HCO₃⁻ secretion in the esophageal submucosal glands

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Although potentially of clinical importance, there is little information about the cellular mechanisms by which esophageal SMG secrete HCO₃⁻. This is because of the technical difficulties of isolating the human esophagus from salivary and gastric contamination for the accurate collection and quantitation of SMG HCO₃⁻ and because of the limited availability of healthy esophageal tissue from esophagectomy specimens. However, the esophagi of some other mammalian species, e.g., pig, opossum, and dog, bear SMG (19), and this affords the opportunity for exploration of the cellular mechanisms of HCO₃⁻ secretion. In the present investigation, we utilized the isolated perfused pig esophagus to quantitate HCO₃⁻ secretion by the SMG in the presence and absence of various agonists and antagonists and supplemented this with immunohistochemical techniques to help define the receptor subtypes and cells of origin. The results show that SMG HCO₃⁻ secretion can be measured in vitro in the isolated perfused esophagus and that this secretion is stimulated by cholinergic agonists in part through activation of M₁ receptors located on both serous acinar cells and duct cells of the SMG. Furthermore, the secretion of HCO₃⁻ by these cells is dependent on the generation of HCO₃⁻ by the action of carbonic anhydrase (CA) II and is partially dependent on serosal membrane-bound extracellularly oriented CA. HCO₃⁻ secretion is inhibited by the removal of basolateral Cl⁻ and is DIDS sensitive. Two HCO₃⁻ transporters, Na⁺-(HCO₃⁻)ₐ (NBC) and Cl⁻/HCO₃⁻ (AE2), were identified in the duct and serous cells of the glands. Ducts and serous cells seem to play an important role in HCO₃⁻ secretion in the esophageal SMG.

METHODS

The Isolated Perfused Esophagus Preparation

Perfusion of isolated pig esophagus in vitro. Pig esophagi were obtained from the slaughterhouse. Immediately upon death of the animal, the tissue was isolated and placed in an ice-cold HEPES-Ringer solution and transferred to the laboratory. The muscularis externa was sharply dissected, leaving behind an intact cylindrical tube of mucosa and submucosa. The SMG are embedded in the submucosa and could easily be visualized under a stereoscope (×60). The esophageal tube was flushed with saline, cannulated at both ends, and mounted in a chamber where it was entirely submerged in...
physiological buffer, HCO$_3^-$-Ringer (solution 1 in Table 1) at 37°C, and bubbled with 95% O$_2$-5% CO$_2$ (Fig. 1). This design permits isolation and independent manipulation of both luminal and serosal perfusates. A cross section of esophageal tissue stained with hematoxylin and eosin shows the SMG (Fig. 2).

Measurement of HCO$_3^-$ secretion. The lumen was perfused with 100 ml of an unbuffered isotonic saline solution (150 mM NaCl) titrated to pH 7.4 with 0.01 N NaOH and continuously bubbled with CO$_2$-free N$_2$. The solution was recirculated in the esophageal tube with a peristaltic pump at 10 ml/min. Total alkaline secretion was recorded continuously by means of an automatic pH-stat system whose pH electrode and acid (0.01 N HCl) titrant are in contact with the luminal perfusate (20). Total alkaline secretion was calculated per unit of time from the amount (volume and concentration) of HCl titrant added to the luminal bath to maintain pH 7.4. Alkaline secretion was noted every 10 min and was averaged over the course of 45–60 min in control conditions and in the presence of the agonist or antagonists. All the agonists and antagonists were added to the serosal side of the esophageal tissue because luminal addition of the antagonist may not effectively deliver the agent to the acinar or duct cells. This is because of the fact that the glands are deeply embedded in the submucosa (Fig. 2) and because fluid movement out of the duct into the lumen, would limit the diffusion of antagonist to the apical serosal side of the esophageal tissue because luminal addition of the inhibitors. All the agonists and antagonists were added to the luminal perfusate (20). Total alkaline secretion was calculated per unit of time from the amount (volume and concentration) of HCl titrant added to the luminal bath to maintain pH 7.4. Alkaline secretion was noted every 10 min and was averaged over the course of 45–60 min in control conditions and in the presence of the agonist or the inhibitors. All the agonists and antagonists were added to the serosal side of the esophageal tissue because luminal addition of the antagonist may not effectively deliver the agent to the acinar or duct cells. This is because of the fact that the glands are deeply embedded in the submucosa (Fig. 2) and because fluid movement out of the duct into the lumen, would limit the diffusion of antagonist to the apical membranes of these cells. Esophageal alkaline secretion resulting from HCO$_3^-$ secretion was calculated using a modified method of Helm et al. (20, 21). Briefly, 100 ml of an isotonic saline solution (pH 7.4) were recirculated for ~1 h through the esophagus and were not titrated with the pH stat technique. Total alkaline secretion was determined by titrating the perfusate to pH 4.35, the pH at which 99% of HCO$_3^-$ are titrated, and subtracting the contribution of the saline vehicle. The contribution of the saline solution was measured as follows: the perfusate solution was bubbled with CO$_2$-free N$_2$ for 15 min to remove CO$_2$ formed by the reaction of HCO$_3^-$ with H$^+$. The pH of this solution was then raised to its original value by the addition of 0.01 N NaOH and again titrated with 0.01 N HCl to pH 4.35. This second titration, after subtracting the contribution of the saline vehicle treated similarly, yields the non-HCO$_3^-$ component of secretion. Alkaline secretion in the pig esophagus was found to be dominated (82 ± 6%, n = 3) by HCO$_3^-$ secretion.

Immunohistochemistry

Sections of esophageal tissue were stained by the immunoperoxidase technique for labeling with specific antibodies. Multiple tissues from at least four different animals were used for labeling with each antibody. The primary antibodies used in this study were the following: rabbit anti-CA II against human erythrocyte polyclonal antibody, rabbit anti-M$_1$ muscarinic ACh receptor affinity purified polyclonal antibody (Chemicon, Temecula, CA), rabbit anti-rat kidney NBC [rkNBC (38); generously provided by Dr. Walter Boron], and rabbit anti-SA6 antibody against the COOH-terminal of mouse AE2 amino acids 1224–1237 (generously provided by Dr. Seth Alper). The

Table 1. Composition of solutions

<table>
<thead>
<tr>
<th></th>
<th>Control HCO$_3^-$ (solution 1)</th>
<th>0 Cl$^-$ HCO$_3^-$ (solution 2)</th>
<th>HEPES Ringer (solution 3)</th>
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<tr>
<td>Na$^+$</td>
<td>140</td>
<td>140</td>
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<tr>
<td>K$^+$</td>
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<tr>
<td>Mg$^{2+}$</td>
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<td>132.3</td>
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<tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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Composition given in mM unless otherwise indicated.
sections were fixed overnight in 10% formalin, dehydrated, and embedded in paraffin blocks. Thin sections (5 μm) were cut and mounted on slides. Slides were baked overnight at 50°C. For staining with NBC antibody (38), additional specimens were fixed in periodate-lysine-paraformaldehyde (PLP) and were cryoprotected overnight in 30% sucrose in PBS and frozen in liquid nitrogen. Cryosections (5 μm thickness) were cut on a Reichert cryostat and mounted on gelatin-coated slides. The tissues were exposed to different concentrations of the primary antibody to determine the optimal concentration needed. For negative controls, the primary antibodies were reacted with the fusion protein for the individual antibody or were incubated without the primary antibody.

**Immunoperoxidase method.** Paraffin sections were dewaxed in xylene and hydrated in a series of graded alcohol solutions followed by PBS. Cryosections were hydrated in PBS. All sections were incubated in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase and then washed in PBS. For labeling with anti-AE2CT, sections were treated with 1% SDS in PBS to enhance the staining (11). Sections were then incubated with normal serum to block nonspecific binding. They were then incubated with the primary antibody followed by incubation with biotinylated secondary antibody against the IgG of the species providing the primary antibody. The sections were then treated with avidin-biotinylated enzyme complex (Vector Laboratories, Burlingame, CA). Peroxidase activity was detected using SIGMA FAST 3,3′-diaminobenzidine (Sigma, St. Louis, MO) as a substrate. Specimens were counterstained with hematoxylin, dehydrated, and mounted for study by light microscopy using a Zeiss Axiosplan 2 or an Olympus IMT2 microscope. Pictures were taken using a digital charge-coupled device camera or an Olympus camera using Kodak 100 ASA film.

**Solutions**

The composition of the Ringer solutions is given in Table 1. Benzolamide (BNZ) was provided by Dr. E. R. Swenson. DIDS and all other chemicals were obtained from Sigma. Inhibitors insoluble in aqueous solutions were dissolved in a small volume of DMSO and added to the solution. The concentrations used were based on the concentrations required to achieve maximal inhibition of the transporters or of CA, as reported previously in other preparations. The concentration of DMSO never exceeded 0.1% of the final solution.

**Statistical Analysis**

Data are presented as means ± SE. Data were analyzed using the two-tailed paired Student’s t-test unless otherwise indicated; n is the no. of experiments.

**RESULTS**

**Measurement of HCO₃⁻ Secretion**

Pig esophagi were perfused using a recirculated unbuffered luminal solution connected to a pH-stat system as described in METHODS. After being mounted and equilibrated, basal HCO₃⁻ secretion recorded in 19 esophagi averaged 0.21 ± 0.04 μeq·h⁻¹·cm⁻² with a range of 0.01 to 0.65 μeq·h⁻¹·cm⁻². When the cholinergic agonist carbachol, 10 μM, was added to the serosal bathing solution, it resulted in a doubling of the rate of HCO₃⁻ secretion over the course of 15–20 min from 0.14 ± 0.05 to 0.27 ± 0.04 μeq·h⁻¹·cm⁻² (n = 5, P < 0.01). Subsequent addition of atropine (10 μM), a general cholinergic antagonist, to the serosal bath abolished both basal and carbachol-stimulated HCO₃⁻ secretion (0.015 ± 0.01 μeq·h⁻¹·cm⁻², n = 5; Fig. 3).

**Identification of Cholinergic M₁ Receptors**

Because the stimulation of SMG secretion by carbachol and its complete inhibition by atropine indicated that cholinergic receptors were important in both basal and stimulated secretion, we sought to identify the receptor subtype by performing similar experiments using pirenzipine, a blocker of the cholinergic M₁ receptor subtype (12). Pirenzipine (0.1 mM), added serosally, decreased HCO₃⁻ secretion from a control value of 0.22 ± 0.04 μeq·h⁻¹·cm⁻² to a value of 0.15 ± 0.06 μeq·h⁻¹·cm⁻² (Fig. 4, n = 6, P < 0.05). Subsequent addition of 10 μM carbachol to the serosal bathing solution failed to increase HCO₃⁻ secretion, which remained at 0.09 ± 0.05 μeq·h⁻¹·cm⁻² (n = 6). These experiments suggest that most of the cholinergic receptors are of the M₁ subtype. Even though pirenzipine did not totally abolish basal HCO₃⁻ secretion like atropine, carbachol did not cause any increase in HCO₃⁻ secretion in the presence of pirenzipine. Confirmation and localization of the M₁ receptors in the SMG was obtained by immunohistochemistry using a rabbit anti-M₁ muscarinic ACh receptor affinity-purified polyclonal antibody. As shown in Fig. 5, both the serous demilunes and the ductal epithelium stained positive, indicating the presence of M₁ receptors on these structures. This suggests that these components of the SMG are important in the regulation of HCO₃⁻ secretion. When the primary antibody was omitted or reacted with the specific binding protein (1.5 μg/ml antibody to 5 μg/ml binding protein), tissue sections stained negative, indicating the specificity of the staining to the M₁ receptor protein.

**Identification of HCO₃⁻ Transport**

To define the nature of the membrane transporters involved in HCO₃⁻ secretion by SMG, we first established that HCO₃⁻ was responsible for the alkaline secretion detected in our system. This was done by serosal replacement of HCO₃⁻ Ringer with a HCO₃⁻-free HEPES-buffered Ringer (solution 3 in Table 1), a maneuver that gradually abolished alkaline secretion; mean value declined from 0.18 ± 0.05 to 0.006 ± 0.004 μeq·h⁻¹·cm⁻² (n = 4, P < 0.05). Subsequent addition of carbachol (10 μM) did not cause any increase in HCO₃⁻ secretion. This suggests that one or more HCO₃⁻ transporters are present in the SMG. Among the possibilities are the NBC
family of transporters (7) and AE (4). Because transporters belonging to both families are irreversibly inhibited by stilbene derivatives, we added 0.1 mM DIDS to the serosal solution while measuring basal HCO₃⁻ secretion. DIDS inhibited HCO₃⁻ secretion from a control value of 0.22 ± 0.04 to 0.09 ± 0.04 μeq·h⁻¹·cm⁻² (n = 4, P < 0.02). Furthermore, when 10 μM carbachol was added serosally to the preparation, there was no increase in HCO₃⁻ secretion. (Fig. 6). These data suggest the presence of DIDS-sensitive membrane transporter(s) involved in the secretion of HCO₃⁻ from SMG.

The data obtained with HCO₃⁻-free Ringer and with DIDS support the possibility that either NBC, Cl⁻/HCO₃⁻ exchanger, or both are involved in HCO₃⁻ secretion. To investigate the presence of these transporters in the SMG, we immunostained the esophageal tissue for NBC using a rabbit polyclonal antibody to rkNBC (KIA immune serum), which recognizes the COOH-terminal portion (last 46 residues) of rkNBC, rat pancreas NBC, and rat brain NBC (36, 38). We also immunostained the tissue for AE2 using a rabbit polyclonal column affinity purified anti-SA6 antibody against the COOH-terminal of mouse AE2 amino acids 1224–1237 (5).

NBC. Immunoperoxidase staining with anti-NBC was done in both PLP fixed cryosections and in sections from formalin-fixed paraffin-embedded tissues. The results from the immunostaining with anti-NBC in paraffin sections are shown in Fig. 7 and were not noticeably different from those obtained in the cryosections. There was a strong labeling of the serous demilunes (Fig. 7A) and of the intralobular (Fig. 7B) and interlobular ducts, and this staining looked more pronounced on the luminal side of the cells. There was very faint labeling in the mucous acinar cells. When the primary antibody was omitted or reacted with the fusion protein (maltose-binding fusion protein MBP-K1A, at 15 μg/ml) before its application to the tissue, the labeling was negative (Fig. 7C). These data suggest that an NBC transporter is present in the serous and in the duct cells of SMG and support the functional data above that NBC is likely to play an important role in HCO₃⁻ secretion in pig esophagus.

AE2CT. Immunoperoxidase staining with anti-SA6 (AE2 antibody) was done in sections from formalin-fixed paraffin-embedded tissues. The tissues were pretreated with 1% SDS as described in Methods. Inter- and intralobular ducts stained strongly positive to anti-SA6 antibody (Fig. 8A, A and B, respectively). The staining of the serous cells to anti-SA6 antibody was faint, whereas the acinar mucous cells did not stain at all (Fig. 8B). When the antibody was reacted with the SA6 (AE2CT) fusion protein, the staining was negative (Fig. 8C). Competition of the antibody with SA35 (AE2 NT) unrelated in sequence to SA6 yielded positive staining and was used as a positive control in competition experiments.

Role of Serosal Cl⁻

The presence of Cl⁻/HCO₃⁻, revealed by immunohistochemistry, indicates a possible role for Cl⁻ in HCO₃⁻ secretion. To investigate the role of serosal Cl⁻ in HCO₃⁻ secretion, we deleted Cl⁻ from the serosal bathing solution (Cl⁻ was replaced with cyclamate, solution 2 in Table 1). In six tissues, removal of Cl⁻ caused HCO₃⁻ secretion to decline from 0.10 ± 0.01 to 0.02 ± 0.01 μeq·h⁻¹·cm⁻² (n = 6, P < 0.05). Figure 9 shows a plot of the changes in HCO₃⁻ secretion upon removal of serosal Cl⁻. It is to be noted that, upon removal of Cl⁻, there was an initial slight and transient increase in HCO₃⁻ secretion followed by a sharp decrease in secretion. The transient increase in HCO₃⁻ secretion is consistent with the presence of a Cl⁻/HCO₃⁻ exchanger on the basolateral side of the cell, which is initially reversed by the removal of serosal Cl⁻. This leads to the accumulation of HCO₃⁻ in the cell, which can transiently increase secretion. Prolonged exposure to a Cl⁻-free solution (~80 min) eventually leads to the inhibition of HCO₃⁻ secre-

Fig. 4. Effect of pirenzipine, an antagonist of M₁ muscarinic subtype receptor, on esophageal HCO₃⁻ secretion. Pirenzipine inhibited basal HCO₃⁻ secretion by ~50% (n = 6, P < 0.05) and also inhibited the stimulation by carbachol (10 μM).

Fig. 5. Photomicrographs showing immunolocalization of the M₁ muscarinic receptor using the immunoperoxidase technique in the serous cells or demilunes (A, arrow) and in the ducts (B, arrow). m, Mucous acinus where the labeling was negative. Rabbit anti-M₁ muscarinic ACh receptor affinity purified polyclonal antibody was used on formalin-fixed paraffin-embedded tissue sections. The incubating medium of the primary antibody contained 0.05% Tween and 0.3% Triton. The antibody was used at a dilution of 1.5 μg/ml.
transport across the cell membrane. It has been reported that the cytoplasmic COOH-terminal domain of Cl_-/HCO_3^- cotransporter (NBC) and Cl^-/HCO_3^- exchange (AE2), on esophageal HCO_3^- secretion. The addition of DIDS (0.1 mM) to the serosal bath inhibited basal HCO_3^- secretion and the increase in HCO_3^- secretion observed with the addition of carbachol (10 μM; n = 4, P < 0.02).

Fig. 6. Effect of DIDS, an inhibitor of HCO_3^- transport mechanisms, including Na^+-HCO_3^- cotransporter (NBC) and Cl^-/HCO_3^- exchange (AE2), on esophageal HCO_3^- secretion. The addition of DIDS (0.1 mM) to the serosal bath inhibited basal HCO_3^- secretion and the increase in HCO_3^- secretion observed with the addition of carbachol (10 μM; n = 4, P < 0.02).

Role and Identification of CA

CA is an enzyme that catalyzes the reversible reaction CO_2 + H_2O ↔ HCO_3^- + H^+ and can therefore generate HCO_3^- for

Fig. 7. Photomicrographs showing immunoperoxidase localization of NBC using a rabbit polyclonal antibody to rat kidney NBC (rkNBC, K1A immune serum), which recognizes the COOH-terminal portion (last 46 residues) of rkNBC, rat pancreas NBC, and rat brain NBC. NBC immunostaining was strong in the serous cells or demilunes (A, arrow) and in the ducts (B, arrow) and faint in the mucous cells (m in A and B). This experiment was performed on sections from formalin-fixed paraffin-embedded tissues. Tween (0.1%) was added to the incubating medium of the primary antibody. When the primary antibody was omitted or reacted with the fusion protein (maltose-binding fusion protein MBP-K1A, at 15 μg/ml) before its application to the tissue, the labeling was negative (C). Magnification ×150.
zolamide (MTZ; see Ref. 24), a set of four tissues was exposed to MTZ. Basal secretion was 0.22 ± 0.06 μeq·h⁻¹·cm⁻². Two tissues were exposed to 1 mM MTZ and two other tissues to 0.2 mM MTZ. In both sets of tissues, HCO₃⁻ secretion stopped totally in 20 min and subsequent carbachol addition did not cause any additional HCO₃⁻ secretion.

Cytosolic CA II, a cytoplasmic variety of the enzyme, has been previously identified in human esophageal SMG (14), in salivary glands of different mammalian species (6, 30, 31). We therefore examined its presence in the SMG tissue of pig esophagus by immunolabeling with an antibody to CA II (rabbit anti-CA II, human erythrocyte polyclonal antibody). The staining was done in formalin-fixed paraffin-embedded tissue sections. Serous demilunes (Fig. 11A), interlobular ducts, and intralobular ducts (Fig. 11B) stained intensely positive for CA II, whereas the mucous cells stained negative. Figure 11C shows a control section where only the primary antibody was omitted from the experiment.

DISCUSSION

The present study examined the cellular mechanisms of HCO₃⁻ secretion by esophageal SMG. For this purpose, experiments were conducted on the isolated, perfused pig esophagus, an organ that serves as a good model for human esophagus in many aspects (13). Like the human esophagus, the pig esophagus bears SMG and is capable of secreting significant amounts of alkali. These SMG are seromucous glands containing mucous acini with serous demilunes (18, 22). In addition, this preparation has several distinct advantages: 1) it allows accurate measurements of luminal secretion of alkali, 2) it allows independent manipulations of luminal and basolateral solutions in the intact esophagus, and 3) alkali secretion can be assessed independently of the influence of the central nervous system.

Fig. 8. Photomicrographs showing immunolocalization of AE2 using the immunoperoxidase technique and a rabbit polyclonal column affinity purified anti-SA6 antibody against the COOH-terminal of mouse AE2 amino acids 1224–1237. The tissues were pre-treated with 1% SDS as described in METHODS. The interlobular (arrow in A) and intralobular (arrow in B) ducts stained strongly positive to the antibody. The serous cells stained very weakly, whereas staining in acinar mucous cells ("m" in B) was absent. When the antibody was reacted with the SA6 (AE2CT) fusion protein, the staining was negative (C). Magnification ×150.

Fig. 9. Effect of removal of Cl⁻ from the serosal bath on HCO₃⁻ secretion. Upon Cl⁻ removal, there was a small transient increase followed by a sharp decrease in HCO₃⁻ secretion. Values are means ± SE from 6 tissues.

Fig. 10. Effect of the carbonic anhydrase (CA) inhibitor ethoxzolamide (ETXZ) on esophageal HCO₃⁻ secretion. The addition of ETXZ (0.2 mM) to the serosal bath inhibited basal HCO₃⁻ secretion and the increase in HCO₃⁻ secretion observed with carbachol (10 μM); n = 3, P < 0.05.
Our data indicate that there is a significant basal (nonstimulated) secretion of alkali by the SMG-bearing esophagus. The basal alkali secretion averaged $0.21 \pm 0.04 \mu$eq h$^{-1}$ cm$^{-2}$, which was readily doubled by the addition of the cholinergic agonist, carbachol, to the serosal bathing solution. Alkali secretion in the esophagus is a product of the SMG, since the squamous epithelium does not secrete alkali (1, 20). Moreover, our data established that the major component of this alkali secretion is HCO$_3^-$.

To further investigate the pathway of alkali secretion, we documented that SMG HCO$_3^-$ secretion was not only markedly stimulated by serosal carbachol but abolished by the addition of the cholinergic antagonist atropine. The inhibition of basal HCO$_3^-$ secretion by atropine suggests that SMG secretion is HCO$_3^-$-dependent. This was confirmed by the following results. 1) Alkali secretion was dominated by HCO$_3^-$ secretion (82 ± 6%). 2) Alkali secretion, basal and stimulated by carbachol, was abolished by the removal of HCO$_3^-$ from the serosal bath. 3) Alkali secretion, basal and stimulated, was inhibited by DIDS, a known inhibitor of HCO$_3^-$ transport.

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The mechanisms by which the esophageal SMG secrete HCO$_3^-$ are unknown. One possible model is that secretion in the SMG, like in the similarly structured salivary glands, occurs in two stages (25, 42, 46). The acini secrete a fluid isotonic to plasma, which is subsequently modified in the duct system by reabsorption of Na$^+$ and Cl$^-$ and secretion of K$^+$ and HCO$_3^-$.

Inhibition of basal and carbachol-stimulated secretion by DIDS indicates a major role of HCO$_3^-$ transporters in the generation of secreted HCO$_3^-$

This was confirmed by the immunolocalization of NBC using an antibody that recognizes the COOH-terminal portion (last 46 residues) of rKNBC, rat pancreas NBC, and rat-brain NBC (38). This transporter was localized to the duct cells (intra- and interlobular) and to the serous demilunes. These experiments indicate the presence of at least one isoform of NBC in the esophageal SMG. Recent studies on the rat salivary glands have demonstrated the presence of NBC on the basolateral side of acinar and duct cells of the parotid gland. In the submandibular glands, NBC is present on the basolateral and in some instances apical side of the duct cells (37). Further experiments are needed to determine the...
homology between the esophageal isoform and the kidney, pancreas, or brain isoforms and to determine the apical or basolateral location of this transporter.

Another HCO₃⁻ transport mechanism known to play an important role in pH regulation and HCO₃⁻ transport in a variety of cells is AE2. This exchanger is present in the salivary glands (26, 44). Several isoforms of this exchanger have been identified (2). AE2 is the most ubiquitously expressed form of the transporter and in most epithelial cells is located on the basolateral side of the cell (3). Because the electrochemical gradient of the cell usually favors Cl⁻ entry in the cell, this exchanger allows the influx of Cl⁻ and the efflux of HCO₃⁻. Our experiments indicate that AE2 is strongly expressed in the cell membranes of interlobular and intralobular ducts. On the other hand, the serous demilunes stained faintly for AE2, whereas staining in the mucous cells was negative.

Some of the issues to be addressed are the relative contribution of NBC and AE2 to HCO₃⁻ secretion and whether they play a direct role in this process. The main questions in this respect are whether these transporters are located at the basolateral membrane or the luminal membrane and the specific stoichiometry of NBC. Considering NBC, the presence of the transporter on the luminal membrane in a 3:1 configuration (HCO₃⁻:Na⁺) leads to HCO₃⁻ efflux and therefore a direct role in HCO₃⁻ secretion in the duct. If NBC is localized to the basolateral membrane, then only a 2:1 isoform (or possibly the electroneutral form of NBC) could lead to HCO₃⁻ influx in the cell and ultimately secretion of HCO₃⁻. Alternatively, a basolateral 3:1 configuration of NBC favors HCO₃⁻ efflux, which does not point to a direct role in HCO₃⁻ secretion via this transporter. As for AE2, most studies indicate its presence at the basolateral membrane, where it transports HCO₃⁻ out of the cell. As such, AE2 does not directly secrete HCO₃⁻ in the duct, and its likely role in these cells would be in regulation of intracellular pH. Further functional and molecular studies are needed to elucidate these mechanisms.

CA II and IV (cytosolic and membrane bound, respectively) play a role in the regulation of cellular HCO₃⁻ and recently have been shown to bind to HCO₃⁻ transporters like AE2 and kNBC to form a transport metabolon that enhances the transporter activity by providing it with HCO₃⁻ (34, 35, 40, 41, 45). The inhibition of HCO₃⁻ secretion by ETXZ and MTZ, two permeable CA inhibitors, indicates a major role of CA in generating HCO₃⁻ in the esophageal SMG. However, the effect of the membrane-permeable CA inhibitors is not restricted to the cytosol but results in the inhibition of both cytosolic and extracellular CA. The role of extracellular CA was determined by the use of BNZ, a membrane-impermeable CA inhibitor that only partially inhibited basal HCO₃⁻ secretion but abolished the effect of carbachol. The very high permeability of MTZ corresponded to a complete inhibition of HCO₃⁻ secretion. ETXZ inhibited HCO₃⁻ secretion by 76%, whereas the less permeable BNZ only inhibited HCO₃⁻ secretion by 29%. It can be concluded from our experiments that basal and carbachol-stimulated HCO₃⁻ secretion are dependent on extracellular CA. Extracellular CA, possibly CA IV, which is present in several epithelial cells of the alimentary tract including the esophagus (15, 33), has a limited role in basal secretion but seems to be implicated in carbachol-stimulated secretion. The role of basolateral membrane-bound CA could be in generating HCO₃⁻ in the vicinity of a putative HCO₃⁻ transporter (NBC) bringing HCO₃⁻ from the serosal side to the cell.

The removal of serosal Cl⁻ caused an initial slight and transient increase in HCO₃⁻ secretion, consistent with the presence of Cl⁻/HCO₃⁻ exchange on the basolateral side of the cell. This was followed by a sharp decrease and almost total inhibition of secretion, indicating a major role of Cl⁻ in generating HCO₃⁻ secretion or in the maintenance of ion gradients necessary for that secretion. The role of luminal Cl⁻ transport is more difficult to address and needs to be further elucidated. In our experiments, removal of luminal Cl⁻ would only deplete Cl⁻ from the esophageal lumen and will not guarantee its removal from the lumens of the glands. This is because the glands are embedded in the submucosa and are several hundred (~1,600) micrometers away from the luminal surface of the esophagus (Fig. 2).

The presence of M₁ receptors, NBC, and CA in the same cells (of serous demilunes and in the ducts) indicates that secretion of HCO₃⁻ in the SMG occurs in these cells. This likely involves a cascade of events involving the activation of M₁ receptor, the generation of HCO₃⁻ in the cell by CA, and the transport of HCO₃⁻ in and/or out of the cell by an NBC transporter.

In conclusion, this study established that alkali secretion by esophageal SMG is mostly the result of HCO₃⁻ generation and transport. HCO₃⁻ secretion is stimulated by cholinergic agonists and mediated by M₁ receptors. The generation of HCO₃⁻ by CA is an important step in this process. DIDS-sensitive HCO₃⁻ transporter(s) NBC and/or AE2 contribute to HCO₃⁻ transport, which is also inhibited by the removal of serosal Cl⁻. The ducts and serous cells of the glands seem to play an important role in HCO₃⁻ transport and secretion.

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