ION TRANSPORT MECHANISMS LINKED TO BICARBONATE SECRETION IN THE
ESOPHAGEAL SUBMUCOSAL GLANDS

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ABSTRACT

The esophageal submucosal glands (SMG) secrete HCO$_3^-$ and mucus into the esophageal lumen, where they contribute to acid clearance and epithelial protection. This study characterized the ion transport mechanisms linked to HCO$_3^-$ secretion in SMG. We localized ion transporters using immunofluorescence and we examined their expression by RT-PCR and in situ hybridization. We measured HCO$_3^-$ secretion using pH-stat and the isolated perfused esophagus. Using double-labeling with Na,K-ATPase as a marker, we localized Na-coupled bicarbonate transporter (NBCe1) and Cl$^-$/HCO$_3^-$ exchanger (SLC4A2/AE2) to the basolateral membrane of duct cells. Expression of cystic fibrosis transmembrane regulator channels (CFTR) was confirmed by immunofluorescence, RT-PCR and in situ hybridization. We identified anion exchanger SLC26A6 at the ducts’ luminal membrane and Na-K-2Cl (NKCC1) at the basolateral membrane of mucous and duct cells. pH-stat experiments showed that elevations in cAMP induced by forskolin or 3-isobutyl-1-methylxanthine (IBMX) increased HCO$_3^-$ secretion. Genistein, an activator of CFTR, which does not increase intracellular cAMP, also stimulated HCO$_3^-$ secretion, whereas glibenclamide, a Cl$^-$ channel blocker, and bumetanide, a Na-K-2Cl blocker, decreased it. CFTR$_{inh}$-172, a specific CFTR channel blocker, inhibited basal HCO$_3^-$ secretion as well as stimulation of HCO$_3^-$ secretion by IBMX. This is the first report on the presence of CFTR channels in the esophagus. The role of CFTR in manifestations of esophageal disease in cystic fibrosis patients remains to be determined.

Running Title: HCO$_3^-$ secretion and Cl$^-$ transport in esophageal submucosal glands

Keywords: pH stat, CFTR, Na-coupled bicarbonate transporter, Cl$^-$/HCO$_3^-$, Na-K-2Cl, SLC26A6, cAMP.
INTRODUCTION

In humans, esophageal submucosal glands (SMG) secrete HCO_3^- (and mucin) in amounts sufficient to neutralize residual volumes of acid left in the esophagus after bolus clearance. This HCO_3^- secretion can approach the HCO_3^- output of salivary glands at rest (38). Unlike the extensive studies of secretions by salivary glands, stomach or the duodenum, little is known about the mechanisms of SMG secretions in the esophagus. This is mainly due to the technical difficulties of isolating secretions of the human esophagus from salivary and gastric contamination and the limited availability of healthy esophageal tissue from esophagectomy specimens. We resolved this latter issue by using tissues from specific mammalian species, such as pig and opossum, whose esophagi bear SMG.

Using pH microelectrodes, we previously demonstrated a lumen-to-surface pH gradient in SMG-containing esophageal sections. Surface pH increased upon stimulation with carbachol, a cholinergic agonist (1). These data suggest that the lumen-to-surface pH gradient is a dynamic process that can be enhanced under conditions when vagal (cholinergic) activity is high. In another study we demonstrated that alkali secretion by esophageal SMG is mostly due to HCO_3^- generation and transport. HCO_3^- secretion is stimulated by cholinergic agonists and mediated by muscarinic M_1 receptors. Secretion of HCO_3^- is DIDS-sensitive, is inhibited by the removal of serosal Cl^- and is blocked by carbonic anhydrase inhibitors (2).

The esophageal SMG consist of a mix of cell types, mostly mucin-producing cells (3; 5; 27), with some demi-lunes or caps of serous cells, arranged in acinar formation. The SMG secrete their HCO_3^- -rich fluids in a manner that is likely similar to secretory mechanisms of other exocrine glands such as pancreas and salivary glands. A number of models exist for how these digestive exocrine glands secrete their HCO_3^- -rich fluids. The secretory products are transported through a system of ducts into the organ’s lumen. In these glands, secretion is likely produced in two stages: the first step is the formation of an ion-rich acinar fluid derived from blood and the second step is the enrichment of this fluid with HCO_3^- (39).

In this study we aimed to identify ion transport mechanisms involved in HCO_3^- secretion and determine their cellular localization in SMG. We investigated the presence of CFTR, its involvement in the secretion process and the effect of cAMP on HCO_3^- secretion in this tissue. We used pig esophagus, which presents several similarities with the human esophagus including
the presence of SMG (19). Our data provide functional and/or immunohistochemical evidence for the presence of sodium-coupled bicarbonate transporter, NBCe1 (member of SLC4 family), chloride-bicarbonate exchanger (Cl-HCO$_3^-$, SLC4A2/AE2), Na-K-2Cl (NKCC1), cystic fibrosis transmembrane regulator, CFTR (ABCC7) and the anion exchanger SLC26A6 (solute carrier family 26, member 6) in this tissue. The presence and role of CFTR in esophageal SMG may explain in part the high incidence of gastroesophageal reflux disease (GERD) in cystic fibrosis (CF) patients. (21; 25; 40)
METHODS

1. The isolated esophagus preparation

a) Perfusion of isolated pig esophagus in vitro: Pig esophagi obtained from the slaughterhouse were transferred to the laboratory in ice-cold HEPES Ringer and dissected for cannulation and perfusion as described previously. (2).

b) Measurement of HCO$_3^-$ secretion: HCO$_3^-$ secretion was measured using the pH stat technique as described previously (2). The lumen was perfused with 100 ml of recirculated unbuffered isotonic saline solution (150 mM NaCl, pH adjusted to 7.4 with 0.01N NaOH and continuously bubbled with CO$_2$-free N$_2$). Bicarbonate secretion, which constitutes most of the alkaline secretion in this tissue (2), was calculated per unit time from the amount of HCl titrant added to the luminal bath to maintain pH 7.4.

2. Immunofluorescence (IF)

Tissues from at least 4 different animals were used for labeling with each antibody. Tissues were placed in O.C.T. compound (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen. Cryosections (5μ thickness) were cut on a Reichert cryostat and mounted on gelatin coated slides. Sections were fixed for 10 minutes in 4% methanol-free formaldehyde and rehydrated in PBS immediately prior to staining. Sections were pretreated with 1% sodium dodecyl sulfate (SDS) for 5 minutes in PBS to enhance the staining; they were then washed and blocked with serum. The primary and secondary antibodies used in this study are listed in Table 1. Sections were counterstained with the nuclear marker 4’,6-diamino-2-phenylindole, dihydrochloride (DAPI) and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Different concentrations of the primary antibody were used to determine the optimal concentration. For negative controls, primary antibodies were reacted with their respective fusion protein prior to staining or, alternatively, sections were incubated without the primary antibody. Micrographs were obtained using a Nikon Eclipse 80i microscope and a Spot RT digital camera or a Leica DMIRE2 confocal microscope.

3: Total RNA isolation, reverse transcription and amplification of mRNA:
Porcine SMG were resected under a magnification of 60x from the cephalic and middle area of the esophagus where the glands abound and were frozen in liquid nitrogen. Frozen tissues were homogenized and total RNA was isolated using the Ultraspec-II RNA Isolation System (Biotecx, Houston, TX) according to manufacturer’s instructions. Detection of expected 18S and 28S ribosomal RNA on a denaturing agarose gel was used to determine the quality of purified total RNA, and a porcine β-2-microglobulin mRNA specific RT-PCR was performed (not shown). Primers were obtained from Integrated DNA Technologies (Coralville, IA). The primers for CFTR were: 5’– GAG GAC AGT TGT TGG CAG TT –3’ and 5’– TTG GCA CGC TTT GAT GAC –3’, for SLC26A6: 5’– GGA GAG CAC CGG GGG CAA CAC A –3’ and 5’–TGA GGC GGT CCA CAT CCA CAC –3’ and for NBCe1-B: 5’-GAA TGG GGA CAC GCC CCA CG -3’ and 5’-AGC TGG CAT CGG TGG CAA CC -3’. Reverse transcription and amplification of target sequences was performed using SuperScript III One-Step RT-PCR System for end point detection (Invitrogen, Carlsbad, CA) according to manufacturer’s instruction. RT-PCR products were resolved by electrophoresis using a 1.5% agarose gel containing 10 µg/mL ethidium bromide. The approximate size of each product was determined by comparison to a DNA ladder (Invitrogen). PCR products were purified and sequenced using an ABI Prism 3130xl sequencer to confirm gene identity (Tulane Medical Center Core Facility)

4. In Situ Hybridization (ISH)

Synthetic oligonucleotides (~36 bp) labeled with fluorescein, corresponding to the tandem repeat sequences of CFTR (sus scrofa NM_001104950.1) were used. The anti-sense sequence was 5’– AAG TGA CGC TGC TGA TGG GGC TGC TGT GGG AGT TGT–3’. The sense sequence was used as a negative control and a fluorescein tagged poly-dT oligonucleotide was used as a positive control. Tissues were fixed in 10% phosphate buffered formalin and embedded in paraffin. Sections (10µM) were dried overnight at 56°C, deparaffinized, rehydrated in a series of alcohols and treated with RNAase inhibitor (Protect RNA, Sigma, St Louis). Proteinase K digestion (7µg/mL in 0.02 M Tris HCl, pH 7.5) was performed for 15 min at 37 °C. Samples were fixed with 4% paraformaldehyde (PFA) for 15 min and treated with 0.1 M triethanolamine, pH 8, and 0.5% acetic anhydride for 10 min. After pre-hybridization in 4xSSC (standard saline citrate) buffer, sections were hybridized overnight at 65°C with fluorescein labelled oligonucleotides (200 ng/mL) diluted in 4xSSC, 10% dextran sulfate, 2x Denhardt’s, 50%
formamide, and tRNA (250 μg/mL) (Poly-dT slides were hybridized at room temperature). Post-
hybridization washes were performed at 37°C (poly-dT slides were washed at room temperature)
stepwise from 4xSSC to a final wash with 0.1xSSC. Sections were then blocked using ISH
blocking solution (Vector Laboratories) and stained with alkaline phosphatase anti-fluorescein
antibody (Vector). Alkaline phosphatase was developed using 5-bromo-4-chloro-3-
indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) in 100 mM Tris–HCl, pH 9.5 with Levamisole
added to block endogenous alkaline phosphatase (Vector) pH 9.5. Slides were counterstained
with Nuclear Fast Red and mounted in Vectamount.

5. Solutions
The composition of Ringer’s solutions is in mM: Na⁺, 140; Cl⁻, 119.8; K⁺, 5.2; HCO₃⁻, 25; Ca²⁺,
1.2; Mg²⁺, 1.2; HPO₄²⁻, 2.4; H₂PO₄⁻, 0.4; glucose, 10, (osmolality 290 mOsm/kg H₂O, pH 7.4
when gassed with 95% O₂ – 5% CO₂ at 37ºC). Chemicals were obtained from Sigma (St. Louis,
MO). CFTRinh-172, 3-isobutyl-1-methylxanthine (IBMX), forskolin, bumetanide, genistein, or
glibenclamide were dissolved in a small volume of dimethyl sulfoxide and added to the solution.
The concentration of dimethyl sulfoxide never exceeded 0.2 % of the final solution. To test
whether DMSO had any effect on the results, HCO₃⁻ secretion was measured in three different
tissues, (0.12±0.04 μEq/hour.cm²), the addition of DMSO at a concentration of 0.2% did not
alter basal HCO₃⁻ secretion which stayed at 0.13±0.03 μEq/hour.cm² (p>0.3).

6. Statistical Analysis
Data are presented as mean ± SEM. Data were analyzed using two-tailed paired Student t-test
unless otherwise indicated. n is the number of experiments.
RESULTS

**Immunolocalization of NBCe1 and Na,K-ATPase**
We double-labeled cryosections of esophageal tissue with NBCe1 (rat kidney NBC, rkNBC) antibody, and an antibody to the α subunit of the Na,K-ATPase, an enzyme located at the basolateral membrane of the majority of epithelial cells. The antibody to NBC we used recognizes the C-terminal portion (last 46 residues) of several NBC isoforms including rat kidney, and pancreas NBC. We used two different fluorescent secondary antibodies to study the co-localization of the two transporters in the same tissue.

The interlobular duct cells of SMG stained intensely with the antibody to Na,K-ATPase (red), and the staining clearly delineated the basolateral membrane (Fig 1A). Similarly, the staining with the NBCe1 antibody (green) was prominent on the basolateral cell membrane of the duct cells (Fig 1B). Figure 1D shows the overlap between the two antibodies as well as the nuclei counterstained blue with DAPI (Fig 1C). Similarly, the intralobular duct cells showed an identical pattern of staining when the same antibodies and nuclear stain were used (data not shown).

In the serous cells (s) or demi-lunes, the staining to Na,K-ATPase (Fig 2A-red) and NBC (Fig 2B-green) was intense without showing any polar distribution (Figs 2A & 2B). Fig 2C shows the nuclei, counterstained blue with DAPI. The overlap between the staining with the antibodies to Na,K-ATPase and NBCe1 was clearly evident in the serous cells (yellow) when figures 2A, 2B & 2C were merged (Fig 2D). In mucous (acinar) cells (m), the staining with Na,K-ATPase and NBCe1 antibodies was positive and clearly delineated the basolateral membrane (Figures 2A & 2B). In all sections when the primary antibody was omitted or reacted with the fusion protein (MBP-K1A at 15 μg/ml) prior to its application to the tissue, the labeling was abolished (Fig 1S supplementary material)

**Identification of NBC by RT-PCR**
We further confirmed the presence of NBC by RT-PCR. Using coding sequence primers for NBCe1-B, RT-PCR amplification of mRNA extracted from SMG tissue showed a positive band at the expected product size of ~479 bp as shown in Fig 2E (lane 2). The sequence of the PCR
product is shown in Supplementary Material. A BLAST of this sequence showed 99% homology to SLC4A4 (sus scrofa, NM_001030533.1) (NBCe1-B).(16)

**Immunolocalization of AE2 and Na,K-ATPase**

To localize the Cl⁻-HCO₃⁻ transporter (SLC4A2/AE2) in SMG cells, we performed double labeling immunofluorescence experiments with an antibody to AE2 (carboxy-terminus CT) and an antibody to the α subunit of Na,K-ATPase. In the intralobular duct cells, the staining to Na,K-ATPase was intensely positive (red) in the basolateral membrane (Fig 3A). The staining with AE2CT antibody was also positive in the basolateral cell membrane (green, Fig 3B). The tissues were counterstained with DAPI (Fig 3C). There was complete overlap between the staining with AE2CT and Na,K-ATPase, evident as yellow stain in the merged Fig 3D. The staining with Na,K-ATPase and AE2CT antibodies showed a similar distribution in interlobular duct cells. Mucous and serous cells of the acini stained strongly positive to Na,K-ATPase antibody, while the staining to AE2CT antibody was positive in serous cells and faint in mucous cells (data not shown).

To check the specificity of the labeling to AE2, we performed an experiment in which the antibody to AE2CT was reacted with the fusion protein (SA6) and applied to the tissue. Reacting with the fusion protein (SA6) completely abolished the staining for (Supplementary data, Fig 2S).

**Immunolocalization of CFTR**

We labeled cryosections of esophageal tissue with a panel of four different antibodies to CFTR as listed in Table 1. All antibodies showed positive staining in the acini, interlobular and intralobular ducts of the SMG. The labeling with CFTR antibody raised against the C-terminus of the protein (peptide corresponding to amino acids 1468-1480 of human CFTR,), gave the best results and is shown in Fig. 4. Figure 4A showed intense labeling of Na,K-ATPase (red) in mucous (acinar) cells and duct cells as observed earlier. In mucous cells, (Fig. 4B) the staining for CFTR was positive (green) but did not localize to a specific cell membrane. However, CFTR staining was more defined in the cell membranes of duct cells (Fig 4B). Co-localization with Na,K-ATPase (merged picture, Fig 4D) clearly indicates that in the duct cells immediately adjacent to the lumen the staining was mostly present on the luminal membrane (labeled “l” in
Fig 4D). In the basal layers of duct cells there was some co-localization with Na,K-ATPase staining (yellow, Fig 4D). Figure 4C shows nuclear staining with DAPI. In experiments where the primary antibody was reacted with the fusion protein no staining to CFTR was observed (negative control, Fig 3S supplementary data). Labeling with another CFTR antibody raised against the N-terminus (amino acids 24-35, human clone MM13-4) showed a similar distribution and is shown in Fig 4S (supplementary data). The labeling with an antibody against the N-terminus of human CFTR (N-20) and another one against human N-terminus (amino acid 1-182) was also positive but the intensity was less pronounced (data not shown).

**CFTR mRNA detection by in-situ hybridization and RT-PCR**

In situ hybridization (ISH) was performed using fluorescein tagged oligonucleotides probes. Positive ISH signals (blue stain) were observed in the sections incubated with the anti-sense nucleotide probe to mRNA of CFTR, as shown in Figure 5A. The sections incubated with the sense probe (Fig 5B) or without any probe (Fig 5D) did not show ISH signals. The sections incubated with poly-dT, as a positive probe for RNA, showed strong positive staining (Fig 5C). To further determine the presence of mRNA coding for CFTR we performed RT-PCR on glandular tissues.

Using gene specific primers, RT-PCR amplification of mRNA extracted from dissected SMG tissue showed a positive band at the expected product size of ~229 bp (Fig 5E lane 1). Lane 2 showed a similar band with RNA extracted from trachea and used as a positive control. The sequence of the PCR product obtained from esophageal SMG as described is shown in Supplementary Material. A BLAST of this sequence shows 95% homology to Sus scrofa CFTR (NM_0011049950.1, ATP binding cassette subfamily member 7).

**Role of CFTR in HCO₃⁻ secretion**

To study the role of CFTR in SMG secretion, we measured esophageal HCO₃⁻ secretion in response to different agents that affect the activity of CFTR channels. HCO₃⁻ secretion was measured in the isolated perfused esophagus using a recirculated unbuffered luminal solution connected to a pH-stat system. Following mounting and equilibration, basal (unstimulated) HCO₃⁻ secretion was recorded every 10 minutes and was averaged over the course of 60 minutes. Basal HCO₃⁻ secretion from 30 tissues averaged 0.10±0.016 μEq/hour.cm². Specific agents
and/or agonists were then added to the serosal bath, and the measurements were repeated and averaged over the next 60 minutes so that each tissue served as its own control. HCO$_3^-$ secretion in the presence of agonists or inhibitors was normalized (%) to the basal secretion for that condition expressed as 100%.

**Effect of stimulation of CFTR:** We used IBMX (100µM), a phosphodiesterase inhibitor which increases cAMP levels in the cell and is a known activator of CFTR. As shown in Fig 6A, the addition of IBMX to the serosal bath of the isolated perfused esophagus increased HCO$_3^-$ secretion by 57±7% (n=6, p<0.01).

We also investigated the effect of *forskolin*, an adenylyl cyclase activator and another activator of CFTR. As shown in Fig 6B, when forskolin (10µM) was added to the serosal bath, HCO$_3^-$ secretion increased by 166±23% (n=6, p<0.01) over the course of 60 minutes.

**Activation by genistein:** Genistein, an isoflavone phytoestrogen and a tyrosine protein kinase inhibitor, has been reported to bind directly to CFTR channels to activate them without affecting cAMP (28). Genistein (20µM) added to the bath increased HCO$_3^-$ secretion by 225±100% (Fig 6C) over the course of 60 minutes (n=7, P<0.005).

**Effect of inhibition of CFTR:** Glibenclamide is a sulfonylurea cell permeable compound (53) that blocks CFTR channels (10; 46; 56). As shown in Fig 7A, the addition of glibenclamide to the bath of the isolated perfused esophagi resulted in a reduction of basal HCO$_3^-$ secretion by approximately 47±23% over the course of 60 minutes (n=6, P<0.005).

Lastly we used CFTR$_{inh}$-172, a thiazolidinone derivative reported to inhibit CFTR channels (34). CFTR$_{inh}$-172 is membrane permeable and has been shown to inhibit submucosal gland secretions in whole tissue preparations of pig bronchi (51). Addition of CFTR$_{inh}$-172 (10 µM) to the basolateral side of the tissue inhibited basal secretion of HCO$_3^-$ by approximately 63% (n=4, P<0.05) over the course of 60 minutes. Subsequent addition of IBMX to the bath in the continued presence of CFTR$_{inh}$-172 did not elicit any stimulation of HCO$_3^-$ (n=4, P>0.05). These data are summarized in Fig 7B.

**Immunolocalization of SLC26A6 and SLC26A3.**

In the pancreas and other tissues, CFTR is reported to interact with members of the SLC26 family of anion exchangers to regulate HCO$_3^-$ secretion into the lumen (31; 49). To investigate
the presence of these transporters in the SMG and their co-expression with CFTR we labeled cryosections of esophageal tissue with an antibody to SLC26A6 or to SLC26A3. The staining with SLC26A3 antibody was negative in the glands. On the other hand, the staining with SLC26A6 antibody was positive and clearly delineated the luminal membrane of the ducts (Fig. 8A and 8C). Figure 8B shows nuclear labeling with DAPI. This finding is consistent with the presence of SLC26A6 at the luminal membrane of those cells. SLC26A6 staining was positive but diffuse in the acinar mucous cells (data not shown).

**Identification of SLC26A6 by RT-PCR**

Expression of SLC26A6 was also confirmed in the SMG tissue by RT-PCR. As shown in Fig. 8D amplification using primers for *sus scrofa* SLC26A6 yielded a predicted 494 base pairs product in SMG (lane 1). A similar band was observed in trachea (lane 2) used as a positive control. The sequence of the PCR product thus obtained from SMG esophageal glands is shown in Supplementary Material. A BLAST of this sequence shows 99% homology to Sus scrofa anion exchanger SLC26A6 (NM_001012298.1)

**Immunolocalization of Na-K-2Cl**

Na-K-2Cl cotransporter (NKCC1) is reported to play a major role in glandular secretion via the ability to mediate influx of Cl^−, K^+ and Na^+ into cell. (33). We used an antibody to NKCC1 to immunolocalize the transporter in SMG tissue. Staining with this antibody showed intense labeling of the mucous cells, clearly delineating the basolateral membrane (Fig 9A and 9B). In the duct cells, the basolateral membrane also stained positive to the antibody, indicating the presence of the transporter at this membrane (Fig. 9C and 9D). The nuclei are stained blue with DAPI Fig 9B and 9D).

**Role of Na-K-2Cl in HCO₃⁻ secretion**

We lastly demonstrated the role of Na-K-2Cl in esophageal SMG secretion. To do so, we measured HCO₃⁻ secretion in the absence and presence of bumetanide (0.1 mM), a known inhibitor of this transporter, in the isolated perfused esophagus. As shown in Fig 9E, the addition of bumetanide to the serosal bath decreased HCO₃⁻ secretion by approximately 37±14% (n=3, p<0.005).
DISCUSSION

Like the human esophagus, the pig esophagus bears SMG that are capable of secreting significant amounts of mucus and HCO$_3^-$ into the esophageal lumen. The secreted mucus and HCO$_3^-$ contribute to the formation of a buffered unstirred surface layer that protects the esophagus from damage caused by acidic exposure (1, [Abdulnour-Nakhoul, 2008 #2194]). In a previous study, we showed that HCO$_3^-$ secretion by esophageal SMG is stimulated by cholinergic agonists and inhibited by DIDS, the removal of Cl$^-$ and carbonic anhydrase inhibitors (2). In the present study we characterized the cellular mechanisms involved in this process and localized the transporters that are likely involved in HCO$_3^-$ secretion. Figure 10 is a schematic diagram of an epithelial duct cell of the esophageal SMG showing apical and basolateral transporters linked to HCO$_3^-$ secretion for which we have obtained histological and/or functional data in our study.

**NBC and AE2**

NBC staining in SMG ducts overlapped with Na,K-ATPase staining, indicating the presence of Na$^+$(HCO$_3^-$)$_n$ co-transporter (NBCe1) on the basolateral side of duct cells. The staining pattern of NBC in the SMG (Figs 1& 2), positive in the ducts and weak in the acini, is similar to the pattern in the pancreas, (35). The antibody we used was raised against the terminal 46 aa of rat kidney NBC (45) and can recognize the variants NBCe1-A (kidney proximal tubule) and NBCe1-B (pancreatic NBC). Using primers to *Sus scrofa* NBCe1-B we obtained a 472 bp PCR product which had 99% homology to NBCe1-B. At the molecular level, the full sequence of this NBC isoform of the esophagus has not yet been cloned. Further experiments are needed to determine the homology between the esophageal isoform(s) of NBC and the kidney, pancreas and brain isoforms. The presence of NBC in serous and duct cells indicates that those cells are the likely source of HCO$_3^-$ secreted by esophageal SMG. To generate HCO$_3^-$ in the duct lumen, basolateral NBC must bring Na$^+$ and HCO$_3^-$ from the blood side into the cell. NBC isoforms leading to influx (rather than efflux) of Na$^+$ and HCO$_3^-$ should have a configuration of (HCO$_3^-$ :Na$^+$) of 2:1 or 1:1. Such an NBC has been reported in other secretory epithelia (4; 44).

Double immunostaining with AE2CT and Na,K-ATPase antibodies localized AE2 to the basolateral side of duct cells only (Fig 3). This is consistent with findings in other glandular and epithelial tissues where AE2 is also restricted to the basolateral membrane of the cells (7).
Because the electrochemical gradients always favor Cl⁻ entry into the cell, this exchanger allows the influx of Cl⁻ and the efflux of HCO₃⁻. It cannot therefore contribute directly to HCO₃⁻ uptake from the blood side for secretion into the lumen. However, the role of AE2 in the secretory process remains significant for at least two reasons. First, a likely role of AE2 is to regulate intracellular pH (pHᵢ). The presence of the transporter (albeit faint) in mucous cells, as well as in serous and duct cells, suggests a ubiquitous role as needed for pHᵢ regulation. A second role of AE2, and probably a more significant one, is to contribute to the transcellular transport of Cl⁻ ions. We have previously demonstrated that the presence of Cl⁻ on the basolateral side of the tissue is critical for HCO₃⁻ secretion (2).

**Na-K-2Cl**

Our immunostaining experiments indicate the presence of Na-K-2Cl (NKCC1) at the basolateral membrane of mucous and duct cells, a finding consistent with the distribution of this transporter in other exocrine glands (22; 32). Our functional data in the isolated perfused esophagus indicate that the addition of bumetanide, an inhibitor of Na-K-2Cl transporter, to the basolateral side of the tissue decreases HCO₃⁻ secretion significantly. This observation, and the fact that HCO₃⁻ secretion in this tissue is dependant on serosal Cl⁻ (2), suggests that Cl⁻ entry across the basolateral membrane is a necessary step for HCO₃⁻ secretion. The residual basal secretion in the presence of bumetanide is due either to incomplete inhibition of Na-K-2Cl, or to the presence of other Cl⁻ transport mechanisms on the basolateral membrane, such as AE2, that can compensate for reduced Cl⁻ influx by Na-K-2Cl.

**CFTR**

A novel finding of our study is demonstrating that CFTR is localized in the esophageal SMG and that it likely contributes to mediating HCO₃⁻ secretion by these glands. In several glandular tissues, CFTR can act either as a Cl⁻ channel or as a channel capable of transporting HCO₃⁻ (41). CFTR is reported to play a major role in HCO₃⁻ secretion in tracheobronchial glands (9) (18) and pancreas (24). CFTR channel activity can also serve as a mediator for activation of Cl⁻-HCO₃⁻ exchange (31; 48). The activation of this channel by cAMP transduction pathway is one of its identifying properties (47).

We used four different antibodies to immunolocalize CFTR. Two antibodies gave the best results: first a polyclonal antibody against peptide corresponding to amino acids 1468-1480 of
human CFTR (Fig. 4), second, a monoclonal antibody against N-terminus amino acids 24-35 (Fig 3S, supplementary data). The distribution of CFTR, diffuse in mucous cells and more localized to the luminal membrane in the ducts, is in agreement with its distribution in salivary glands and pancreas (56). CFTR was prominently expressed in apical membrane of cells immediately lining the ducts. Interestingly, some basolateral labeling of CFTR was observed in basal cells of the ducts (cells not directly lining the lumen). The presence of CFTR in the basolateral membrane of glandular duct cells has been observed in other glandular tissues (42). Because immunolabeling of CFTR has been an issue of controversy in some studies (17; 37), we further tested the presence of CFTR by RT-PCR. Using gene specific primers we confirmed the presence of CFTR product in isolated SMG. Lastly, we used in-situ hybridization as a third approach to verify the presence of CFTR in SMG. As shown in Fig. 5, CFTR transcript was positively labeled in SMG tissues.

Functionally, there is no ideal way to selectively activate CFTR. However, one hallmark of CFTR activation is an increase in cAMP. In our experiments the increase in intracellular cAMP caused by forskolin, an adenylyl cyclase activator, or IBMX, a phosphodiesterase inhibitor, caused a large increase in basal HCO₃⁻ secretion. However, the elevation in intracellular cAMP could activate other ion transport mechanisms, among which are K⁺ channels, Na-K-2Cl and Na-(HCO₃⁻)ₙ (8). To confirm that the increase in HCO₃⁻ secretion was indeed due to CFTR stimulation and not to secondary effects of cAMP, we used genistein, a flavonoid which is a potent activator of CFTR channels (6; 23; 29; 36). Genistein does not operate through activation of protein kinase A (PKA), protein kinase C (PKC), or PKG because it does not cause increases in cAMP, Ca²⁺, or cGMP (11). Our experiments indicate that the activation of CFTR channel by genistein caused an increase in HCO₃⁻ secretion. Our findings were further supported by the use of glibenclamide, an inhibitor of CFTR (46), which decreased HCO₃⁻ secretion. In another set of experiments on the isolated perfused esophagus, we used another specific CFTR channel blocker, CFTRinh-172 to block CFTR (34), and demonstrated that basal as well as IBMX-induced HCO₃⁻ secretion were significantly inhibited.

The presence of CFTR in the esophagus has been questioned by few studies. In human fetal tissue CFTR expression was reported in most of the GI tract but was absent in the esophagus (52). However, it is not clear whether expression by in-situ-hybridization was done on esophageal tissues containing SMG. Rochelle et al. (43) did not detect CFTR expression in
murine esophagus, but it is known that murine esophagus is devoid of SMG which our studies indicate are the primary location of CFTR expression in the esophagus. In a third study, published as an abstract, Joo et al (30) reported pharmacological results on mucus secretion by porcine SMG consistent with our findings on \( \text{HCO}_3^- \) secretion but could not detect CFTR expression by PCR. However, our data confirmed CFTR presence in SMG by ISH, PCR and immunolabeling in addition to the contribution of CFTR to a physiologic response measured as \( \text{HCO}_3^- \) secretion.

Our studies explain important characteristics of the cellular transport pathways involved in \( \text{HCO}_3^- \) secretion in SMGs. These properties are derived from the functional and immunohistochemical data of this study and our previous studies on \( \text{HCO}_3^- \) secretion in the glands (2). Figure 10 is a schematic diagram of a SMG duct cell presenting a likely model of \( \text{HCO}_3^- \) secretion by these glands. Similar to the salivary glands (55), the acini possibly secrete a plasma-derived fluid that is then modified and enriched with \( \text{HCO}_3^- \) in the duct system. As shown in Fig 10, the basolateral entry step for \( \text{HCO}_3^- \) seems to be predominantly mediated by NBCe1-B. Basolateral \( \text{Cl}^-\text{HCO}_3^- \) (AE2) identified in this study, does not contribute directly to \( \text{HCO}_3^- \) secretion but may be significant for intracellular pH regulation and \( \text{Cl}^- \) transport. The luminal \( \text{HCO}_3^- \) exit step is likely mediated by \( \text{Cl}^-\text{HCO}_3^- \) exchanger SLC26A6. We have established a critical role of \( \text{Cl}^- \) in \( \text{HCO}_3^- \) secretion with CFTR and basolateral Na-K-2Cl contributing to this process. Apical CFTR may have dual roles in \( \text{HCO}_3^- \) secretion. On one hand, CFTR may serve as a channel that is permeable to \( \text{HCO}_3^- \) and therefore can contribute directly to \( \text{HCO}_3^- \) secretion (41; 50). Alternatively, CFTR may serve predominantly as a channel for apical \( \text{Cl}^- \) efflux, a fraction of which may be shunted to drive apical \( \text{Cl}^-\text{HCO}_3^- \) exchanger (SLC26A6) leading to \( \text{HCO}_3^- \) secretion. The mode of regulation of these transporters and their relative contribution to \( \text{HCO}_3^- \) secretion in the SMG remain to be determined.

In human esophagus the expression of CFTR is yet to be determined. However, several observations indicate that a role of CFTR in the esophagus is likely. A great majority of CF patients have GERD; ~25% in infants and ~80 % in adults (20; 54). In CF patients, proximal esophageal acid exposure is significantly higher than control with more frequent and longer episodes (14; 15; 54). Using impedance-pH monitoring and esophageal manometry in children and adult CF patients, two recent studies by Blondeau suggest that GERD is a primary phenomenon and it is not secondary to cough (12; 13). Heine et al (26) demonstrated that GERD
in infants was present before radiological lung disease was established. Accordingly, it is possible that impaired HCO$_3^-$ and mucus secretions from the esophageal glands, caused by defective Cl$^-$ channels, might be a factor contributing to the epithelial damage in the esophagus.

**Perspectives and Significance**

The esophageal submucosal glands (SMG) are well positioned anatomically and functionally to play an important role in the defense of the esophagus against acid injury. Mechanisms that mediate and regulate esophageal SMG secretions remain largely unknown. Characterizing these mechanisms is important to understand the physiology and pathophysiology of the esophagus. Our study investigated the ion transport mechanisms involved in SMG bicarbonate secretion. We have immunolocalized several HCO$_3^-$ and Cl$^-$ transport mechanisms to the membranes of SMG duct cells. Na-coupled bicarbonate transporter, NBCe1-B, chloride-bicarbonate exchanger SLC4A2/AE2 (Cl$^-$-HCO$_3^-$) and NKCC1 (Na-K-2Cl) are present at the basolateral membrane whereas the anion exchanger SLC26A6 and cystic fibrosis transmembrane regulator, CFTR, are present at the luminal membrane. The basolateral entry step for HCO$_3^-$ is likely mediated by NBCe1-B. This is the first report on the presence of CFTR and SLC26A6 in the esophagus. We have demonstrated that CFTR activity is important for HCO$_3^-$ secretion. As such CFTR may act as a channel that mediates HCO$_3^-$ efflux and/or activates luminal SLC26A6. Our study could explain the high incidence of esophageal disease in cystic fibrosis patients.

**ACKNOWLEDGMENTS**

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**Disclosures**

None.
Bibliography


FIGURE LEGENDS

Figure 1. Confocal immunofluorescence micrographs showing localization of NBCe1 and Na,K-ATPase in SMGs duct cells: Na,K-ATPase staining (red) was intense and delineated the basolateral membrane of duct cells (A). NBCe1 staining (green) was positive in the basolateral membrane (B). Nuclei were counterstained with DAPI (C). Co-localization of NBCe1 and Na,K-ATPase is shown when (A) (B) and (C) were merged (D). bl=basolateral . (bar=50μ)

Figure 2. Immunofluorescence localization of NBCe1 and Na, K-ATPase in SMGs acini: Na,K-ATPase staining (red) was intense in serous demi-lunes and the basolateral membrane of the mucous cells (A). NBCe1 staining (green) was positive in the serous demi-lunes and the basolateral membrane of mucous cells (B). Nuclei were counterstained blue with DAPI (C) NBCe1 and Na-K-ATPase stainings co-localized (yellow) when (A, B and C) were merged (D). l=luminal, bl=basolateral. Bar=10μm.
(E) Shows amplification of NBCe1-B product from mRNA isolated from SMG tissue, at the expected product size of ~472 bp (lane 2). DNA ladder is shown in lane 1.

Figure 3. Immunofluorescence localization of the Cl-HCO₃⁻ exchanger AE2 (SLC4A2) and Na,K-ATPase in intralobular ducts of SMGs: Na,K-ATPase staining (red) was intense and delineated the basolateral membrane of duct cells (A). AE2 staining (green) was positive at the basolateral membrane only (B). Nuclei were counterstained with DAPI (C). AE2 and Na,K-ATPase stainings co-localized (yellow) when (A, B, and C) were merged (D). bl=basolateral. Bar=10μm.

Figure 4. Confocal micrographs showing immunofluorescence localization of cystic fibrosis transmembrane conductance regulator (CFTR) and Na,K-ATPase in SMGs: Duct cells showed intense staining for Na,K-ATPase at the basolateral membrane of the cells (A). CFTR staining was positive and diffuse in the acini but was clearly delineating the luminal membrane of duct cells lining the lumen (B). Nuclei were counterstained with DAPI (C). The merged image (D) showed that staining to CFTR was clearly positive at the luminal membrane of duct cells. Bar=10μM.
**Figure 5. In situ hybridization (ISH) and RT-PCR amplification of CFTR in esophageal tissues:**

(A) shows positive ISH signals (blue staining; arrows) in paraffin sections incubated with the anti-sense nucleotide probe to mRNA of CFTR. The sections incubated with the sense probe (B) or without any probe (D) did not show ISH signals. Sections incubated with poly-dT, as a positive probe for RNA, showed strong positive staining (C). Nuclei were counterstained with Nuclear Fast Red. (E) shows RT-PCR amplification of CFTR product from mRNA isolated from esophageal SMG (1) or trachea (2) used as a positive control. The expected product size was obtained in both cases.

**Figure 6. Effects of IBMX, forskolin and genistein on esophageal HCO$_3^-$ secretion:**

(A) 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, increased HCO$_3^-$ secretion significantly (n=6, p<0.005). (B): Forskolin, an adenylyl cyclase activator, more than doubled HCO$_3^-$ secretion (n=6, p<0.001). (C): Genistein an isoflavone phytoestrogen and a potentiator of CFTR increased HCO$_3^-$ secretion significantly (n=7, p<0.005). (*) indicates significance compared to basal secretion.

**Figure 7. Effects of glibenclamide and CFTRinh-172 on esophageal HCO$_3^-$ secretion.**

(A) Glibenclamide, a Cl$^-$ channel (CFTR) blocker, inhibited basal HCO$_3^-$ secretion significantly (n=6, p<0.05). (B) CFTRinh-172 a thiazolidinone derivative and potent CFTR channel blocker inhibited basal HCO$_3^-$ secretion significantly and inhibited stimulation of secretion by IBMX. (*) indicates significance compared to basal secretion.

**Figure 8. Immunofluorescence localization and RT-PCR amplification of the luminal anion exchanger SLC26A6 in SMGs.**

Staining of SLC26A6 (red) was positive at the luminal membrane (A). Nuclei were counterstained with DAPI, (B). (C) is a merged image showing expression of the anion exchanger at the apical membrane of an interlobular duct.

(D): SLC26A6 expression was confirmed by RT-PCR using mRNA isolated from dissected SMG (lane 1) and trachea (lane 2) as positive control. The 494 bp band was the predicted product size.
Figure 9. Immunofluorescence localization of Na-K-2Cl in the SMGs using a mouse monoclonal antibody. In mucous cells (A & B), (A) shows intense staining (red) for Na-K-2Cl. (B) is a merged image of an acinus showing Na-K-2Cl and nuclei, counterstained blue with DAPI. These figures indicate that Na-K-2Cl clearly delineated the basolateral membrane (bl). m=mucous cells. In duct cells (C & D), (C) shows the localization of Na-K-2Cl (red) in interlobular duct cells of SMGs. (D) is the merged image of a duct showing Na-K-2Cl and nuclei stained blue with DAPI. The figures indicate that Na-K-2Cl staining (red) was intense and clearly delineated the basolateral membrane (bl). Bar=5μM. (E): Bumetanide a Na-K-2Cl blocker decreased HCO_3^- secretion significantly (n=3, p<0.005). (*) indicates significance compared to basal secretion.

Figure 10. Cell model of a duct cell depicting ion transport mechanisms involved in HCO3-secretion in esophageal SMGs. Basolateral HCO_3^- entry is mediated by Na^+/HCO_3^- co-transporter (NBCe1). The anion exchanger Cl^-/HCO_3^- (AE2) mediates Cl^- uptake by the cell and could play a role in intracellular pH regulation. Basolateral Na-K-2Cl (NKCC1) mediates Na^+, K^+ and Cl^- entry into the cell. HCO_3^- efflux at the apical membrane is mediated by SLC26A6. Apical CFTR may serve as a channel that is permeable to HCO_3^- and therefore can contribute directly to HCO_3^- secretion. Alternatively, CFTR may serve predominantly as a channel for apical Cl^- efflux, a fraction of which may be shunted to drive apical Cl^-/HCO_3^- exchanger (SLC26A6) leading to HCO_3^- secretion. This model is based on data from this study and a previous study from our lab (2) where the role of different transport inhibitors including carbonic anhydrase inhibitors on HCO_3^- secretion was evaluated.
SUPPLEMENTARY FIGURES

Figure 1S. Photomicrographs of an experiment where NBCe1 antibody was reacted with its fusion protein (MBP-K1A, 15μg/ml), showing the staining was negative (A). (B) shows nuclear staining with DAPI.

Figure 2S. Photomicrographs of an experiment where Cl⁻-HCO₃⁻ (AE2CT) antibody was reacted with AE2CT fusion protein (SA6). (A): Na-K-ATPase staining (red) in the same sections was intense and delineated very clearly the basolateral membrane of duct cells. (B): staining to AE2CT was negative when it was pre-adsorbed with the fusion protein (1:50) (C) shows nuclear staining with DAPI and (D) is the merged image of (A) (B) and (C).

Figure 3S. Photomicrographs of an experiment where CFTR antibody was reacted with its fusion protein (1:10), showing the staining was negative (A). (B) shows nuclear staining with DAPI.

Figure 4S. Immunofluorescence localization of CFTR in SMGs using mouse monoclonal antibody against the N-terminus (amino acids 24-35, human clone MM13) of human CFTR. Mucous cells (A-C): (A): Staining for CFTR (red) is positive but diffuse; (B): nuclei counterstained with DAPI, (C): Merged image of (A) and (B). Duct cells (D-F): (D): Staining for CFTR (red) is positive at the luminal membrane; (E) nuclei counterstained with DAPI; (F): Merged image of (E) and (F) (arrow). Bar=10μM. Negative control in duct cells (G-I): (G): Primary antibody was omitted from the incubation medium and no staining was observed (negative control) (H): nuclei counterstained with DAPI (I): Merged image of (G) and (H)
Table 1: Primary antibodies, the concentrations used, the homology of immunogen to pig protein, secondary antibodies and the relative labeling intensity in different cells of the SMGs.

<table>
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<th>Host-Antibody</th>
<th>Immunogen-clone, source</th>
<th>Homology to pig protein %</th>
<th>Concentration used</th>
<th>Secondary antibodies Alexa Fluor (Invitrogen)</th>
<th>Immunolocalization in SMG</th>
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<td>1/100</td>
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**Fig 1**

**A**
- NBCe1
- Lumen
- Acini

**B**
- Na,K-ATPase
- Bl
- Duct

**C**
- DAPI
- Lumen

**D**
- Merged
Fig 3

AE2CT

B

lumen

bl

Merged

D

lumen

bl

Na,K-ATPase

A

lumen

bl

DAPI

C

lumen
Fig 5

A. CFTR-anti-sense
B. CFTR-sense
C. poly-dT
D. No probe
E. Gel:

- 229
A

Basal secretion

IBMX (100 μM)

HCO₃⁻ secretion (%)

B

Basal secretion

Forskolin (10 μM)

HCO₃⁻ secretion (%)

C

Basal secretion

Genistein (20 μM)

HCO₃⁻ secretion (%)

Fig 6
Fig 7

A

HCO₃⁻ secretion (%)

0 20 40 60 80 100 120 140

Basal secretion Glibenclamide 100 μM

B

HCO₃⁻ secretion (%)

0 20 40 60 80 100

Basal secretion CFTR inh-172 10 μM CFTR inh-172+IBMX

*
Fig 8

DAPI

SLC26A6

MERGED
**Fig 9**

- **Na-K-2Cl**
- **Mucous Cells**
- **SMG duct**

**E**

- Basal secretion
- HCO$_3^-$ secretion (%)

Bar graph showing the effect of Bumetanide (100 μM) on basal secretion.
Duct cell

SEROSA

HCO₃⁻ → Cl⁻ → Na⁺ → (HCO₃⁻)ₙ → Cl⁻ → Na⁺ → K⁺ → 2Cl⁻ → CO₂ → K⁺

LUMEN

CO₂ + H₂O ↔ CA → H₂CO₃ ↔ H⁺ + HCO₃⁻

Glibenclamide
CFTRₐₙh-172

SLC26A6