Lumen-to-surface pH gradients in opossum and rabbit esophagi: role of submucosal glands

SOLANGE ABDULNOUR-NAKHOUL, NAZIH L. NAKHOUL, AND ROY C. ORLANDO
Departments of Medicine and Physiology, Tulane University School of Medicine, and Veterans Administration Medical Center, New Orleans, Louisiana 70112-2699

Lumen-to-surface pH gradients in opossum and rabbit esophagi: role of submucosal glands. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G113–G120, 2000.—The opossum esophagus, like that of humans, contains a network of submucosal glands with the capacity to secrete bicarbonate ions into the esophageal lumen. To evaluate the role of these glands in protecting the epithelial surface from acid insult, we measured the lumen-to-surface pH gradient in opossum esophagus at different luminal pH and compared it to that of rabbit esophagus, an organ devoid of submucosal glands. Sections of opossum and rabbit esophageal epithelium were mounted luminal side up in a modified Ussing chamber. pH-sensitive microelectrodes, positioned within 5 µm of the epithelial cell surface, were used to monitor surface pH during perfusion with solutions of different pH. At luminal pH 7.5, the pHs of both opossum and rabbit were similar (pHs = 7.5). Lowering luminal pH from 7.5 to 3.5 in opossum decreased pHs to 4.2 ± 0.16, a value significantly higher than pH of perfusate, whereas in rabbit this maneuver decreased pHs to 3.69 ± 0.08, a value not significantly different from pH of perfusate. In opossum but not in rabbit, addition of carbachol to the serosal solution increased basal pHs, to 7.8 ± 0.1 and significantly blunted the decline in pHs on perfusion with acidic Ringer solution (pH 3.5), with pHs falling to 6.0 ± 0.45. The effect of carbachol on surface buffering was inhibited by prior treatment with atropine. Luminal acidification to pH 2.0 in opossum (as in rabbit) abolished the lumen-to-surface pH gradient even after addition of serosal carbachol. We conclude that the presence of submucosal glands in esophagus contributes through bicarbonate secretion to creation of a lumen-to-surface pH gradient. Although this gradient can be modulated by carbachol, its capacity to buffer (and therefore to protect) the epithelial surface against back-diffusing H+ is limited and dissipated at pH 2.0.

Animal and tissue preparation. American opossums (North-eastern Wildlife, South Plymouth, NY) and New Zealand White rabbits were killed by administration of an intravenous overdose of pentobarbital sodium (60 mg/ml). The esophagus was excised, opened longitudinally, and pinned mucosal side down in a paraffin tray containing ice-cold oxygenated Ringer. The muscle layers were lifted up with forceps, and the underlying mucosa was dissected free with a scalpel. The sheet of mucosa obtained was cut, and a section was mounted apical side up, horizontally in a modified Ussing chamber with an aperture of 1.13 cm². The chamber allowed continuous and independent perfusion of the apical and the serosal sides of the tissue. The fluid for the perfusion of the tissue was delivered by gravity, and all solutions were placed at the same height from the chamber. The perfusion solutions could be switched quickly and with minimal dead space by means of a combination of six-way rotary valves and four-way pneumatically activated slider valves (Rainin, Emeryville,
The chamber was kept at a fixed level. The turnover rate in the lower side of the chamber was 2 ml/min; however, only part of this fluid (\( \sim 1 \) ml/min) entered the chamber, the rest leaving through a drain that was kept at a fixed level. The turnover rate in the lower side of the chamber was \( \sim 15 \) s. The solutions were prewarmed and delivered to the chamber at 37°C.

Electrodes. Transepithelial potential difference (V\(_{TE}\)) was measured as the voltage difference between a free-flowing KCl electrode (tip \( \sim 10 \) µm) placed in the bath fluid of the serosal side and a similar electrode placed in the bath fluid of the apical side. Both electrodes were fitted with an Ag-AgCl wire, and the leads were connected to the amplifier of a voltage clamp (Physiologic Instruments, San Diego, CA). The voltage clamp was also used to deliver a direct-current pulse of 5–15 µA via platinum wires located in each side of the chamber. This allowed us to determine the transepithelial resistance (R\(_{TE}\)) from the voltage deflection (\( \Delta V_{TE} \)) as follows:

\[
R_{TE} = \frac{\Delta V_{TE}}{I}
\]

where I is current. Given the use of free-flowing KCl electrodes to measure V\(_{TE}\), junction potentials produced during exposure to solutions of varying composition, including pH 2 Ringer solution, were negligible and therefore were not corrected for.

The pH-sensitive microelectrodes were of the liquid ion exchanger type. Alumino-silicate glass tubes (1.2 mm OD \( \times \) 0.86 mm ID; Frederick Haer, Brunswick, MD) were pulled on a vertical microelectrode puller (David Kopf, Tujunga, CA) to a tip of \( \sim 1–2 \) µm and dried in an oven at 200°C for 2 h. The electrodes were exposed in a closed vessel to tri-n-butylchlorosilane fumes for 2 min, after which the silane fumes were vented, and the electrodes were left in the oven for an additional 30 min. The exchanger (Fluka, Hydrogen Iono- phore Il, cocktail A) was then introduced into the tip of the electrodes by means of a very fine glass capillary. The electrodes were then backfilled with a buffer solution containing 0.04 M KH\(_2\)PO\(_4\), 0.023 M NaOH, and 0.015 M NaCl, pH 7.0 (3) and calibrated in standard buffer solutions of pH 2, 4, and 7. The average slope was 57.8 \( \pm \) 0.4 mV/pH unit (n = 15).

For measurements of pH at the epithelial surface (pH\(_{e}\)), the pH-sensitive electrode was advanced using a motorized micromanipulator (Merhauzer, Fine Science Tools) and under microscopic control at \( \times 140 \) (Stereozoom7, Leica) to a position immediately above, but without touching, the tissue. The pH electrode was then further advanced using the micromanipulator until the electrode was observed microscopically to touch the apical membrane of a surface cell, an event that was accompanied by an increase in the magnitude of the direct-current pulses. The tip size of the microelectrode was big enough not to allow intracellular impalements of the epithelial cells. The pH electrode was then withdrawn in steps of 1–2 µm until the direct-current pulse returned to that observed in free solution (12). This permitted localization of the pH electrode within 5 µm of the epithelial cell surface, a position that was maintained throughout the course of the experiments. All electrical and pH readings were recorded on a three-channel strip chart recorder (Kipp & Zonen, Bohemia, NY).

Solutions. The composition of the solutions is shown in Table 1. The chemicals were obtained from Sigma.

### Table 1. Composition of solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>pH 7.5</th>
<th>pH 3.5</th>
<th>pH 2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+)</td>
<td>140</td>
<td>139.7</td>
<td>130</td>
</tr>
<tr>
<td>K(^+)</td>
<td>5.1</td>
<td>5.1</td>
<td>5.1</td>
</tr>
<tr>
<td>Mg(^2+)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Ca(^2+)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>H(^+)</td>
<td>&lt;0.1</td>
<td>0.3</td>
<td>10</td>
</tr>
<tr>
<td>Cl(^−)</td>
<td>119.8</td>
<td>147.22</td>
<td>148.72</td>
</tr>
<tr>
<td>HCO(_3^−)</td>
<td>25</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>H(_2)PO(_4^−)</td>
<td>0.5</td>
<td>2.68</td>
<td>1.18</td>
</tr>
<tr>
<td>HPO(_4^{2−})</td>
<td>2.3</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>H(_2)PO(_4^−)</td>
<td>0</td>
<td>0.12</td>
<td>1.62</td>
</tr>
<tr>
<td>CO(_3^−), %</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O(_2^−), %</td>
<td>95</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Concentrations are in mM unless otherwise indicated.

Statistical analysis. The results are presented as means \( \pm \) SE. Data were analyzed using the two-tailed paired Student’s t-test unless otherwise indicated; n is the number of observations. The initial rates of pHe change were determined from the slope of a linear regression fit of pHe vs. time. Only the initial portion of pHe change was taken, which amounted to 30–40 s, during which the data could be tightly fit to a straight line.

### RESULTS

Both the opossum and rabbit esophagi are lined by a moist stratified squamous epithelium; however, the opossum esophagus contains an extensive network of tubulocinar SMGs (8, 13, 14), whereas that of the rabbit is completely devoid of SMGs (Fig. 1). Using pH-selective microelectrodes positioned within 5 µm of the esophageal epithelium, we measured pHe in both rabbit and opossum esophagi and monitored the changes during luminal perfusion with acidic Ringer solutions of pH 3.5 or 2.0 (Table 1). R\(_{TE}\) and V\(_{TE}\) were monitored as well.

Resting condition. Esophageal tissues obtained from six opossums and five rabbits were perfused with control Ringer (pH 7.5) in the lumen and in the serosal bath. Under basal conditions in opossum, pHe was 7.5 \( \pm 0.03 \). V\(_{TE}\) was \(-7.6 \pm 0.94 \) mV, and R\(_{TE}\) was \(1.616 \pm 214 \Omega \cdot \text{cm}^2\) (n = 13, 1–3 tissues were used from each animal). In the rabbit, pHe was 7.5 \( \pm 0.04 \), V\(_{TE}\) was \(-12.0 \pm 0.94 \) mV, and R\(_{TE}\) was \(2.001 \pm 157 \Omega \cdot \text{cm}^2\) (n = 7). Both R\(_{TE}\) and V\(_{TE}\) were significantly higher in the rabbit than in the opossum (P < 0.004, unpaired t-test).

Effect of luminal acidification to pH 3.5. After baseline recordings, luminal pH was lowered by switching the luminal solution to acidic Ringer, pH 3.5. In the opossum, this maneuver resulted in a small depolarization of V\(_{TE}\) (1.9 \( \pm \) 0.4 mV, P < 0.001) but no significant change in R\(_{TE}\). However, acidification of the lumen resulted in a progressive decline in pHe, over 7.4 \( \pm 1.14 \) min, to a new pHe of 4.2 \( \pm \) 0.16 (Fig. 2, segment ab). The initial rate of decline in pHe during perfusion with pH 3.5 was 1.78 \( \pm \) 0.48 pH/min, and ΔpHe was 3.3 \( \pm \) 0.15 pH units. Thus pHe at the new steady state was 0.64 \( \pm \) 0.16 pH units higher than that of the luminal perfusate pH 3.5 (n = 13, P < 0.002). When the luminal bathing solution was switched back to bicarbonate-Ringer solu-
tion, pH 7.5, the changes were reversible and pHs returned to its previous baseline value (Fig. 2, segment bc).

In the rabbit esophagus, perfusion of the lumen with acidic Ringer, pH 3.5 (solution 2), produced no change in $V_{TE}$ ($-12.8 \pm 1.25$ mV) or $R_{TE}$ ($1,928 \pm 180$ $\Omega \cdot cm^2$; $n = 7, P > 0.05$). However, pHs declined at an initial rate of $2.58 \pm 0.78$ pH/min, by $3.81 \pm 0.07$ units ($n = 6$) to reach a plateau value of $3.69 \pm 0.08$ (Fig. 3, segment ab). This latter pHs value was not significantly different from pH 3.5 of perfusate (plateau reached within $5.1 \pm 1.01$ min). These changes were reversible on switching back the luminal solution to a control Ringer solution (Fig. 3, segment bc).

Effect of carbachol on pHs. Carbachol, a cholinergic agonist, has been reported to stimulate bicarbonate secretion in the opossum esophagus (10). Therefore, the effect of serosal carbachol on pHs was studied in the opossum and, for comparison, in the rabbit. Perfusing the serosal side of opossum esophagus with a Ringer solution containing carbachol ($10^{-7} M$) increased pHs by $0.3 \pm 0.11$ pH units (Fig. 2, segment de), decreased $R_{TE}$ by $236 \pm 49$ $\Omega \cdot cm^2$, and had no effect on $V_{TE}$ ($n = 9, P < 0.01$). These data are summarized in Fig. 4. In

---

**Fig. 1.** Light micrographs ($\times 100$) of opossum (A) and rabbit (B) esophageal mucosa. Although both esophagi possess stratified squamous epithelia (uppermost layer), only the opossum esophagus shows an extensive network of submucosal glands (arrows).
The rate of acidification was slower, at 0.58 ± 0.6 \( \text{pH/min} \), in opossum than in its absence (1.78 ± 0.48 \( \text{pH/min} \)), and the time it took to reach the new steady-state value for \( \text{pH}_s \) was longer (11.5 ± 0.5 min vs. 7.4 ± 1.14 min) than in the absence of carbachol (\( n = 8, P < 0.03 \) for each comparison). \( \text{pH}_s \) values at defined time points of 1, 3, and 5 min after acidification are shown in Table 2. Luminatal luminal exposure to pH 3.5 in the presence or absence of carbachol resulted similarly in a depolarization of \( V_{TE} \) of 2.32 ± 0.77 mV and no change in \( R_{TE} \).

In contrast to the opossum, luminal exposure to pH 3.5 in the rabbit, in the presence of serosal carbachol, resulted in a decrease of \( \text{pH}_s \) to 3.9 ± 0.18 (Fig. 3, segment ef). A value not significantly different from that observed in the absence of carbachol (\( \text{pH}_s = 3.7 ± 0.1 \)) or from that of the luminal perfusate (\( n = 5, P > 0.05 \) for both comparisons; Fig. 5). Also, in the rabbit, there was no difference in the initial rate of decline of \( \text{pH}_s \) at luminal pH 3.5 (2.58 ± 0.78 \( \text{pH/min} \)) or time to plateau (4.9 ± 1.12 min) in the presence of carbachol compared with the values recorded in its absence (\( P > 0.05 \) for each comparison; Fig. 5).

Because serosal carbachol had a marked effect on the lumen-to-surface pH gradient in opossum, we assessed the effect of atropine, a cholinergic antagonist, on this parameter. This was done by simultaneously exposing the tissue to both serosal carbachol and atropine (10\(^{-6} \) M) and then acidifying the luminal bath with Ringer at pH 3.5. As shown in Fig. 2, the ability of carbachol alone to reduce the rate and degree of decline in \( \text{pH}_s \) with luminal acidification (segment ef) was markedly reduced by the addition of atropine to the serosal solution (segment hi). Indeed, in the presence of atropine, the change of \( \text{pH}_s \) and the rate of acidification in response to pH 3.5 in the lumen were similar to the changes obtained in control conditions. Thus, in tissues from four opossums when the lumen was acidified to pH 3.5, \( \text{pH}_s \) decreased to 4.0 ± 0.09 in control, 6.1 ± 0.54 in the presence of carbachol (\( n = 4, P < 0.05 \)), and 5.1 ± 0.6 for atropine plus carbachol. This is reflected in different \( \Delta \text{pH}_s \) shown in Fig. 6. Also observed is that the initial rate of acidification in the presence of atropine (1.1 ± 0.61 \( \text{pH/min} \)) was not significantly different from control. This is in contrast to the findings in the opossum (see Fig. 2).
different from the rate of 1.3 ± 0.4 pH/min in the control period (Fig. 6).

Effect of luminal acidification to pH 2.0 in the absence and presence of carbachol. Because the opossum esophagus maintained a significant lumen-to-surface pH gradient during luminal perfusion at pH 3.5, we investigated its ability to handle an increased acid load by perfusing the lumen with an acidic Ringer at pH 2.0, in both the presence and absence of serosal carbachol. In the opossum, when the luminal perfusate was switched to an acidic Ringer at pH 2.0, there was a rapid decline in pHs, which reached a value similar to that of the perfusate (ΔpHs of 5.43 ± 0.15 units) over a period of 2.5 ± 0.67 min (Fig. 7, segment ab). Although the presence of carbachol was able to reduce the initial rate of surface acidification (1.70 ± 0.5 pH/min); compared with its absence (3.45 ± 0.9 pH/min; n = 4, P < 0.05), acidifying the luminal solution to pH 2.0 in the presence or absence of carbachol resulted in similarly substantial declines in pHs (5.45 ± 0.2 units vs. 5.6 ± 0.28 pH units, respectively; n = 4, P > 0.05) such that both reached new steady-state values that were not significantly different from the luminal perfusate of pH 2.0 (Fig. 7, segment ef). These data are summarized
Table 2. Surface pH in the opossum at defined time points after luminal acidification to pH 3.5 in the absence and presence of carbachol

<table>
<thead>
<tr>
<th>Time After Acidification to pH 3.5</th>
<th>pHs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1 min</td>
<td>6.29 ± 0.22</td>
</tr>
<tr>
<td>3 min</td>
<td>4.90 ± 0.39</td>
</tr>
<tr>
<td>5 min</td>
<td>4.37 ± 0.34</td>
</tr>
<tr>
<td>Steady state</td>
<td>4.20 ± 0.24</td>
</tr>
</tbody>
</table>

Values are means ± SE. Steady state after acidification to pH 3.5 is achieved after 7.4 ± 0.1 min in control and after 11.5 ± 0.9 min in carbachol (n = 8). *P < 0.05.

As shown during luminal acidification at pH 3.5 of the rabbit esophagus, the absence of SMGs results in little to no capacity to protect the epithelium from being accessed by luminal acid even at this relatively modest level of acidity. Thus luminal acidity at pH 3.5 resulted in the rapid decline in pHs until it reached a value similar to that of the luminal perfusate.

In contrast to rabbit esophagus, the opossum esophagus responded to luminal acid perfusion of pH 3.5 with a slower rate of acidification and a longer time to reach a steady-state pHs value that was higher than that of the rabbit esophagus and significantly above that of the luminal perfusate by a little more than one-half a pH unit. Because neither the rabbit nor the opossum stratified squamous esophageal epithelium secretes mucus or bicarbonate, this indicates that the superior lumen-to-surface pH gradient maintained by the opossum esophagus is due either to secretion of bicarbonate and/or mucus, see below) by the submucosal glands or to greater passive paracellular diffusion of bicarbonate from blood to lumen. This latter possibility cannot be ignored, given that the transepithelial electrical resistance of opossum esophageal epithelium is significantly lower than that of rabbit and that this parameter is dominated by the ion permeability across the paracellular route (19).

In the present investigation, pH-sensitive microelectrodes were used to measure pHs in two species, the opossum (submucosal glands bearing) and the rabbit (submucosal glands free), under two conditions: 1) in the presence of two different levels of luminal acidity (pH 3.5 and 2.0), and 2) in the presence of a known stimulant of bicarbonate secretion by the submucosal glands.

**DISCUSSION**

Documentation that secretion from the esophageal submucosal glands is a major contributor to the lumen-to-surface pH gradient (and so preepithelial defense) in opossum was established by serosal exposure to carbachol, a known stimulant of submucosal gland secretion. Notably, the addition of carbachol both raised the resting pHs at luminal pH 7.5 and markedly enhanced the magnitude of the lumen-to-surface pH gradient at luminal pH 3.5 in opossum esophagus. In contrast, carbachol neither raised the resting pHs at luminal pH 7.5 nor did it improve the ability of the rabbit esophagus to establish a lumen-to-surface pH gradient in the presence of luminal acid of pH 3.5. Carbachol has no effect on esophageal stratified squa-
mous epithelium (5), and so these observations indicate that the source of the enhanced lumen-to-surface pH gradient in opossum but not rabbit is due to its ability to stimulate secretion (bicarbonate) by the SMGs. Furthermore, bicarbonate, not mucin, is the likely factor in the secretion to be responsible for these observations, since mucin secretion, by creating a larger diffusion barrier, might slow the rate of H⁺ diffusion but would not probably change the ultimate pHs reached on luminal acidification. In effect, the ability of carbachol to produce a new higher steady-state value for pHs over that of the perfusate indicates that the secretion of bicarbonate from the submucosal glands was the likely component accounting for protection. Indeed, from the change in pHs, the increase in buffer capacity produced by carbachol can be estimated at 1.75 times over basal levels. This increase in buffering power can be calculated using the formula

\[ \frac{\beta_{\text{carb}}}{\beta_{\text{control}}} = \frac{\Delta \text{pH}_{\text{carb}}}{\Delta \text{pH}_{\text{control}}} \]

where \( \beta_{\text{carb}} \) and \( \beta_{\text{control}} \) indicate the buffering power of the surface layer in the presence of carbachol and in control respectively. Moreover, Hamilton and Orlando (9) have shown that carbachol stimulation increases the basal rate of bicarbonate secretion in the opossum esophagus from 0.39 ± 0.03 to 0.74 ± 0.07 µeq·h⁻¹·cm⁻². Assuming that this increase results in a proportionate increase in bicarbonate concentration in the surface unstirred layer, on the basis of the Henderson-Hasselbach equation, this increase in secretory rate could account for an increase in pHs of ~0.27 pH units. Thus
our experiments document that the presence of submu-
ccosal glands can contribute to and modulate the protective
capacity of the preepithelial defense against lumi-
nal acid and that such modulation by cholinergic
agonists is clearly absent in esophagi devoid of such
glands. Moreover, because atropine could block the en-
hancement in the gradient produced by carbachol, it is
evident that cholinergic muscarinic receptors in the
tissue mediate the response to carbachol in vitro just as
they have been documented to do in vivo (15).

The effect of exposing the rabbit and opossum
esophagi to luminal pH 2.0 was also of interest. In the
absence of carbachol, luminal pH 2.0 resulted in a
similar pattern of acidification in the opossum as in the
rabbit esophagus, i.e., there was rapid equilibration of the
pHs to that of the luminal solution. When stimu-
lated by carbachol, however, the opossum esophagus
was able to reduce the initial rate of acidification but
not stop the ultimate decline in pH. Nonetheless, to-
gether, these data suggest that, under both basal and
stimulated conditions, the SMG-bearing esophagus
(opossum) has a limited capacity to resist acidification
as is the case in the rabbit. In humans, the capacity of the glandular secretion to maintain a
preepithelial (buffering) layer protects against luminal acidosis, and so the capacity for protection
against gastroesophageal reflux disease is

In summary, this study establishes that the presence of submucosal glands in the esophageal wall is an
important contributor to the preepithelial (buffering)
defense against luminal acid. This defense is generated in
large measure through the ability of these glands to
secrete bicarbonate, and so the capacity for protection
against luminal acidity varies and is dependent in part
on the degree of glandular secretion to protect the
epithelial surface against back-diffusing H+. However,
the capacity of the glandular secretion to maintain a
lumen-to-surface pH gradient is limited. In humans, whose
SMG-bearing esophagus is often exposed to
luminal pH ≤ 2.0, additional intrinsic (epithelium
proper) and extrinsic (acid clearance mechanisms)
esophageal defenses are necessary for protection against
gastroesophageal reflux disease.

This work was supported by National Institute of Diabetes and
Digestive and Kidney Diseases Grant DK-36013.

Received 24 June 1999; accepted in final form 6 October 1999.

REFERENCES
1. Allen, A., and A. Garner. Mucus and bicarbonate secretion in the
13. Krause, W. J., J. H. Cutts, and C. R. Leeson. The postnatal development of the alimentary canal in the opossum I. Oesopha-
19. Tobey, N. A., C. Caymaz-Bor, S. S. Hosseine, M. S. Awadaya, and R. C. Orlando. Circuit analysis of cell membrane and junctional resistances in healthy and acid-damaged rabbit esophaga-