EFFECT OF LUMINAL ACIDITY ON THE APICAL CATION CHANNEL IN RABBIT ESOPHAGEAL EPITHELIUM

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ABSTRACT

Esophageal epithelial cells contain an apical cation channel that actively absorbs sodium ions (Na\(^+\)). Since these channels are exposed \textit{in vivo} to acid reflux, we sought the impact of high acidity on Na\(^+\) channel function in Ussing-chambered rabbit epithelium.

RESULTS: Serosal nystatin abolished short-circuit current (Isc) and luminal pH titrated from pH 7.0 to pH \geq 2.0 had no effect on Isc. Circuit analysis at pH 2.0 showed small, but significant, increases in apical and shunt resistances. At pH < 2.0, Isc increased while resistance (R\(_T\)) decreased along with an increase in fluorescein flux. The change in Isc, but not R\(_T\), was reversible at pH 7.4. Reducing pH from 7.0 to 1.1 with H\(_2\)SO\(_4\) gave a similar pattern, but higher Isc values, suggesting shunt permselectivity. A 10:1 Na\(^+\) gradient after nystatin increased Isc by \(\sim 4\ \mu\text{Amps/cm}^2\) and this declined at pH \(\leq 3.5\) until it reached \(\sim 0.0\) at pH 2.0. Impedance analysis on acid-exposed (non-nystatin-treated) tissues showed compensatory changes in apical (increase) and basolateral (decrease) resistance at modest luminal acidity that were poorly reversible at pH 2.0 and associated with declines in capacitance, a reflection of lower apical membrane area. CONCLUSIONS: In esophageal epithelium apical cation channels transport Na\(^+\) at gradients as low as 10:1 but do not transport H\(^+\) at gradients of 100,000:1 (luminal pH 2.0). Luminal acid also inhibits Na\(^+\) transport via the channels and abolishes it at pH 2.0. These effects on the channel may serve as a protective function for esophageal epithelium exposed to acid reflux.

Keywords: Nystatin, Ussing chamber, impedance, sodium transport.
INTRODUCTION:

The esophagus is a muscular tube lined by a moist stratified squamous epithelium that like that of frog skin and toad urinary bladder, is electrically tight and actively transports sodium ions (Na\(^+\)) from lumen to blood (24). Investigations of Na\(^+\) transport in frog skin have documented that luminal Na\(^+\) crosses the apical membrane through a Na\(^+\) channel and exits the cell in exchange for K\(^+\) across the basolateral membrane via the action of the enzyme, NaK,ATPase. K\(^+\) then is recycled across the basolateral membrane via a barium (Ba\(^{2+}\))-sensitive K\(^+\) channel (34). Further, the rate limiting step for Na\(^+\) absorption in tight epithelia is Na\(^+\) movement through the apical Na\(^+\) channel, a channel characterized by a high Na\(^+\) selectivity and high sensitivity to inhibition by amiloride (11). In 1994, Canessa and colleagues cloned the Na\(^+\) channel and established that it consisted of 3 functional subunits known as \(\alpha\), \(\beta\), and \(\gamma\) EnaC (6). More recently, Awayda and colleagues used antibodies to specific EnaC subunits to show by Western Blotting that the Na\(^+\) channel in native rabbit esophageal stratified squamous epithelium (ESSE) possessed the same 3 subunits and that the amino acid sequence for \(\alpha\)ENaC in ESSE was the same as that of the Na\(^+\) channel cloned from kidney (2, 3). Despite structural similarities, the Na\(^+\) channel in ESSE like that in sheep rumen, differs functionally from the classic Na\(^+\) channel in that it is neither Na\(^+\)-selective nor amiloride-sensitive (2, 16). With respect to selectivity, the Na\(^+\) channel in ESSE was observed to support a similar Isc when Na\(^+\) was substituted with either K\(^+\) or Li\(^+\).

Another notable aspect of the Na\(^+\) channel in ESSE is that \textit{in vivo} in humans it is exposed to high levels of acidity due to repeated daily exposure to refluxates from the stomach whose pHs may attain values as low as pH ~1.0. Moreover, esophageal pH
monitoring has documented that acid reflux extends well beyond the distal esophagus and into proximal esophagus in both healthy subjects (adults and children) and subjects with gastroesophageal reflux disease (GERD) (5, 7, 36) - with proximal reflux being much more common in the post prandial period and when ambulatory rather than sedentary (10). In one recent report, 39% of 130 patients suspected of having laryngopharyngeal reflux had pathologic proximal esophageal acidity on pH monitoring (35). What effect such acidity has on the function of the Na\(^+\) channels in ESSE is unknown. Moreover, and of clinical interest, is to what extent these channels permit the passage of H\(^+\), the smallest of cations, and ones present in high luminal concentrations due to acid reflux. In effect, the question to be considered is whether the apical Na\(^+\) channel serves as a direct pathway for H\(^+\) to traverse the apical membrane and acidify the cell cytosol – thereby promoting the development of reflux esophagitis. This concern takes on added significance because of the apical membrane location of the channel where it is exposed to luminal content and because of its established lack of cation selectivity (18, 29, 31). Nonetheless, while this scenario is of concern, Khalbuss et al impaled esophageal surface cells with pH-sensitive microelectrodes and showed that luminal acidity as low as pH 3.0 had no effect on intracellular pH (pHi) and that lowering luminal pH to 2.0 resulted in only a small decline in pHi of ~ 0.2 pH units (14). Further, these observations were all the more surprising given that the (rabbit/human) esophagus, unlike the stomach or duodenum, is devoid of a surface coat of mucus and has minimal capacity to buffer back diffusing luminal H\(^+\) toward the surface of the epithelium (9, 20, 25). For instance, Abdulnour-Nakhoul et al showed that at luminal pH 2.0, the modest gradient maintained between luminal acidity and surface acidity in rabbit esophagus at pHs of 3.5, collapsed when luminal acidity was
lowered to pH 2.0 – i.e. at pH 2.0 luminal and surface pH became the same (1). In effect
the data indicate that free diffusion of H⁺ directly from the lumen into the cell cytosol does
not occur in ESSE even when apical membranes contain a nonselective Na⁺ channel. This
suggests that environmental pH may regulate ion transport through this channel in ESSE
as has been previously reported for the classic Na⁺ channel (3, 8, 13, 18, 22, 37), and that
this may serve a protective function in vivo against acid reflux injury to the esophageal
epithelium in humans.

For the above reasons, we investigated the effects of luminal acidity on the function of
the apical Na⁺ channels in ESSE – and did so using two Ussing chamber techniques. One
was done by mounting ESSE in Ussing chambers and abolishing the spontaneous
short-circuit current (Isc) by permeabilizing the basolateral membranes of ESSE to
monovalent cations by serosal exposure to nystatin. In this model, then, the Isc generated
by ion gradients across ESSE can be used to monitor ion transport via the apical Na⁺
channel. The second method was by performance of impedance analysis on ESSE
mounted in Ussing chambers but not exposed to nystatin. This technique enables ion
transport across the apical and basolateral membranes to be separated and characterized
non-invasively under varying environmental conditions such as changes in acidity.
MATERIALS AND METHODS:

Ussing Chamber Technique. Healthy New Zealand White male rabbits weighing between 8-9 lbs were killed by administering intravenously an overdose of pentobarbital (60 mg/ml). The esophagus was excised, opened, and stripped of its muscle layers in a paraffin tray containing ice-cold oxygenated normal or HEPES (N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid) Ringer solution so that a sheet of tissue was obtained consisting of stratified squamous epithelium and a small amount of underlying connective tissue. From this tissue, four sections were cut and mounted as flat sheets between Lucite half-chambers with an aperture of 1.13 cm² for measurements of potential difference (PD), short-circuit current (Isc), and calculation of transepithelial electrical resistance (Rₜ). Tissues were bathed with either normal Ringer (composition in mmol/L) Na⁺ 140, Cl⁻ 119.8, K⁺ 5.2, HCO₃⁻ 25, Ca²⁺ 1.2, Mg²⁺ 1.2, HPO₄²⁻ 2.4, H₂PO₄⁻ 0.4, 268 mosmol/kg H₂O, pH 7.5 when gassed with 95% O₂/5% CO₂ at 37°C or in HEPES Ringer solution (composition in mm/L Na⁺ 140, Cl⁻ 130, K⁺ 4.8, HEPES 25, Ca²⁺ 1.2, Mg²⁺ 1.2, HPO₄²⁻ 2.4, and H₂PO₄⁻ 0.4, and gassed with 100% O₂, pH 7.4, osmolality 288 mOsm/kg H₂O. Mucosal and serosal solutions were connected to calomel and Ag/AgCl electrodes with Ringer agar bridges for measurements of PD. Tissues were in the short-circuited state except for 5-10 sec when the open circuit PD was read. Rₜ was then calculated using Ohm's Law by dividing the open circuit PD by the Isc (or from the current deflection to imposed voltage). Junction potentials were determined for acidic conditions according to the method of Read and Fordtran (26). At pH ≥ 2, junction potentials were <1 mV, so no corrections were made in the observed readings. For pH < 2.0, junction potentials exceeded 1 mV and these values used to correct the observed PD and Isc values. For pH 1.6, the junction potential was +6.3 mV and for pH 1.1, it was +16.7 mV.
Circuit Analysis. Circuit analysis was performed on Ussing chamber-mounted rabbit esophageal epithelium to obtain estimates of apical membrane resistance (Ra), basolateral membrane resistance (Rb), transcellular resistance (Rcell) and shunt (paracellular) resistance (Rs). This was done, as previously reported (30) using the following formula: $R_T = \frac{(Ra+Rb)(Rs)}{Ra+Rb+Rs}$. Briefly, after recording the initial PD and Isc and calculating $R_T$, component resistances – i.e. Ra, Rb and Rs - were determined by serosal exposure to nystatin 0.1 mM for 1hr to permeabilize the basolateral membrane and in so doing eliminate the contribution of Rb to $R_T$. By then changing both luminal and serosal bathing solutions to a Na⁺-free HEPES solution (isosmolar substitution of NMDG-Cl for NaCl and KCl in standard HEPES Ringer) transcellular ion transport is largely eliminated so that recorded $R_T$ now approximates Rs. From these data, resistances for Ra, Rb and Rs are calculated from the formula and Rcell calculated by adding Ra and Rb.

Fluorescein Flux. The flux of fluorescein, 300 MW, (Sigma, St. Louis, MO) was determined by adding 1 mM fluorescein to the luminal bath. The luminal bath was then sampled to obtain the initial concentration. The serosal bath was sampled at zero time and at periodic intervals before, during and after experimental manipulation of the tissue. Samples were read for the presence of fluorescein using a fluorometer (SLM Amico SPF 500, SLM Instruments, Inc. Urbana IL) and the fluorescence in the serosal samples divided by the fluorescence in the luminal bath x 100 used to calculate the flux as percent (%) of initial fluorescein concentration. [Note: the values for fluorescence over the range measured were linear.]

Impedance Analysis. Impedance analysis was carried out on short-circuited tissues in modified Ussing chambers that are designed to minimize edge damage and allow continuous
perfusion of either the apical or basolateral solutions (4). Impedance was continuously monitored by imposing 5 distinct voltage sinusoids and a low frequency (sub Hertz) voltage command signal. The resulting current signal, along with the imposed voltage were digitized, Fourier transformed, and used to calculate the magnitude of the transepithelial conductance (gm), and capacitance (Cm). Data were collected every 10 s and plotted as a function of time along with the short-circuit current (Isc). These parameters are well-accepted means for non-invasively monitoring the activity of membrane ion channels as well as its area, respectively. Moreover, in the kHz range, the measured capacitance is predominantly due to the apical membrane area and can therefore be used to monitor potential trafficking events at this membrane.

Following each experimental manipulation an impedance spectrum was also measured. This procedure involved imposing a complex voltage command sinusoid containing 103 different frequencies with frequencies ranging from 1 Hz to 19.4 kHz. The current and voltage signals were digitized, Fourier transformed and used to calculate the complex impedance as a function of frequency. Data were plotted (see Fig. 9) using a Nyquist format of real (Real Z_T) versus imaginary (Imag Z_T) components of the impedance. In this representation, the impedance of each membrane describes a semi-circle owing to the parallel arrangement of resistance and capacitance. Under conditions, as observed in esophageal epithelia, where the apical membrane resistance and capacitance are markedly different than that of the basolateral membrane, the resulting Nyquist representation yields two semi-circles. In this case, and owing to the higher basolateral than apical area, the low frequency semi-circle reflects the basolateral impedance, while the higher frequency semi-circle reflects that of the apical impedance.
Statistics: Statistical significance was determined using Student's t-test for parametric data and the Mann-Whitney test for nonparametric data (injury assessment). All data were reported as the mean ± standard error.

Animal Experiments. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC).
RESULTS

To investigate cation permeation through the NSCC in esophageal epithelia, tissues were mounted in Ussing chambers and exposed serosally to nystatin. Following nystatin permeabilization of the basolateral membrane, Isc fell to zero creating an apical cell membrane-junctional complex barrier model that enables subsequent changes in Isc with imposed gradients to reflect lumen-to-serosal (transepithelial) cation flux. H⁺ diffusion across the epithelium was then investigated in this model at varying luminal pH. As shown in Figure 1A, after nystatin abolished Isc, lowering luminal pH from 7.0 to 2.0 with 3N HCl had no effect on Isc, i.e. Isc remained zero, and this correlated with lack of change in RT (Figure 1B). Further, circuit analysis after luminal pH 2.0 for 30 min revealed that Ra and Rs increased significantly while Rb declined a small but insignificant amount (Figure 2). Also, fluorescein flux after pH 2.0, though increased, was not significantly different from that of tissues exposed to normal Ringer solution (Figure 3). When luminal acidity was further increased to pH < 2.0 - i.e. pH 1.6 then pH 1.1 - Isc increased to 5 uAmps/cm² and 16 uAmps/cm² and RT decreased by 106 ohms cm² and 368 ohms cm², respectively (Figures 1A and B). Further, these changes were accompanied by a significant increase in mucosal-to-serosal fluorescein flux as illustrated for exposure to pH 1.1 for 30 min (Figure 3). [Note: serosal-to-mucosal fluorescein flux after exposure to pH 1.1 (data not shown) was similar to that shown for mucosal-to-serosal flux in Figure 3.] Finally, following restoration of luminal acidity to neutral pH either by replacement with normal Ringer, pH 7.4, or by titration of luminal pH with N-methyl-D-glucamine, Isc rapidly reverted to zero (Figure 4). In a similar set of experiments the lumen was acidified with 3N H₂SO₄ instead of 3N HCl. This resulted in a similar pattern of changes in Isc (and RT) as observed for luminal exposure to HCl over the
entire pH range (data not shown). The only notable difference was that at pH values < 2.0 the magnitude of the increase in Isc with H$_2$SO$_4$ was significantly greater than for HCl at pH 1.6 (Figure 5).

To study Na$^+$ permeation through the NSCC, a 10:1 lumen-to-serosal gradient for Na$^+$ was created (by reducing serosal bathing solution Na$^+$ to 14 mM while maintaining luminal Na$^+$ at 140 mM) in nystatin-treated tissues. At neutral pH, the 10:1 Na$^+$ gradient produced a rise in Isc that stabilized at ~ 5 $\mu$Amps/cm$^2$ above baseline (Figure 6) – a value similar to that observed with a lumen-to-serosal gradient for H$^+$ of ~ 500,000:1 (Figure 1A). [Note: Figure 6 also shows that the rise in Isc generated by the imposed Na$^+$ gradient in a nystatin-treated tissue reflects cation movement through the NSCC as opposed to the paracellular pathway because of the lack of effect on Isc of a similar Na$^+$ gradient in tissues that were not pretreated with nystatin.] The effect of acidity on NSCC was then assessed in the presence of the 10:1 Na$^+$ gradient by monitoring the change in Isc while titrating the pH of either the luminal or serosal bathing solution with 3N HCl (Figure 7A and 7B). As illustrated at pHs as low as 4.5, there was no discernable effect on the Isc created by the Na$^+$ gradient. However, when either luminal or serosal acidity was reduced to pH 3.5, Isc began to decline. For luminal acidity, Isc declined to ~ +1 $\mu$Amp/cm$^2$ at pH 2.0 and then dramatically rose to ~ +4 $\mu$Amps/cm$^2$ at pH 1.6, the latter a reflection of a break in the barrier (decline in R$_T$ – data not shown) and generation of a H$^+$ diffusion gradient from lumen-to-serosal side through the paracellular pathway (see Figure 1A). For serosal acidity, Isc declined to a greater extent than for luminal acidity, reaching zero at serosal pH 2.0 and decreasing to ~ -3 $\mu$Amps/cm$^2$ at serosal pH 1.6. [Note the change in sign for Isc at serosal pH 1.6 that reflects a break in the barrier as with luminal pH 1.6 and generation of a H$^+$ diffusion gradient but from serosal-to-luminal side
rather than vice versa for luminal pH 1.6.] Also following restoration of a neutral bathing solution pH after exposures to luminal or serosal pH 2.0, Isc rose but reached by 1 hr only 50-60% of the Isc observed in simultaneously studied non-acidified Na\(^+\) gradient controls (data not shown).

Impedance analysis was also performed on intact esophageal epithelia mounted in Ussing chambers and exposed to varying luminal pH. Consistent with the above findings, acidification of the luminal solution down to pH 5.0 had minimal effects on ion transport (Figure 8). However, when luminal pH was reduced to 3.0, there was a small but consistent decrease of the Isc and a further decrease to pH 2 resulted in an increase in Isc. This latter increase is likely due to stimulation of basolateral membrane transport as evidenced by the decline in basolateral membrane impedance and by the absence of a change in Isc in nystatinized tissues where the basolateral membranes are eliminated. These effects were reversible upon washout of the luminal solution to pH 7.4, and both gm and the Isc stabilized to values below baseline, indicating potential sustained inhibitory effects on apical membrane transport.

Acidification of both apical and basolateral solutions to pH 2 resulted in transient changes of Isc and gm, followed by stabilization of the Isc at values not different from zero. These transient changes and the observed negative Isc are attributed to the mixing kinetics of the chamber and the slightly different flow rates that resulted in an initial reverse current through the paracellular pathway. The essentially complete inhibition of the Isc with bilateral acidification to pH 2 was not reversible upon return of the apical and basolateral solutions to pH 7.4. The decrease of gm was also irreversible within the 60 min washout period indicating a sustained inhibition of cellular transport, and most likely apical membrane transport.
Consistent with this conclusion is the observed sustained inhibition of capacitance at apical pH 2, and the larger sustained decrease in symmetrical pH 2. Thus, one possible interpretation of these data is that acidification irreversibly inhibits the apical membrane cation channel via decrease of channel density mediated via endocytosis leading to a lower Cm.

To further test the above hypothesis on the effects of pH on the apical membrane, an impedance spectrum was collected. As shown in Figure 9, the Nyquist presentation yields a clearly demarcated apical and basolateral membrane impedance. As mentioned in Methods, the basolateral membrane impedance is dominant at low frequencies, while the apical membrane dominates at high frequency. The resistance of both membranes is obtained from the intercepts with the real axis at low and high frequency. It is clear from Figure 9 that apical membrane resistance is increased as the luminal solution is acidified. This response is enhanced with either basolateral pH 2 alone (data not shown) or with symmetrical (apical and basolateral) pH 2 (Figure 9), indicating a potential role of intracellular pH rather than extracellular. This finding is similar to that observed in Fig. 7 where apical or basolateral acidification produced inhibition of the Isc at pH 2. In both cases, however, the increase of apical resistance is not reversible with washout to pH 7.4, again consistent with a second messenger intracellular pH mediated effect.

An interesting effect observed in Figure 9 was that the changes of apical and basolateral membrane impedances were opposite in direction. In this case, the presumed intracellular acidification is causing an inhibition of the apical membrane, a finding that is also consistent with the observed decrease of capacitance (Figure 8) accompanied by a stimulation of the basolateral membrane conductive pathway. One potential explanation would be an inhibition of the number or open probability of the apical membrane NSCC, accompanied by a
stimulation of the basolateral membrane K\(^+\) channel. In this case, the resulting effect on Isc and transepithelial transport (at least under some conditions) may be fortuitously negligible or underestimated due to the increased driving forces for Na\(^+\) entry by basolateral membrane hyperpolarization. At the extreme, as with symmetrical pH 2, this compensation cannot fully counteract the large inhibition of the apical membrane and leads to inhibition of the Isc to values not different from 0.
DISCUSSION.

The present experiments were designed to assess in esophageal epithelium the ability of H\(^+\) to traverse the NSCC at low extracellular pH and to characterize the effects of low extracellular pH on the ability of Na\(^+\) to traverse the NSCC. The studies were carried out using rabbit esophageal epithelium mounted in Ussing chambers and exposed serosally to nystatin. Nystatin was used to permeabilize the basolateral cell membranes to monovalent cations and by so doing to abolish active ion transport (I_sc approached zero) and negate the contribution of the basolateral membrane to the transepithelial electrical resistance. This maneuver resulted in a model of a chambered epithelium whose barrier function was predominantly due to parallel resistances of the apical cell membranes and intercellular junctional complexes and whose I_sc could be used to reflect passive diffusion of ions across the barrier during the presence of an applied lumen-to-serosal (or vice versa) ion gradient. Alternatively, impedance analysis was used to assess the contribution of each membrane in intact epithelia.

When a lumen-to-serosa H\(^+\) gradient was produced by lowering luminal bathing solution pH from 7 to 2.0, the latter yielding an H\(^+\) gradient of ~100,000:1, no change in either I_sc or R_T was observed – I_sc remaining zero and R_T stable at baseline levels. In addition circuit analysis showed that both apical and shunt resistance rose in tissues exposed to pH 2.0 for 30 min. The likely explanation for these observations was that the barrier created by the apical membranes and junctional complexes remained intact at these levels of luminal acidity and that H\(^+\) diffusion across the apical membranes and its NSCC and/or junctional complex was insufficient to produce a measurable change in current. Alternatively, most of these same observations could be explained if luminal acidity was never translated into similar acidity at the level of the surface cells within the epithelium – the latter protected as it were by a surface
buffer zone comprised of mucus and unstirred water layers containing bicarbonate ions as is present in gastroduodenal epithelium. This, however, is not the case since the esophagus unlike the stomach and duodenum has a surface without a defined viscoelastic mucus layer and its surface cells are devoid of the capacity for bicarbonate secretion. In addition the rabbit esophagus unlike the opossum, pig or human esophagus, has no bicarbonate-secreting submucosal glands. Consequently, the absence of an effective pre-epithelial buffer defense means that luminal acidity has easy access to the esophageal epithelium proper. Indeed and irrespective of whether the model was in the gland-free rabbit or gland-bearing opossum esophagus it was shown by pH microelectrodes that at luminal pH of 2.0 in both species the existing surface buffer zone completely collapsed so that luminal pH 2.0 produced an epithelial surface pH of 2.0.

In contrast to pH = 2.0, when luminal pH was reduced to < 2.0, Isc rose and R_T fell – reflecting a break in the barrier and H^+ diffusion across the epithelium. That H^+ diffusion was the mobile species was evident in the abolition of Isc by either bathing solution neutralization with NMDG or by replacement of the acidified solution with one of neutral pH. Further, since coincident with a decline in R_T, there was an increase in fluorescein flux [in an epithelial model without cell necrosis – data not shown (30)], the break in the barrier produced a leak in the shunt pathway and it is through this pathway that H^+ diffusion likely took place.

Additional evidence for a paracellular route of H^+ movement is provided by comparing the effects of acidification with H_2SO_4 and HCl (see Fig. 5). The ratio of the H^+-induced Isc with pH 1.1 and 1.6 is ~ 3.2 for both acids. This ~ 3.2 fold increase of the Isc is the same fold increase in [H^+] (calculated as simply the anti-Log of pH 1.1 and 1.6). This indicates that the Isc is simply a passive property of the paracellular [H^+] concentration gradient. This also
suggests that the paracellular pathway retains its selectivity for Cl\(^-\) over SO\(_4\)\(^{2-}\) even at these high [H\(^+\)]. These data are also consistent with previously published data from circuit analysis on esophageal epithelium indicating a break in the barrier (fall in R\(_T\)) by acid at pH < 2.0, accompanied by a decrease of shunt resistance (30). Taken together, these findings indicate that H\(^+\) are unable to freely diffuse through the NSCC in the apical membranes of esophageal epithelium and that even under acidic stress sufficient to break the barrier, H\(^+\) passage is via the shunt rather than cellular pathway.

To gain further insight into the effect of luminal acidity on the function of NSCC in esophageal epithelium, we utilized the nystatin model to monitor Na\(^+\) diffusion through the NSCC by creating a lumen-to-serosa Na\(^+\) gradient – a gradient that provided a stable rise in Isc of ~4 µAmps/cm\(^2\). That the observed Isc rise reflected Na\(^+\) movement via the NSCC and not the shunt pathway was evident by the fact that the same Na\(^+\) gradient across esophageal epithelium not pretreated with nystatin showed no sustained change in Isc. Consequently, the Isc generated by the Na\(^+\) gradient in nystatin-treated tissue enabled the function of the NSCC to be monitored while varying bathing solution acidity. As shown in results, acidification of the luminal or serosal bathing solution to levels of pH < 3.5 resulted in a progressive decline in Isc such that at pH 2.0 Isc approached zero. These findings are consistent with those above in that H\(^+\) didn’t traverse the NSCC; moreover they showed that luminal or serosal acidity inhibits all cation movement (as illustrated by Na\(^+\)) via the NSCC. Also, serosal acidity was somewhat more rapid and effective in inhibiting the NSCC than luminal acidity which suggests that inhibition of the channel was likely mediated by intracellular H\(^+\) rather than extracellular H\(^+\). This is the case since it has been previously reported that H\(^+\) more readily
enters esophageal cells across the basolateral cell membrane than across the apical cell membrane (14, 32, 33).

An inhibitory effect of acid on Na⁺ absorption in electrically tight epithelia such as frog skin and toad bladder has been previously reported (13, 22); and the mechanism for this effect reported to be due to acid’s inhibitory effects on the apical membrane sodium channels. Moreover the effect of acid on the channel was found to be mediated via changes in intracellular and not extracellular pH. Further Palmer and Frindt (23) observed that low intracellular pH inhibited sodium channel activity on Na⁺ channels from rat cortical collecting tubule by reducing its open probability while Zeiske et al (37) reported that low intracellular pH inhibited the channel by reducing open channel density in A6 cells. In addition using a cloned epithelial Na⁺ channel (ENaC) expressed in Xenopus oocytes, Chalfant et al (8) observed that decreases in intracellular pH rapidly (within minutes) inhibited ENaC activity while similar changes in extracellular pH were without appreciable effect. Moreover, inhibition of ENaC activity was found to be specifically mediated by interaction of H⁺ with the α ENaC subunit and not by an action on other regulatory proteins. It was further suggested that protonation of key amino acids is the means by which H⁺ alters channel conformation which shifts the equilibrium between the open and closed state to the closed state (8). Zeiske et al. also suggested that the total number of channels could vary and that a fraction of channels disappear as a consequence of acidification, either by becoming permanently closed or by endocytic removal.

In our studies, impedance analysis revealed that Isc was largely irreversible. A decrease of the high frequency capacitance is observed indicating a decrease in apical membrane area. This effect is consistent with the poor reversibility and the increase in apical
membrane resistance. Furthermore, luminal acidification at levels that have little or no effects on junctional permeabilities (pH>2), produced large changes of basolateral membrane resistance, suggesting an intracellular mechanism of H⁺ actions. Therefore, the simplest interpretation for these findings is that luminal acidification, via effects on intracellular pH, inhibits the apical membrane NSCC via endocytosis leading to a decrease in area, an increase in resistance, and poor reversibility. Nonetheless, further experiments are needed in order to confirm the presence of endocytosis under these conditions. On the other hand [H⁺] at pH 2 alters the junctional permeability and transverses the epithelium at rates high enough to be observed as a reverse current, only via the paracellular pathway.

The data in the manuscript support serosal acidity having a greater effect on apical NSCC Na⁺ conductance than does luminal acidity (Figure 7) and consequently we suggested that intracellular pH is more important than extracellular (luminal) pH in regulating NSCC. We also concluded that H⁺ are not transported into the cell via the apical NSCC. These results, which may appear contradictory, are reconciled by recognizing that luminal H⁺ gains access to the cell cytosol by first diffusing through the paracellular (shunt) pathway to access the more acid-permeable basolateral membrane. H⁺ then crosses the basolateral membrane to gain access to the cell cytosol (and NSCC) rather than directly accessing the cell cytosol by crossing the apical cell membrane. Support for this conclusion is also derived from a study in which a luminal cell in a chambered sheet of rabbit esophageal epithelium is impaled with a pH microelectrode for monitoring of intracellular pH (pHᵢ) (14). When luminal pH was lowered to acidic levels > 2.0, there was little or no change in pHᵢ and no change in Rₜ. However, when luminal pH was lowered to < 2, pHᵢ declined significantly, i.e. by approximately 1 unit from 7.5 to 6.5. Since the drop in pHᵢ occurred in association with an
increase in shunt permeability as evident by a decline in $R_T$, this suggested that acid lowered pH$_i$ either by: a) its direct diffusion across the apical membrane into the cell with the lowering of pH$_i$ then producing the fall in $R_T$, or b) after damaging the tight junctions and lowering $R_T$, acid diffused through the shunt pathway to gain access to the more acid-permeable basolateral membrane. H$^+$, then, entered the cytosol and lowered pH$_i$ by crossing the basolateral membrane. The latter route is supported by the fact that lowering serosal pH to 4.0 is sufficient to reduce the pH$_i$ of the surface cell by ~1 pH unit (7.5 to 6.5) and does this without a concomitant change in $R_T$. Hence, the change in $R_T$ at luminal pH $< 2.0$ was not caused by cell acidification but the fall in $R_T$ was instead a prerequisite for cell acidification. This concept is also supported by circuit analyses showing that at luminal pH $< 2.0$, shunt resistance falls while apical membrane resistance remains essentially unchanged (30).

How luminal acidity at pH values $< 2.0$ alters the intercellular junctional complex to lower shunt resistance in esophageal epithelium remains unknown, though it is likely through changes in protein conformation and/or degradation. Indeed the nature of the proteins that regulate permeability via its paracellular route have only recently been addressed. For instance, m-RNA or microarray analyses and immunohistochemistry have demonstrated that the junctional complex contains the following bridging proteins: occludin, claudins 1,2,3,4,7 and E-cadherin (12, 15, 17, 27, 28). To date only E-cadherin has been subjected to functional analysis- this by using the calcium-switch technique. Removing bathing solution calcium results in an ~ 50% decline in shunt resistance and one that is reversible upon restoration of calcium. Restoration or ‘resealing’ of the junction with calcium replacement is effectively blocked by antibodies or small peptides to the extracellular domain of E-cadherin (28).
In summary, the apical NSCC in esophageal epithelium are impermeant to H\(^+\) even at gradients of 1:100,000 (while permitting Na\(^+\) passage at gradients as low as 10:1). Moreover, luminal acidity inhibits Na\(^+\) passage via apical channels, accounting in part for acid-induced inhibition of active Na\(^+\) transport. The ability of luminal acidity to regulate apical membrane NSCC permeability has potentially important clinical applicability – serving as a protective mechanism by which the esophagus protects itself against injury from exposure to refluxed gastric acid. This protection is afforded both by directly preventing H\(^+\) permeation through the NSCC and into the cytoplasm and second, by lowering PD (via inhibition of Na\(^+\) transport) which reduces the driving force for Cl\(^-\) diffusion into the tissue. The fact that this impermeance provides protection against high levels of luminal acidity, e.g. pHs as low as 2.0, is evident by the minimal change in pH\(_i\) at luminal pH 2.0 (14) and absence of gross or histologic damage to the tissue during prolonged exposure to pH 2.0 (32). Finally, H\(^+\) entry into the epithelium as evidenced by the rise in Isc at low pH\(_o\) reflects diffusion via the paracellular rather than transcellular pathway since protection against cell necrosis is afforded by serosal buffers, even when the buffer, e.g. HEPES, is impermeant to the cell cytosol (33).
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FIGURE LEGENDS

Figure 1. Effect of luminal acidity on (A) the short-circuit current (Isc) and (B) the transepithelial electrical resistance (R_T) in rabbit esophageal epithelium. Tissues are mounted in Ussing chambers and initially pretreated with serosal nystatin for 30 min to abolish active transport. Isc, now a reflection of ion diffusion, remains zero until luminal acidity is reduced to pHs < 2.0. At pH 1.6 and pH 1.1, Isc incrementally rises as a reflection of a H⁺ diffusion current as compared to the non-acidified tissues where Isc remains unchanged at zero. Coincident with the increase in Isc at pHs < 2.0 are declines in R_T, a reflection of increases in tissue permeability that occurs in acidified but not non-acidified tissues. N=3 for control and N=6 for experimental group. Values are mean ± SEM. *p < 0.05 compared to non-acidified controls.

Figure 2. Circuit analysis was performed on rabbit esophageal epithelium mounted in Ussing chambers and exposed to normal Ringer solution, pH 7.4, or HCl, pH 2.0, for 30 min. The values for transcellular resistance (Rcell), apical membrane resistance (Ra), basolateral membrane resistance (Rb) and shunt (paracellular) resistance (Rs) were calculated and presented as percent (%) of initial transepithelial resistance (R_T) for the tissues. Acidity at pH 2.0 is shown to significantly raise apical and shunt resistances. Values are mean ± SEM. *p < 0.05 compared to non-acidified controls. N=6 per group. [Absolute values for R_T are for pH 7.4 - 2019±251 and for pH 2.0 - 2109±289].

Figure 3. The mucosal-to-serosal flux of fluorescein (300 molecular weight) is shown for rabbit esophageal epithelium exposed to normal Ringer solution, pH 7.4, or luminal acidity,
HCl pH 1.1, for 30 min. Luminal acidity, pH 1.1, is shown to markedly increase flux of this non-ionic marker of shunt (paracellular) permeability. pH 2 also increases the flux of fluorescein but this did not reach statistical significance. N = 8 for each group. Values are mean ± SEM (standard error of the mean). *p < 0.05 compared to non-acidified controls.

Figure 4. The reversibility of the rise in the short-circuit current (Isc) in rabbit esophageal epithelium exposed to luminal acidity following washout and restoration of luminal pH to 7.4. Tissues are mounted in Ussing chambers and initially pretreated with serosal nystatin for 30 min to abolish active transport. Isc which at this time is a reflection of ion diffusion remains zero until the lumen is acidified to pH 1.1. Isc rises as a reflection of a H⁺ diffusion current and this reversed by replacement of the luminal bath with normal Ringer solution, pH 7.4. This same effect is also achievable by neutralization of luminal acid with NaOH (data not shown). Values are mean ± SEM, n=6.

Figure 5. Comparative effect of luminal acidity from pH 3.0 to pH 1.1 on the short-circuit current (Isc) of rabbit esophageal epithelium exposed to either HCl or H₂SO₄. Tissues are mounted in Ussing chambers and initially pretreated with serosal nystatin for 30 min to abolish active transport. Isc, now a reflection of ion diffusion, remains zero until luminal acidity is reduced to pHs < 2.0 for both HCl and H₂SO₄. Note that the rise in Isc with exposure to H₂SO₄ is greater than for HCl at comparable pHs, i.e. pH 1.6 and pH 1.1. This suggests that the diffusion of H⁺ across the tissue is in part dependent on the nature of the accompanying anion. Non-acidified control tissues exposed only to Ringer solution, pH 7.4, serves as control.
N=3 for control and N=6 for each experimental group. Values are mean ± SEM. *p < 0.05 for HCl versus H2SO4.

**Figure 6.** Effect of a 10:1 lumen-to-serosal sodium (Na⁺) gradient on the short-circuit current (Isc) in rabbit esophageal epithelium. Tissues are mounted in Ussing chambers and one set left alone as untreated controls and the others initially pretreated with serosal nystatin for 60 min to abolish active transport. In the nystatin-treated tissues, Isc, now a reflection of ion diffusion, remains zero until the Na⁺ gradient is imposed. Isc then rises and plateaus at ~ 4 µAmps/cm². In the control tissues that were not treated with nystatin, imposition of the same gradient is shown to have no sustainable effect on Isc. This indicates that the rise in Isc in nystatin-treated tissues is a reflection of Na⁺ diffusion through the apical membrane cation channels. N=4 for control and N=5 for experimental group. Values are mean ± SEM. *p < 0.05 compared to values prior to Na⁺ gradient.

**Figure 7.** Effect of (A) luminal acidity or (B) serosal acidity on the short-circuit current (Isc) produced by a 10:1 lumen-to-serosal sodium (Na⁺) gradient in rabbit esophageal epithelium. Tissues are mounted in Ussing chambers and pretreated with serosal nystatin for 60 min to abolish active transport. Isc, now a reflection of ion diffusion, remains zero until the Na⁺ gradient is imposed. After Isc rises and stabilizes at ~ 4 µAmps/cm², luminal or serosal acidity is titrated while for controls bathing solution pH remains at 7.4. Titration of luminal or serosal pH as low as 4.5 had no effect on Isc. At luminal or serosal pH 3.5, however, Isc begins to decline and either approaches one (luminal acidity) or reaches zero (serosal acidity) at pH 2.0. At pH of 1.6, a value associated with a significant decline in transepithelial electrical
resistance (data not shown), Isc increases with both luminal or serosal acidity; however the Isc increase with luminal acid is positive with respect to the serosal bath and the Isc increase with serosal acid is negative with respect to the serosal bath – reflecting the direction of H⁺ diffusion. N=3 for control and N=4-5 for experimental groups. Values are mean ± SEM. *p < 0.05 compared to controls.

**Figure 8.** Effects of apical acidification on transepithelial properties. Experiments were carried out on continuously perfused tissues and therefore allowed for the ease of addition and washout of various solutions. Top panel; effects on membrane capacitance. Data were summarized at two frequencies. Owing to the arrangement of the apical and basolateral membrane impedances in these tissues, the capacitance at both of these frequencies is essentially a reflection of apical capacitance. Addition of apical pH 2 caused a small and irreversible decrease of Cm. A larger but similar decrease was also observed with symmetrical pH 2. No effects were observed at pH 5 or 3 apically. Middle panel, effects on transepithelial conductance. Effects were minimal at pH 5 and 3, and are likely due to compensatory changes of apical and basolateral membrane conductances (see Fig. 2 and text). Upon washout from pH 2 (apical or symmetrical) membrane conductance stabilized to values below control indicating sustained inhibition of the cellular pathway. Bottom panel; effects on the short-circuit current (see text for details). Data representative of 5 experiments.

**Figure 9.** Compensatory inhibition of apical and stimulation of basolateral impedances with acidification. Data plotted as real and imaginary components of the impedance (Nyquist presentation). Top panel; acidification of the apical solution to pH 5 and 3 caused a small
increase of apical resistance and a small decrease of basolateral resistance. The net effect was a small increase in transepithelial resistance. At pH 2, this effect is more marked, and is accompanied by a small decrease of transepithelial resistance. Bottom panel; washout of apical and symmetrical acidification to pH 2. Washout of apical pH 2 caused an increase of transepithelial resistance with no apparent recovery of the inhibition of apical resistance. A small but largely exaggerated response is observed with symmetrical pH 2, where the apical resistance continued to dominate and eventually lead to the near disappearance of the basolateral membrane impedance. Data representative of 5 experiments. Ra and Rbl denote apical and basolateral membrane resistance of the impedance under control pH 7.4.
Figure 1A
Figure 1B
Figure 3

Fluorescein Flux (% luminal cone)
Figure 5
Figure 6
Figure 7A
Figure 7B