$, whereas Zn$^{2+}$-free solutions (“Before” and “After”) contained 1 mM EGTA. The numbers above each bar show the number of observations; error bars are standard errors; *significant differences at a given pH$_o$ in the observations; error bars are standard errors.

Table 1. Results of the statistical analysis to determine the effects of Zn$^{2+}$ and pH$_o$ on $t_{\text{min}}$, $\tau$, $pH_{\text{min}}$ and $pH_{\text{fin}}$ for tissue in K-Ringer

<table>
<thead>
<tr>
<th>$t_{\text{min}}$, min</th>
<th>$\mu$</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$0.462 \pm 0.052$</td>
<td>2.51</td>
<td>0.0046</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$7.9 \pm 1.1$</td>
<td>1.87</td>
<td>0.43</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$6.524 \pm 0.030$</td>
<td>0.9793</td>
<td>0.0064</td>
<td>1.0368</td>
<td>0.0069</td>
</tr>
<tr>
<td>$7.045 \pm 0.040$</td>
<td>1</td>
<td>1.0582</td>
<td>0.0095</td>
<td>1</td>
</tr>
</tbody>
</table>

Data in Fig. 5 were fitted with Eq. 5, a–d, as well as various reduced models obtained by setting $\alpha$, $\beta$, or $\gamma$ to unity. “Before” and “After” data were pooled. The best-fit, minimum-parameter models were chosen by F-tests at the 5% level. The table gives the parameter estimates for these models with SE. $\mu$ is the value of $x$ when extracellular pH (pH$_o$) = 7.4 and [Zn$^{2+}$] = 0, $\alpha$ is the factor by which $x$ is changed by Zn$^{2+}$ at pH$_o$ = 7.4, $\beta$ is the factor by which $x$ is changed by a change in pH$_o$ (7.4 → 8.0) when [Zn$^{2+}$] = 0, and $\gamma$ allows for the possibility that the effect of Zn$^{2+}$ is different at different pH$_o$ (and vice versa). A value of unity for $\alpha$ or $\beta$ implies no effect of Zn$^{2+}$ or pH$_o$, respectively. Since $\gamma$ was always unity, there was no interaction between the effects of Zn$^{2+}$ and pH$_o$, i.e., the value of $x$ when pH$_o$ = 8.0 and [Zn$^{2+}$] > 0 is simply $\mu(\beta)$. $pH_{\text{min}}$, limiting value to which the pH apparently recovers as $t \to \infty$; $t_{\text{min}}$, the time to reach the pH minimum at the end of phase C; pH$_{\text{fin}}$, pH at that minimum; $\tau$, time constant of pH recovery.

Fig. 6. The pH$_o$ recovery time constants in the presence and absence of Zn$^{2+}$ ($\tau_{Zn}$ and $\tau_{\text{EGTA}}$, respectively) were significantly correlated (Fig. 6), which means that the errors on estimates of $\tau_{Zn}/\tau_{\text{EGTA}}$ can be reduced relative to those obtained from unpaired data. Estimates of $\tau_{Zn}/\tau_{\text{EGTA}}$ obtained from paired data were significantly greater than unity at both pH$_o$ 7.4 (2.34 ± 0.36, $n = 4$) and pH$_o$ 8.0 (2.52 ± 0.40, $n = 5$).

The statistical analysis also confirmed that pH$_{\text{fin}}$ is reduced in the presence of Zn$^{2+}$, at least at pH$_o$ 7.4, and there was a clear effect of pH$_o$, such that pH$_{\text{fin}}$ was higher at pH$_o$ 8.0 than at pH$_o$ 7.4 (Fig. 5C). There was no statistically significant effect of Zn$^{2+}$ on pH$_{\text{fin}}$, i.e., pH$_o$ recovered to about the same level in the presence or absence of Zn$^{2+}$ (albeit over a longer time course in the presence of Zn$^{2+}$). As one might expect, pH$_{\text{fin}}$ was significantly higher at pH$_o$ 8.0 than at pH$_o$ 7.4 (Fig. 5D).

The outcome of statistical analysis of the effects of Zn$^{2+}$ and pH$_o$ on acid loading and pH$_o$ recovery is illustrated by the
graphs in Fig. 7, A and B, which shows plots of pH in vs. t predicted by the best-fit models in Table 1 (see Fig. 7 legend for details). The curves in Fig. 7A illustrate that Zn2+ caused a similar relative slowing of recovery (increase in τ) at both pHo 7.4 and pHo 8.0 but had no significant effect on the final level of pH recovery (pHfin). In Fig. 7B the early portions of the curves in Fig. 7A are shown on an expanded time scale. The increased acid load (lower pHmin) and delayed recovery (larger tmin) in the presence of Zn2+ are evident.

Maximum H+ fluxes in K-Ringer. The curves in Fig. 7A allow us to calculate a quantity qJH (Fig. 8) that is proportional to the Zn2+-sensitive H+ flux (JH), which we attribute to H+ transport through voltage-gated proton channels (q is the apical surface area to volume ratio of the monolayer (≈1/monolayer thickness). qJH was calculated as

\[ q_{JH} = B_1 \left( \frac{dpH}{dt} \right)_{Zn} - B_1 \left( \frac{dpH}{dt} \right)_{EGTA} \]

(8)

where \( (dpH/dt)_{Zn} \) and \( (dpH/dt)_{EGTA} \) are the rates of change of pH in the presence and absence of Zn2+ respectively, and B1 is the (pH-dependent) intracellular buffer capacity. In this formulation, proton efflux is a negative quantity. To employ Eq. 8, \( (dpH/dt)_{Zn} \) and \( (dpH/dt)_{EGTA} \) were determined at the same pHi, and calculations were restricted to times \( t > 3\tau_1 \) (see Eq. 3) so as to be clear of the pHi minimum (so that changes in pHi reflect JH rather than NH3 and NH4+ fluxes). Values of B1 were taken from Fig. 1 in Lubman and Crandall (26). Although these values pertain to 22°C rather than 37°C, we are really only seeking an order of magnitude calculation here. In Fig. 8 qJH is plotted vs. pH (solid curves). It can be seen that at both pHi, the maximum qJH approaches 2 mM/min. If one assumes a monolayer thickness of 1–5 μm (i.e., q = 0.2–1/μm), this implies a maximum equivalent proton-current density across the apical membrane of 3–16 fA/μm2. This calculation assumes that 1) the JH observed in the presence of Zn2+ persists unaltered and simply adds to JH when Zn2+ is removed and 2) the rate of production of H+ by metabolism is the same in the presence and absence of Zn2+.

Boyrarsky et al. (4, 5) concluded that the high K+-nigericin calibration technique leads to overestimates of pHi. As discussed in the MATERIALS AND METHODS, this error does not affect the conclusions regarding tmin, τ, pHmin, and pHfin but would affect the estimates of qJH. From Boyarsky et al. (5) the true value of qJH is given by

\[ q_{JH, true} = q_{JH, nigericin} \times 10^\text{pHerror} \]

(9)

where \( q_{JH, nigericin} \) is the value of qJH calculated under the assumption that the high-K+-nigericin calibration technique is unbiased, and pHerror is the amount by which pH is overestimated. Assuming pHerror = 0.0–0.3 (5), the calculated maximum

Fig. 7. Idealized pH recovery time courses reconstructed statistically from all of the experimental data. A and B: plots of pHi vs. t predicted by the best-fit models in Table 1. To produce these plots, we generated predicted mean values of tmin, τ, pHmin, and pHfin by substituting the parameter values from Table 1 into Eq. 5, a–d. Using these values, expressions for tmin, τ, pHmin, and pHfin derived from Eq. 3 were then solved simultaneously for τ1, ΔpHi, and ΔpH0, and τ2 was set equal to τ. Finally, pH(0) was set to its mean value, and the curves in A and B were generated with Eq. 3. The curves in A illustrate how Zn2+ caused a similar relative slowing of recovery (increase in τ) in K-Ringer at both pHi, (7.4 and 8.0) but had no significant effect on the final level of pH recovery (pHfin). In B the early portions of the curves in A are shown on an expanded time scale. The increased acid load (lower pHmin) and delayed recovery (larger tmin) in the presence of Zn2+ are evident. C and D: similar plots for recovery in Ringer (pH 7.4); for these plots pH(0) was set to its mean value and the values of tmin, τ, pHmin, and pHfin were taken from Fig. 10 (“Before” and “After” data were pooled).

Fig. 8. The quantity qJH was calculated from the curves in Fig. 7, A and C, according to Eq. 8. JH is the Zn2+-sensitive transapical membrane H+ efflux (considered negative), and q is the apical surface area to volume ratio (≈1/monolayer thickness). Calculations were restricted to times \( t > 3\tau_1 \) to ensure that dph/dt reflects mainly the proton efflux. KR, solid curves; R, dashed curve.
The current density should be increased by a factor of 1–2. So instead of 3–16 fA/μm² as calculated above, a reasonable range might be 3–30 fA/μm². Assuming a membrane capacitance of 1 μF/cm², we can translate this to a normalized H⁺ current (I₉₉) = 0.3–5 pA/pF. As a frame of reference, I₉₉ is typically 10–20 pA/pF during large depolarizations in rat alveolar epithelial cells over a wide pH range (9). The I₉₉ required to produce the pH recovery in this study thus requires activating only a small fraction of the maximum available H⁺ conductance.

**Table 2.** H⁺/K⁺-ATPase inhibitor SCH-28080 had no detectable effect on τ or pHᵢᵣₑᵢⱼ in the absence of Zn²⁺, or on pH₉ᵢᵣₑᵢⱼ

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SCH-28080 (μM)</th>
<th>100 μM</th>
<th>0 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>τ, min</td>
<td>0</td>
<td>6.64±0.67 (7)</td>
<td>7.83±0.26 (3)</td>
</tr>
<tr>
<td>pHᵢᵣₑᵢⱼ</td>
<td>0</td>
<td>7.00±0.062 (8)</td>
<td>6.91±0.10 (3)</td>
</tr>
<tr>
<td>pH₉ᵢᵣₑᵢⱼ</td>
<td>0</td>
<td>6.590±0.035 (10)</td>
<td>6.505±0.072 (5)</td>
</tr>
<tr>
<td>pHᵢᵣₑᵢⱼ</td>
<td>10</td>
<td>6.355±0.077 (7)</td>
<td>6.44±0.10 (5)</td>
</tr>
</tbody>
</table>

The data were insufficient and/or too variable for a meaningful analysis of other parameters (τ in the presence of Zn²⁺, τ₉ᵢᵣₑᵢⱼ, etc.). Mean values are given with SE and the number of observations in parenthesis.

The results of Kemp et al. (24), who found evidence for an anion-transport inhibitor DBDS, 4,4'-disulfonic acid (DBDS, Ref. 38). Adding 0.2 mM DBDS to the recovery solution of K-Ringer (pH 7.4) using 100 mM bafilomycin A₁, and 100 μM SCH-28080, 10 μM ZnCl₂, and 1.5 μM valinomycin. Despite the presence of 100 mM bafilomycin A₁, 10 μM ZnCl₂, and no external Na⁺ (hence no Na⁺/H⁺ exchange), pHᵢᵣₑᵢⱼ recovery still occurred following an acid load. As pointed out by Richard D. Vaughan-Jones (personal communication), the presumed membrane depolarization in K-Ringer would lead to an accumulation of intracellular Cl⁻, which might then result in pHᵢᵣₑᵢⱼ recovery via Cl⁻/OH⁻ exchange. To test whether this mechanism might contribute under the present conditions, we conducted a separate set of experiments with the anion-transporter inhibitor 4,4'-dibenzamidostilbene-2,2'-disulfonic acid (DBDS, Ref. 38). Adding 0.2 mM DBDS to the recovery solution containing all other inhibitors (K-Ringer containing 100 mM bafilomycin A₁, 100 μM SCH-28080, 10 μM ZnCl₂, and 1.5 μM valinomycin) had no significant effect on pHᵢᵣₑᵢⱼ recovery (Table 3). Given the variability of the data, we cannot exclude a small effect, but it is clear that cells still recovered from an acid load in the presence of DBDS. Hence the residual pHᵢᵣₑᵢⱼ recovery is apparently not primarily associated with a DBDS-sensitive anion transporter.

**Table 3.** Anion-transport inhibitor DBDS had no detectable effect on pHᵢᵣₑᵢⱼ recovery following an NH₄Cl prepulse

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DBDS (μM)</th>
<th>0.2 mM</th>
<th>0 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>τ, min</td>
<td></td>
<td>1.09±0.54 (5)</td>
<td>1.08±0.15 (6)</td>
</tr>
<tr>
<td>pHᵢᵣₑᵢⱼ</td>
<td></td>
<td>10.8±3.4 (5)</td>
<td>14.0±4.0 (6)</td>
</tr>
<tr>
<td>pH₉ᵢᵣₑᵢⱼ</td>
<td></td>
<td>6.40±0.11 (4)</td>
<td>6.657±0.029 (6)</td>
</tr>
<tr>
<td>pHᵢᵣₑᵢⱼ</td>
<td></td>
<td>7.60±0.13 (4)</td>
<td>7.490±0.069 (6)</td>
</tr>
</tbody>
</table>

pHᵢᵣₑᵢⱼ recovery was monitored in K-Ringer (pH 8) containing 100 mM bafilomycin A₁, 100 μM SCH-28080, 10 μM ZnCl₂, and 1.5 μM valinomycin. Mean values are given with SE and the number of observations in parenthesis. DBDS, 4,4'-dibenzamidostilbene-2,2'-disulfonic acid; pHᵢᵣₑᵢⱼ, intracellular pH; K-Ringer, potassium Ringer solution.

28. The Ringer solution also contained 100 mM bafilomycin A₁ and 100 μM SCH-28080 (but no valinomycin). It was also of interest to examine the effects of 10 μM ZnCl₂ in this more physiological medium. If the resting membrane potential were more negative in Ringer, this would be expected to decrease Zn²⁺-sensitive J₉₉ via voltage-gated H⁺ channels (9). As usual, Zn²⁺-free Ringer contained 1 mM EGTA plus an additional 1 mM CaCl₂. Fig. 9 shows example pHᵢᵣₑᵢⱼ records with ZnCl₂ applied before (Fig. 9A) or after (Fig. 9B) the EGTA control. Surprisingly, 10 μM Zn²⁺ slowed recovery in Ringer solution. As in K-Ringer, recovery still occurred in Ringer despite addition of the entire gamut of inhibitors.

![Fig. 9](http://ajplung.physiology.org/)
Figure 10 summarizes the mean values of $t_{\text{min}}$, $\tau$, $\text{pH}_{\text{min}}$, and $\text{pH}_{\text{fin}}$ for experiments in Ringer solution. As with the K-Ringer data, these mean values were used to generate the $\text{pH}(t)$ plots in Fig. 7, C and D, and the $q_{\text{H}}$ plot (dashed curve) in Fig. 8 (see legends to Figs. 7 and 8 for details). The overall impression is that recovery in Ringer is generally similar to recovery in K-Ringer at pHo 7.4, both in kinetics and effects of Zn$^{2+}$ (compare Figs. 5 and 10; Fig. 7, A, B and C, D, and the pHo 7.4 curves in Fig. 8). One difference was that Zn$^{2+}$ had no detectable effect on $\text{pH}_{\text{min}}$ in Ringer. As with K-Ringer, there was no effect of Zn$^{2+}$ on $\text{pH}_{\text{fin}}$ (Fig. 10D). One-way ANOVA, as well as analysis of paired data, shows that Zn$^{2+}$ significantly increased both $t_{\text{min}}$ (Fig. 10A) and $\tau$ (Fig. 10B), although the slowing of $\tau$ was less profound than in K-Ringer. In summary, the effects of Zn$^{2+}$ were less pronounced in normal-Na$^+$ Ringer solution, but both the Zn$^{2+}$-sensitive and Zn$^{2+}$-insensitive components of pH$_i$ recovery following an acid load still occurred.

**DISCUSSION**

$pH_i$ in unchallenged alveolar epithelial cells. The average baseline pH$_i$ was 7.13, within the lower range of values in previous studies of cultured rat alveolar type II epithelial cells in nominally HCO$_3^-$-free solutions, 7.17–7.36 (6, 19, 36); a higher value of 7.50 has also been reported (25, 28).

Given the strong regulation of their gating by pH$_i$, one would predict that proton channels would not be open under resting conditions. Voltage-gated proton channels open only positive to the Nernst potential for H$^+$ ($E_H$) (9, 11), with the result that they always extrude acid. Because $E_H$ is normally positive to the resting membrane potential in alveolar epithelial cells (10), proton channels would be expected to be closed in an unchallenged cell. The data confirm this expectation. Addition of 10–100 $\mu$M ZnCl$_2$ had no effect on the baseline pH$_i$. Although a variety of alternative explanations could be suggested, the simplest interpretation is that proton channels are not open under resting conditions.

**Zn$^{2+}$-sensitive pH$_i$ recovery: voltage-gated proton channels.** Several types of evidence support the conclusion that voltage-gated proton channels contribute to pH$_i$ recovery after acid loading of rat alveolar epithelial cells. In Ringer and K-Ringer, respectively, pH$_i$ recovery was slowed on average 1.50-fold and 2.44-fold by the addition of 10 $\mu$M ZnCl$_2$ (Figs. 5B, 6, and 10B). Although Zn$^{2+}$ has effects on many proteins, including many ion channels, it is a potent inhibitor of voltage-gated proton channels. At pH 7, 10 $\mu$M Zn$^{2+}$ severely inhibits proton currents (8) but has relatively little effect on several other ion channels (11), including voltage-gated K$^+$ channels in type II cells (V. V. Cherny and T. E. DeCoursey, unpublished) and cGMP-activated cation channels in type II cells (23). We thus attribute the slowing of pH$_i$ recovery by ZnCl$_2$ to inhibition of proton channels. These effects of Zn$^{2+}$ were observed under conditions designed to prevent the operation of other pH regulating transporters. Two more subtle effects of Zn$^{2+}$ also suggest the involvement of the proton conductance. The time to reach the pH$_i$ nadir ($t_{\text{min}}$) increased (Figs. 5A and 10A), and, at least in K-Ringer, pH$_{\text{fin}}$ was lower in the presence of Zn$^{2+}$ (Fig. 5C). Both of these effects indicate that the proton conductance was activated rapidly, before $t_{\text{min}}$ was reached. The effects of Zn$^{2+}$ were reversible; $t$-tests on parameter estimates obtained before and after treatment with Zn$^{2+}$ were never significant. Finally, the H$^+$ efflux was substantially larger at a given pH$_i$ when pH$_o$ was 8.0 than 7.4 (Fig. 8), which is consistent with the well-established pH$_o$ dependence of proton channels (9, 11). A qualitatively similar effect of pH$_o$ would also be expected for any transporter that is driven by the pH gradient. In summary, the data strongly support the interpretation that the Zn$^{2+}$-inhibitable component of pH$_i$ recovery is mediated by voltage-gated proton channels.

Zn$^{2+}$ had no significant effect on pH$_{\text{fin}}$ (Figs. 5D and 10D). That is, although Zn$^{2+}$ slowed recovery, it did not alter the final level of recovery. This is not surprising given the effects of transmembrane pH gradients on H$^+$ channel gating and the lack of effect of Zn$^{2+}$ on unchallenged cells (Fig. 4). H$^+$

![Figure 10. Mean values of $t_{\text{min}}$, $\tau$, $\text{pH}_{\text{min}}$, and $\text{pH}_{\text{fin}}$ in the presence and absence of Zn$^{2+}$ for tissue bathed in Ringer plus 0.1 mM DMA, 0.1 $\mu$M bafilomycin A$_1$, and 0.1 mM SCH-28080. One-way ANOVA showed that Zn$^{2+}$ significantly increased $t_{\text{min}}$ and $\tau$ (* in A and B) but had no significant effect on pH$_{\text{fin}}$ and pH$_{\text{min}}$ (C and D). However, large standard errors on at least 2 of the pH$_{\text{min}}$ estimates may have precluded detection of a Zn$^{2+}$ effect (C). Zn$^{2+}$ solutions contained 10 $\mu$M ZnCl$_2$ while Zn$^{2+}$-free solutions (Before and After) contained 1 mM EGTA. The numbers above each bar show the number of observations; error bars are standard errors.](http://ajplung.physiology.org/10.22033.32.2017)
channels open only at membrane potentials positive to \( E_{H} \), and the threshold voltage (\( V_{\text{threshold}} \)) for opening is described by the following empirical relation \( (9) \)

\[
V_{\text{threshold}}(\text{mV}) = 20 - 40 \Delta \text{pH},
\]

where \( \Delta \text{pH} = \text{pH}_{e} - \text{pH}_{i} \). For the K-Ringer experiments in the absence of \( \text{Zn}^{2+} \), \( \text{pH}_{\text{fin}} \) was 7.040 ± 0.040 (\( n = 21 \)) and 7.456 ± 0.051 (\( n = 14 \)) at \( \text{pH}_{i} \) 7.4 and 8.0, respectively. So on setting \( \text{pH}_{i} = \text{pH}_{\text{fin}} \), Eq. 10 gives \( V_{\text{threshold}} \) values of 5.6 mV and −1.8 mV at \( \text{pH}_{i} \) 7.4 and 8.0, respectively. Thus at \( \text{pH}_{\text{fin}} \) and if it is assumed the cells were depolarized to near zero, any contribution of voltage-gated proton channels to \( H^{+} \) efflux will be small, even in the absence of \( \text{Zn}^{2+} \). In other words, as \( H^{+} \) efflux increases \( \text{pH}_{i} \), the resulting dissipation of the \( \text{pH} \) gradient shuts off the proton conductance. Accordingly, \( \text{pH}_{\text{fin}} \) will be determined mainly by any \( \text{Zn}^{2+} \)-insensitive \( H^{+} \) transport and the rate of production of \( H^{+} \) by metabolism. For the same reasons, proton channels do not contribute to \( \text{pH}_{i} \) in resting, unchallenged cells (Fig. 4); \( \text{Zn}^{2+} \) did not change baseline \( \text{pH}_{i} \) in cells in Ringer solution. Proton channels are expected to come into play when cells are challenged by periods of intense metabolic activity, membrane depolarization, acid loading, or other stressful conditions. Thus we cannot blithely extend the conclusion regarding proton channel activity to the in vivo situation, because the environment of cultured alveolar epithelial cells in these experiments differs radically from that in vivo, in which there is continuous \( \text{CO}_2 \) flux as well as a large \( \text{pH} \) gradient across the epithelium.

The \( \text{Zn}^{2+} \)-sensitive component of \( \text{pH} \) recovery (Fig. 8) represents proton currents of ~0.3–3 pA/pF or less. Is this flux consistent with known properties and magnitude of the voltage-gated proton conductance? From whole cell patch-clamp studies, the expected normalized \( I_{H} \) is given to a first approximation by

\[
I_{H} = \frac{G_{H_{\text{max}}}(V + 2.303RT\Delta \text{pH}/F)}{1 + \exp[(a + b\Delta \text{pH} - V)/V_{i}]} \tag{11}
\]

where \( V \) is the membrane potential, \( R \) is the universal gas constant, \( T \) is the absolute temperature, \( F \) is Faraday’s constant, \( a \approx 40 \text{ mV}, b \approx -40 \text{ mV}/\text{pH}, \) the slope factor \( V_{i} = 10 \text{ mV}, \) and the normalized maximum \( H^{+} \) conductance (\( G_{H_{\text{max}}} \)) is ~100 pS/pF at 20°C (9). \( G_{H_{\text{max}}} \) at 37°C is at least 3.5 times larger (14). Assuming \( \Delta \text{pH} \approx 1 \) and \( V = 0 \) in K-Ringer, Eq. 11 predicts a proton current, \( I_{H} \approx RTG_{H_{\text{max}}}/F \approx 10 \text{ pA/pF}, \) under “typical” conditions at the peak of the experimental acid load. The \( \text{Zn}^{2+} \)-sensitive \( I_{H} \) calculated above from the observed rate of \( \text{pH}_{i} \) change (Fig. 8) is smaller than this value. However, it is necessary to consider the dynamic nature of events during the acid load and recovery processes. Before the acid load, the \( K^{+} \) conductance may successfully clamp the membrane potential near 0 mV. However, when \( \text{pH}_{i} \) drops to \( \text{pH}_{\text{fin}} \), \( E_{H} \) will shift to −53 mV (for \( \text{pH}_{i} \) 7.4) or −77 mV (for \( \text{pH}_{i} \) 8.0), and proton channels will open. In a nonvoltage-clamped cell in vivo, any \( I_{H} \) will tend to drive the membrane potential toward \( E_{H} \), and thus the proton current is in a sense self-limiting. If no other electrogenic processes intervene, the resting potential during recovery will fall within the Nerst potentials for \( K^{+} \) and \( H^{+} \). Because these two conductances are of similar magnitude (10), they will compete for the privilege of controlling the resting potential. As \( \text{pH}_{i} \) recovers, \( \Delta \text{pH} \) will dissipate, decreasing the open probability of \( H^{+} \) channels and removing the driving force for \( H^{+} \) current.

The effects of 10 \( \mu \text{M} \) \( \text{Zn}^{2+} \) strongly implicate voltage-gated proton channels in \( \text{pH}_{i} \) recovery from an acid load in rat type II alveolar epithelial cells in K-Ringer. Surprisingly, \( \text{Zn}^{2+} \)-inhibited \( \text{pH}_{i} \) recovery in Ringer solution, although to a lesser extent than it did in K-Ringer. One would expect the cells to be depolarized in K-Ringer, but hyperpolarized in Ringer. Because hyperpolarization decreases the open probability of voltage-gated \( H^{+} \) channels, one would expect a smaller \( I_{H} \) in Ringer than in K-Ringer. Two early estimates of the resting membrane potential of rat and rabbit alveolar type II epithelial cells are −27 mV (7) and −63 mV (18), respectively, based on \( K^{+} \) or \( Rb^{+} \) distribution. However, the assumption that the membrane potential is equivalent to the \( K^{+} \) gradient neglects the fact that the voltage-gated \( K^{+} \) channels identified in rat alveolar epithelial type II cells are predominantly Kv1.3 (20), which open only with depolarization above roughly −40 mV (15, 34). If Kv1.3 channels set the resting membrane potential, then it is likely to be in the vicinity of −30 to −40 mV. Then, if one assumes \( \Delta \text{pH} \approx 1 \), Eq. 11 gives \( I_{H} = 0.04 – 0.15 \text{ pA/pF}, \) much less than calculated above for \( V = 0 \). Yet Fig. 8 indicates that the \( \text{Zn}^{2+} \)-sensitive \( I_{H} \) (\( qI_{H} \sim I_{H} \)) was similar in Ringer and K-Ringer at \( \text{pH}_{i} \) 7.4. These calculations are based on whole cell patch-clamp studies; conceivably the gating of \( H^{+} \) channels in intact cells might be different. This raises the intriguing possibility that voltage-gated proton channels may be active in these cells under a wider range of conditions than previously supposed. A more mundane explanation is that the membrane potential may have become depolarized during the acid-loading procedure in Ringer solution. Because decreasing \( \text{pH}_{i} \) inhibits many ion channels including \( K^{+} \) channels (16, 29), some depolarization in response to decreased \( \text{pH}_{i} \) would not be surprising. Finally, part of the explanation must be the hyperpolarization produced by proton current at low \( \text{pH}_{i} \), as discussed above. These questions should be addressed in future studies using membrane potential-sensitive probes and kinetic modeling.

\( \text{Zn}^{2+} \)-insensitive \( \text{pH} \) recovery. To isolate the contribution of proton channels to \( \text{pH}_{i} \) regulation in alveolar epithelial cells, we created conditions to eliminate other transporters that might influence \( \text{pH}_{i} \), especially those that might contribute to cytoplasmic alkalization. The absence of \( \text{Na}^{+} \) in K-Ringer prevents \( \text{Na}^{+}/H^{+} \)-antiport, at least in its normal mode of operation. Bafilomycin \( A_{2} \) was used to inhibit the plasma membrane \( H^{+} \)-ATPase (28). We included SCH-28080 (37) to inhibit any possible \( H^{+}-K^{+} \)-ATPase activity, although this drug had no detectable effect under our conditions. This observation is consistent with the report that \( H^{+}-K^{+} \)-ATPase activity can be detected in guinea pig, but not rat, alveolar epithelium (24). We used nominally \( \text{HCO}_3^{-} \) and \( \text{CO}_2 \)-free conditions to avoid \( \text{HCO}_3^{-} \) transport. The \( \text{Cl}^{-}/\text{HCO}_3^{-} \) exchanger (33) and \( \text{Cl}^{-}/\text{OH}^{-} \) exchange (38) both normally acidify the cytoplasm and thus would not normally contribute to produce recovery from an acid load. However, if intracellular \( \text{Cl}^{-} \) were elevated, for example due to depolarization of the membrane potential by the high \( K^{+} \) in K-Ringer, then reverse operation could conceivably produce alkalization using environmental \( \text{HCO}_3^{-} \) or \( \text{OH}^{-} \) as a substrate. To circumvent this possibility, we also added DBDS, which inhibits anion exchangers in general and \( \text{Cl}^{-}/\text{OH}^{-} \) exchange specifically (38). Finally, to
minimize any K+/H+ exchange (2), we monitored pH recovery in Ringer plus DMA (to inhibit Na+/H+ antiport). Yet, even when all known transporters were inhibited or prevented from working, pH recovery was not prevented. Recovery was slow, with an average time constant of ~20 min in K-Ringer, but complete recovery still occurred.

The mechanism for this residual slow alkalization is not known. Incomplete inhibition of channel-mediated H+ efflux by Zn2+ is unlikely. Zn2+ inhibits H+ currents by shifting \( V_{\text{threshold}} \) to more positive voltages. The shift of \( V_{\text{threshold}} \) by 10 \( \mu \)M Zn2+ would be 59 mV at pH 7.4 and 66 mV at pH 8.0 (8). Given the values of \( \phi_{\text{max}} \) (Figs. 5C and 10C), \( \phi = 1.0 \) and 1.3 at pH 7.4 and 8.0, respectively. From Eq. 10, the corresponding values of \( V_{\text{threshold}} \) are -20 and -32 mV. Hence \( V_{\text{threshold}} \) would be shifted by 10 \( \mu \)M Zn2+ to +39 mV at pH 7.4 and +34 mV at pH 8.0. Assuming that the high [K+]o clamped the membrane potential close to 0 mV, \( V_{\text{threshold}} \) for activating the proton conductance would be well positive to the membrane potential, and hence little \( \Delta \psi \) should occur. Significant H+ permeation through the lipid bilayer is also exceedingly unlikely given the small, pH-independent leakage current measured in these cells (13). Because Cl− was present at ~160 mM in both Ringer and K-Ringer, a recovery mechanism involving Cl− cannot be ruled out, although any such mechanism was apparently insensitive to DBDS.

Conclusions. Voltage-gated proton channels in rat alveolar epithelial cells contribute to pH recovery after an acid load in normal and high-[K+]o solutions. Slow recovery still occurred after all known transporters were inhibited, suggesting the existence of a yet-unidentified acid extrusion mechanism. Whether this mysterious transporter is the same as that deduced by Joseph et al. (22) is an open question. Zn2+ does not change resting pHi, indicating that proton channels are closed under resting conditions, presumably because the membrane potential is negative to \( E_f \). The classical property of proton channels opening only with an outward electrochemical gradient for protons ensures that there is no proton influx under normal conditions; the fundamental problem of pH regulation is acid extrusion.

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