Regulation of vocal fold transepithelial water fluxes

KIMBERLY V. FISHER,1 ALVIN TELSER,2 JONATHAN E. PHILLIPS,3 AND DONOVAN B. YEATES3
1Department of Communication Sciences and Disorders, Northwestern University, Evanston 60208; 2Department of Cell and Molecular Biology, Northwestern University, Chicago 60611; and 3Department of Medicine, University of Illinois at Chicago, and Veterans Affairs Chicago Health Care System, Chicago, Illinois 60612

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Fisher, Kimberly V., Alvin Telser, Jonathan E. Phillips, and Donovan B. Yeates. Regulation of vocal fold transepithelial water fluxes. J Appl Physiol 91: 1401–1411, 2001.—Vocal fold hydration is critical to phonation. We hypothesized that the vocal fold generates bidirectional water fluxes, which are regulated by activity of the Na+–K+–ATPase. Western blots and immunohistochemistry demonstrated the presence of the α-subunit Na+–K+–ATPase in the canine vocal fold (n = 11). Luminal cells, basal and adjacent one to two layers of suprabasal cells within stratified squamous epithelium, were immunopositive, as well as basolateral membranes of submucosal seromucous glands underlying transitional epithelia. Canine (n = 6) and ovine (n = 14) vocal fold mucosae exhibited transepithelial potential differences of 8.1 ± 2.8 and 9.3 ± 1.3 mV (lumen negative), respectively. The potential difference and short-circuit current (ovine = 31 ± 4 μA/cm2; canine = 41 ± 10 μA/cm2) were substantially reduced by luminal administration of 75 μM acetylstrophanthinid (P < 0.05). Ovine (n = 7) transepithelial water fluxes decreased from 5.1 ± 0.3 to 4.3 ± 0.3 μl·min−1·cm−2 from the basal to luminal chamber and from 5.2 ± 0.2 to 3.9 ± 0.3 μl·min−1·cm−2 from the luminal to basal chamber by luminal acetylstrophanthinid (P < 0.05). The presence of the Na+–K+–ATPase in the vocal fold epithelium and the electrolyte transport derived from its activity provide the intrinsic mechanisms to regulate cell volume as well as vocal fold hydration.

VOCAL FOLD HYDRATION MUST be regulated to maintain the ease of phonation in an environment of disparate and rapidly changing humidities. For example, both superficial and intrinsic hydration of the vocal fold are critical for vocal fold oscillation to occur in an ex vivo canine model (8, 14). Challenges that predictably decrease vocal fold hydration, such as dry air and hyperosmolar fluids, also increase the subglottic pressure necessary to achieve and maintain vocal fold oscillation (phonation threshold pressure (8, 14)). Dehydration challenges are thought to inhibit vocal fold oscillation by increasing the viscosity and decreasing the thickness of the vocal fold cover (8, 11). Such volumetric and rheological properties of the vocal fold cover govern the biomechanics of vocal fold oscillation [e.g., body cover theory (13) and mucoviscoelastic-aerodynamic theory (18, 32, 33)]. Whether the vocal fold mucosa generates transepithelial water fluxes toward and/or away from the lumen has not been explored. Similarly, in the vocal fold, any cellular mechanisms that regulate ion and fluid transport and, therefore, volumetric and rheological homeostasis have not been identified.

The vocal fold cover consists of mucosa that contains stratified squamous epithelium, basal lamina, and superficial layer of lamina propria (13). Wet, stratified squamous epithelia of similar structure to that of the vocal fold (e.g., those of the buccal, esophageal, and vaginal mucosae) generate a potential difference (PD) and short-circuit current (Isc) indicative of transepithelial ion fluxes (7, 17, 22, 23) and consistent with transepithelial water fluxes. In the stratified, squamous epithelia of the aerodigestive tract, the Na+–K+–ATPase contributes to the generation of this PD (23, 24); however, any contribution of Na+ transport to vectorial water flux has not been demonstrated. In tracheal epithelia (consisting of pseudostratified, ciliated columnar cells and goblet cells), Phillips et al. (26) have shown that vectorial water transport is coupled to sodium transport and the activity of basolateral Na+–K+–ATPase (39). Thus, in the trachea, where pseudostratified epithelial cells are known to be electrically and structurally polarized, the active Na+–K+–ATPase pump maintains the electrochemical gradients responsible for the PD across the epithelia and supplies most of the energy driving the Isc. We questioned whether stratified vocal fold epithelium might be electrically and polarized and, if so, whether the Na+–K+–ATPase may regulate cation transport and thus vocal fold water flux.

We hypothesized that the Na+–K+–ATPase is present in vocal fold epithelia and contributes to its luminal negative PD, Isc, and transepithelial water flux. We identified the α-subunit protein of the Na+–K+–ATPase in the vocal fold epithelium and submucosal glands of the canine larynx. We subsequently demonstrated the contribution of the Na+–K+–ATPase to the transepithelial...
lial PD, $I_{\text{sec}}$, and water flux by luminal application of acetylstrophanthinidin (a specific Na$^+$/K$^+$-ATPase inhibitor) to native canine and ovine vocal fold epithelium. The results support the presence of an active transport system that regulates transepithelial ion and water flux in the vocal fold. This system may provide the foundation for adaptive cellular mechanisms capable of responding to the osmotically challenging environment of the vocal folds.

METHODS

The identification and localization of α-subunit protein of the Na$^+$/K$^+$-ATPase. In accordance with approved protocols at Northwestern University and University of Illinois at Chicago, the larynx and kidneys were excised from 11 dogs (Canis familiaris, age 3–4 yr, 25–35 kg) that were undergoing other nonsurvival procedures. Surgical anesthesia was induced with pentobarbital sodium (25 mg/kg iv). Vocal fold and kidney tissues were frozen or fixed immediately after excision. Animals were killed by a lethal dose of pentobarbital sodium (100 mg/kg iv) followed by thoracotomy. Masson’s trichrome and hematoxylin and eosin preparations were used to stain the vocal fold tissues. Western blot and immunohistochemistry and immunohistochemistry were used to detect the presence of the α-subunit protein of the Na$^+$/K$^+$-ATPase. For Western blots ($n = 3$), the frozen tissue was rapidly homogenized in the presence of 1% Triton X-100 in 20 mM Tris buffer and 150 mM NaCl, as well as the protease inhibitors benzamidine, leupeptin (5 µg/ml each), and 1 mM phenylmethylsulfonyl fluoride. For histology and immunohistochemistry ($n = 11$), tissues were fixed in 4% paraformaldehyde and embedded in paraffin for subsequent sectioning and staining.

The α-subunit protein of the Na$^+$/K$^+$-ATPase was detected by a mouse monoclonal antibody (no. 5) anti-α-subunit to chicken Na$^+$/K$^+$-ATPase (the kind gift of Drs. Douglas Fambrough, Johns Hopkins University, and Chau Wu, Northwestern University). Anti-mouse IgG conjugated to horseradish peroxidase (Super Sensitive Multilink, BioGenix, San Ramon, CA) was used as the secondary antibody. The localization of this antibody was visualized with 3-amin-9-ethylcarbazole. Kidney tissues were used for positive tissue controls for the Western blots and immunohistochemistry. The negative tissue control was the glomerulus (renal corpuscle) of the renal cortex. As procedural controls, the renal cortex and vocal fold tissues also were processed with the primary antibody replaced by a nonimmune preparation.

To examine the stratified squamous epithelium along the cartilaginous and membranous glottal margin, one vocal fold from each animal was sectioned in the transverse plane at a level 3–5 mm inferior to the superior surface of the vocal fold. The remaining vocal folds were cut in cross section along the coronal plane so that the glands and respiratory epithelium present in the supra- or subglottis could be observed. The cortex of the kidney was sectioned in a sagittal plane.

Sectioned tissue was mounted, deparaffinized, and rehydrated. To inactivate endogenous peroxidases, the preparation was immersed in H2O2 for 15 min at room temperature. The sections were then rinsed in distilled water and placed in citrate buffer (0.01 M, pH 6.0) for 15 min and placed in a 600-W microwave oven for 8 min at high power. Sections were rinsed in PBS three times for 5 min each before application of the primary antibody at 1:250 dilution. The tissue was incubated with the antibody overnight at 4°C under a plastic coverslip. The sections were again rinsed in PBS three times for 5 min each and flooded with the secondary antibody (Super Sensitive Multilink, BioGenix) for 1 h at room temperature. After a further rinse in PBS three times for 5 min each, peroxidase-conjugated streptavidin (BioGenix) was applied for 1 h at room temperature. After rinsing (with PBS 3 times for 5 min each), sections were dipped in 3-amin-9-ethylcarbazole chromogen and carefully monitored for appearance of color. Development was stopped by placing the sections in distilled water. Sections were dipped in hematoxylin (nonalcoholic), rinsed in running tap water, and dipped in lithium carbonate. The resulting sections were bathed in an aqueous mounting medium and protected with a coverslip.

Immunohistological specimens were inspected by three independent observers in a single-blind protocol using light microscopy. Each observer scored and recorded results (positive or negative staining) for vocal fold tissues (thicker and thinner epithelium and submucosal glands) and kidney tissues (glomerulus, proximal, and distal tubules). There were no instances of disagreement among observers regarding positive or negative staining, attesting to the reliability of the results. The specimens were further studied by two of the authors (K. V. Fisher and A. Telser) and photographed using a light microscope (Zeiss Axioskop2) coupled to a digital camera (ProGres 3008).

Contribution of the Na$^+$/K$^+$-ATPase to electrophysiology of native vocal fold mucosa. To perform the electrophysiological experiments, canine ($n = 6$) and ovine ($n = 14$) larynges were obtained. Canine larynges (from mongrel dogs, 45–60 kg, age 3–4 yr) were excised under surgical anesthesia, which was induced with pentobarbital sodium (25 mg/kg iv). Dogs were killed by a lethal dose of pentobarbital sodium (100 mg/kg iv) followed by thoracotomy. Ovine larynges that were larger than the canine larynges were obtained from a local abattoir. As soon as possible after excision, larynges were placed in 4°C Hanks’ balanced salt solution (HBSS) and transported directly to the laboratory.

Under cool HBSS, each larynx was sectioned posteriorly in the median plane to expose the interior larynx. A sheet of vocal fold epithelium was dissected free from the vibratory margin of the vocal fold along the plane of the loosely connected superficial lamina propria. Thus the membrane for study included the epithelium, its associated basal lamina, and superficial layer of lamina propria. Collectively, this tissue has been called the vocal fold “cover” in biomechanical models of phonation (13).

A commercially available Ussing chamber (model 15362, World Precision Instruments) and voltage clamp (EC-825, Warner Instrument) were used to measure the electrophysiological properties of membranes (35). A removable lucite cell (World Precision Instruments, 12-mm diameter chamber) was used to hold the membrane. The two bathing reservoirs, one each for the basal and luminal sides of the tissue, held a volume of 8.0 ml HBSS. A gas-lift (95% O2-5% CO2) pump oxygenated and circulated HBSS past each side of the membrane. The temperature of the baths was maintained at 37°C. Two irreversible, nonpolarizable voltage-sensing electrodes (Ag+/AgCl electrodes with Agar-3 M KCl salt bridges; FKVC cartridge electrodes, World Precision Instruments) were placed 2 mm equidistant from the membrane surfaces. Similarly, two irreversible current electrodes (FKVC cartridge electrodes, World Precision Instruments) were placed 11 mm equidistant from the membrane. Data from a subset of experiments (2 canine, 8 ovine) were acquired and digitized by using PC Multilab analog to digital board (Advantech, PCL-711B) at a 1-Hz sampling rate and stored in a 100-MHz 486 PC. MatLab software (Mathworks) was used to automate the
data-acquisition process and display electrophysiological data.

The Ussing chamber system was filled with HBSS equilibrated at 37°C with no membrane present. Any PD between the voltage electrodes was null, and adjustments were made to compensate for fluid resistance. The system was then drained, and the tissue chamber was removed. The vocal fold membranes were carefully mounted between the half-chambers, each of 12-mm diameter, exposing 1.13 cm² of the epithelium (Ussing, World Precision Instruments) (35). Under open-circuit conditions, the membrane PD stabilized in ~1 h as did the Iac. After stabilization of the membrane PD and Iac, the mucosa was returned to the luminal bath (~50 ml/min past the membrane) and through a quartz tube to facilitate the excitation of ANTS at 394 nm and detection of the green fluorescence (515 nm) by its respective photomultiplier (PMT, H3460–04, Hamamatsu, Bridgewater, NJ). For each photon detected, an emission-coupled logic pulse was produced and counted over consecutive 0.056-s intervals (DT2819, Data Translation, Marlboro, MA). The PD or Iac output of a potentiometer and/or voltage clamp (DVC1000, WPI Sarasota, FL) was simultaneously acquired by an analog-to-digital board (PCL-711b, Advantech, Sunnyvale, CA). The photon counts and the electrophysiological parameters were collected in real time by using data-acquisition software (Real toolbox, Humusoft, Czech Republic; and Matlab, The Mathworks, Natick, MA).

Raw photon count data were conditioned and analyzed by off-line analysis by using Matlab. A median filter removed outliers (spikes caused by transient electromagnetic interference in the environment). Background photon counts for pure HBSS or HBSS-1 mM ANTS were subtracted from each experiment. When in HBSS-1 mM ANTS solution, 2H2O flux from the basal to the luminal chamber caused a temporal decrease in ANTS fluorescence of the luminal chamber (due to the addition of 3H2O to a predominantly 2H2O-HBSS solution) and was used to calculate transepithelial water flux from the basal to the luminal chamber (dIw/dt). Similarly, H2O flux from the luminal chamber to the basal chamber (with predominantly 2H2O-HBSS solution) caused a temporal decrease in fluorescence in the basal chamber that was used to calculate transepithelial water flux from the luminal to the basal chamber (dIw/dt). The temporal change in fluorescent photon counts was determined by linear regression performed every second with the use of a 10-s data window. Sixty slope (dIw/dt) values per minute were calculated for each side of the membrane for 20 min, yielding 1,200 slope values for each side. The conversions of the slopes to fluid fluxes were made by using system calibration curves. The average flux over the 20-min period was taken as the flux value for that measurement period. The PD (during open-circuit conditions) was also recorded during the 20-min measurement periods.

At the beginning of the experimental protocol, the system was filled with HBSS and allowed to equilibrate at 37°C with no membrane present. Any PD between voltage-sensing electrodes was null, and adjustments were made to compensate for fluid resistance. The system was drained. Larynges from large lambs (n = 7) were obtained from the abattoir, as previously described for the electrophysiological experiments. The vocal fold mucosa (including that from the inferior, superior, and cartilaginous glottis) was dissected from the larynx. Tissues, ~2.0 cm², were mounted flat in the flux cell with the vocal fold mucosa covering the open area. Both sides of the system were filled and circulated with 4 ml H2O-HBSS, supplied with 95% O2 and 5% CO₂ at 25 ml/min through gas-permeable silicone tubes within each circulation loop. Each circulation loop contained a quartz tube to facilitate the excitation of ANTS at 394 nm and detection of the green fluorescence (515 nm) by its respective photomultiplier (PMT, H3460–04, Hamamatsu, Bridgewater, NJ). For each photon detected, an emission-coupled logic pulse was produced and counted over consecutive 0.056-s intervals (DT2819, Data Translation, Marlboro, MA). The PD or Iac output of a potentiometer and/or voltage clamp (DVC1000, WPI Sarasota, FL) was simultaneously acquired by an analog-to-digital board (PCL-711b, Advantech, Sunnyvale, CA). The photon counts and the electrophysiological parameters were collected in real time by using data-acquisition software (Real toolbox, Humusoft, Czech Republic; and Matlab, The Mathworks, Natick, MA).

The role of Na⁺-K⁺-ATPase in vocal fold transepithelial water flux. The method used for simultaneous measurement of vectorial water fluxes and electrophysiological parameters has been validated elsewhere in studies of airway mucosa (25, 26). The technique employed 8-aminoisonaphthalene-1,3,6-trisulfonic acid (ANTS; Molecular Probes, Eugene, OR), a fluorescent probe that has a threefold greater quantum yield when in H2O-HBSS than in H2O-HBSS. When 1 mM ANTS was filled with HBSS and allowed to equilibrate at 37°C with no membrane present. Any PD between voltage-sensing electrodes was null, and adjustments were made to compensate for fluid resistance. The system was drained. Larynges from large lambs (n = 7) were obtained from the abattoir, as previously described for the electrophysiological experiments. The vocal fold mucosa (including that from the inferior, superior, and cartilaginous glottis) was dissected from the larynx. Tissues, ~2.0 cm², were mounted flat in the flux cell with the vocal fold mucosa covering the open area. Both sides of the system were filled and circulated with 4 ml H2O-HBSS, supplied with 95% O2 and 5% CO₂ and warmed to 37°C. Electrophysiological parameters stabilized after 45 min. The system was drained. A 4-ml volume of H2O-HBSS-1 mM ANTS was added and circulated to rinse the basal chamber and membrane, whereas a 4-ml volume of H2O-HBSS-1 mM ANTS solution was used to rinse the luminal chamber and membrane. After the 1-min rinse, the system was drained. The basal and luminal circulation loops were filled with 4 ml each of H2O-HBSS-1 mM ANTS and H2O-HBSS-1 mM ANTS, respectively. Both sides of the sys-
tem were supplied with 95% O₂-5% CO₂ and warmed to 37°C. The electrophysiological parameters stabilized during open-circuit conditions over a 1-h period. The transmembrane PD was recorded, and immediately thereafter the membrane was clamped for 5 s to obtain the $I_{sc}$. Next, 75 μM acetylstrophanthidin was added to the luminal bath. PD was recorded during open-circuit conditions for 60 min. The $I_{sc}$ was recorded at the end of a 45-min period and once again after 1 h.

The dependent variables were the simultaneous, unidirectional water fluxes $J_{wB}$ and $J_{wL}$, as well as the electrophysiological PD and $I_{sc}$ that were measured during open- and closed-circuit conditions, respectively. Fully repeated-measures analyses of variance with one between factor was used to assess the probabilities associated with a treatment effect or interaction on the unidirectional water fluxes. Treatment effects on PD and $I_{sc}$ were assessed via separate, paired, one-tailed Student’s $t$-tests. $P < 0.05$ was considered statistically significant.

RESULTS

The identification and localization of α-subunit protein of the Na⁺-K⁺-ATPase. A coronal section of a canine vocal fold is shown in Fig. 1A. The canine vocal fold mucosa (Fig. 1A) overlies the thyroarytenoid muscle complex. It can be seen that the mucosa consists of epithelium and a highly vascularized lamina propria with numerous lymphatic vessels. Glands and their associated ducts are most numerous at the inferior and superior margins of the vocal fold. These ducts as well as lymphatics are shown at higher magnification in Fig. 1, B and C. In all 11 dogs, the epithelium of the vibratory margin of the vocal fold consisted of a non-keratinized, stratified, squamous epithelium of five to eight cell layers, as typified in Fig. 2, A and B. There was a brief transitional segment where the morphology of the...
of the cells and tissue changed from stratified, squamous epithelium to pseudostratified, ciliated, columnar epithelium, with goblet cells that lined the trachea and laryngeal ventricles. This transitional segment of the epithelium was characterized by a gradual increase in cell height (Fig. 2, C and D). There were numerous submucosal glands found beneath the ciliated epithelium of the trachea and laryngeal ventricles (not shown) and less frequently under transitional epithelium lining the inferior vocal fold (Fig. 2C). Numerous glands were seen under transitional epithelium that lined the inferior, posterior, cartilaginous glottis (Fig. 2, E and F). One glandular duct was observed to open onto the stratified, squamous epithelium of the vocal fold (Fig. 1, A and B). Numerous ducts were associated with the transitional region of the epithelium (Fig. 1C). The glands were of the mixed seromucous type with numerous serous demilunes (Fig. 2, E and F).

Immunohistochemical staining of α-subunit protein of the Na⁺-K⁺-ATPase in the renal cortex is shown in Fig. 3. In a low-magnification view of the renal cortex (Fig. 3A), thick ascending limbs of the nephron revealed strong immunopositive staining in the medullary rays, as well as in portions of the distal convoluted tubule. Many, but not all, sections of the proximal convoluted tubule also exhibited immunopositive staining. This staining was of lesser intensity (Fig. 3A), which can be better visualized at higher magnification (Fig. 3B). Figure 3C is from the renal cortex of another dog showing strong, positive staining of distal convoluted tubules and weaker, but distinctly positive staining of proximal convoluted tubules. No staining was seen in control specimens in which normal rabbit serum was used in place of the primary antibody, as illustrated in Fig. 3D. These observations are consistent with the distribution of Na⁺-K⁺-ATPase activity in different segments of the nephron, in which the enzyme activity in the thick ascending limbs has been reported to be about six times that found in the proximal convoluted tubule (12, 15) and absent in the renal corpuscle (12). Western blot analysis of samples of kidney and vocal fold mucosa revealed a band at 97–100 kDa (data not

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Fig. 2. A: stratified squamous epithelium and submucosa of the midmembranous canine vocal fold (magnification ×100). B: inset shown in A magnified ×400. C: transitional epithelium of the inferior glottis (magnification ×100). D: inset shown in C magnified ×400. E: seromucous glands from the posterior, cartilaginous vocal fold (magnification ×100). F: inset shown in E magnified ×400.
shown), which corresponds to the expected molecular mass in the α-subunit of Na\(^{+}\)-K\(^{+}\)-ATPase.

Immunohistochemical staining of the α-subunit of Na\(^{+}\)-K\(^{+}\)-ATPase in the vocal fold mucosa is shown in Fig. 4. Basal epithelial cells (those in contact with the basal lamina) and the immediately adjacent one to two layers of suprabasal epithelial cells (not in contact with the basal lamina) were immunopositive (Fig. 4A). Squamous cells at the luminal surface were also immunopositive (Fig. 4A). No immunopositive staining was seen in a negative control (Fig. 4B). Staining of the basal epithelial cells was punctate, in approximate superposition with the plasma membrane, and thus appeared to encircle the perimeter of the cells (Fig. 4A). The punctate appearance was expected because of the invaginations and extensions on the cell surface (10). Staining in cytoplasmic regions was either the result of tangential sections through plasma membrane and/or intracellular, presumably due to vesicular recycling of the protein. Immunopositive staining of the epithelium was present in all 11 animals. The stain was found in the epithelium covering both membranous and cartilaginous glottis and in sections of thicker and thinner epithelial membranes. The basolateral membranes of cells within the submucosal glands from the posterior, inferior vocal fold also stained positively for the Na\(^{+}\)-K\(^{+}\)-ATPase (Fig. 5, black arrows). In addition, the serous components of glandular cells stained strongly (Fig. 5, yellow arrowheads) for the α-subunit of Na\(^{+}\)-K\(^{+}\)-ATPase.

**Contribution of the Na\(^{+}\)-K\(^{+}\)-ATPase to the electrophysiology of native vocal fold mucosa.** The electrophysiological viability of the tissue preparations was maintained for >3 h. The 14 ovine tissues had a mean baseline PD of 9.3 ± 1.3 mV (lumen negative) and generated an \(I_{sc}\) of 31 ± 4 μA/cm\(^2\). This resulted in a mean baseline tissue resistance of 342 ± 48 Ω·cm\(^2\) for ovine membranes. Similarly, the six canine tissues had a mean PD of 8.1 ± 2.8 mV (lumen negative) and generated \(I_{sc}\) of 41 ± 10 μA/cm\(^2\). This resulted in a mean baseline tissue resistance of 254 ± 81 Ω·cm\(^2\) for the canine specimens. Figure 6 shows an example of the current generated by a selected PD of a tissue from a canine vocal fold. The baseline current-voltage relation, representative of \(R_{m}\), was linear. The offset of the \(I_{sc}\) at zero PD was the baseline \(I_{sc}\) for this membrane.

For ovine tissues (\(n = 14\), acetylstrophanthidin reduced the absolute PD toward 0 mV within 45 min (ANOVA main effect, \(P < 0.001\)). Luminal application of acetylstrophanthidin reduced the PD to 2.9 ± 2.1 mV (\(n = 7\)), an effect significantly greater than that of basal application, which reduced the PD to 6.7 ± 5.1 mV (\(n = 7\)) (ANOVA interaction, \(P = 0.034\)). The time required for the PD to reach 63.3% of its total change with luminal treatment was 1,400 ± 560 s (\(n = 6\)). Similarly, acetylstrophanthidin caused a significant decrease in \(I_{sc}\) within 45 min for the ovine tissues (ANOVA main effect, \(P = 0.002\)). The reduction in \(I_{sc}\) was slightly but not significantly greater for luminal (\(I_{sc}\) reduced to 8 ± 9 μA/cm\(^2\)) rather than basal (\(I_{sc}\) reduced to 20 ± 9 μA/cm\(^2\)) application (ANOVA interaction, \(P = 0.111\)).

The pronounced reduction in PD after luminal treatment of ovine tissues was also observed in canine tissues (cf. Fig. 7, A (canine) and B (ovine)). The mean PD in the canine vocal fold was reduced from 8.1 ± 2.8 to 1.4 ± 1.5 mV (lumen negative) within 45 min of luminal treatment (\(P = 0.031, n = 5\)). The estimated
The canine $I_{sc}$ was reduced to 66 m$\text{A}$ within 45 min of luminal treatment ($P = 0.009, n = 5$). For the canine membrane with basal application of acetylstrophanthidin, neither the PD of 6 mV nor the $I_{sc}$ of 15 m$\text{A}$ decreased during the 45-min posttreatment time frame. The absence of response to basal application in the canine membrane was consistent with the decreased response observed for basal treatment of ovine membranes.

The role of Na$^+$-K$^+$-ATPase in vocal fold transepithelial water flux. The reduction in bidirectional water flux ($P = 0.014$) by luminal acetylstrophanthidin ($n = 7$) is shown in Fig. 8. The baseline $J_{w\text{B} \rightarrow \text{L}}$ of 5.1 ± 0.3 $\mu$L min$^{-1}$ cm$^{-2}$ was reduced by 15% to 4.3 ± 0.3 $\mu$L min$^{-1}$ cm$^{-2}$ within 45 min. The baseline $J_{w\text{L} \rightarrow \text{B}}$ of 5.2 ± 0.2 $\mu$L min$^{-1}$ cm$^{-2}$ was reduced by 25% to 3.9 ± 0.3 $\mu$L min$^{-1}$ cm$^{-2}$ within 45 min. Application of acetylstrophanthidin reduced $J_{w\text{L} \rightarrow \text{B}}$ to a greater extent than $J_{w\text{B} \rightarrow \text{L}}$, as shown by the significant treatment by flux direction interaction ($P = 0.035$). Two post hoc comparisons by Student’s paired $t$-test confirmed that acetylstrophanthidin reduced $J_{w\text{L} \rightarrow \text{B}}$ ($P = 0.005$) to a greater extent than $J_{w\text{B} \rightarrow \text{L}}$ ($P = 0.027$) and showed both responses to be significant. The baseline open-circuit PD of $-8.7 \pm 1.2$ mV and its associated $I_{sc}$ of $44 \pm 11$ $\mu$A were reduced to $-1.3$ mV ± 0.5 mV and $27 \pm 12$ $\mu$A, respectively, replicating results from the electrophysiological experiments.

**DISCUSSION**

In the stratified, squamous epithelium of the vocal fold, the 100-kDa $\alpha$-subunit protein of the Na$^+$-K$^+$-ATPase was observed predominantly in association with the plasma membranes of the basal and adjacent one to two layers of suprabasal epithelial cells and perhaps, to a lesser extent, the luminal surface cells (Fig. 4). In seromucous glands, it also was found predominantly in association with the basolateral membranes of mucous cells, as well as the plasma membranes and cytosol of serous cells (Fig. 5). These glands were located beneath the tracheal epithelium and the transitional epithelium of the inferior and superior lateral vocal fold margins but not beneath the stratified squamous epithelium of the membranous vocal fold (Figs. 1 and 2). The vocal fold mucosa was polarized with the lumen negative, indicating an asymmetric cellular distribution or activity of the Na$^+$-K$^+$-ATPase. As the basal and possibly the luminal epithelial cells stained for the $\alpha$-subunit protein of the Na$^+$-K$^+$-ATPase, these cell layers are likely responsible for much of the observed PD. This PD was reduced by the addition of acetylstrophanthidin, a specific inhibitor of the Na$^+$-K$^+$-ATPase added to the luminal rather than the basal surface. The pronounced luminal response...
Fig. 6. The current-voltage relation for a canine vocal fold epithelium [obtained by clamping the potential difference (PD) of the tissue between -50 and +50 mV]. The current at a selected PD was recorded. This current was normalized to the surface area of the tissue. The data were fit by the line $y = 0.08x - 4.42$ to yield a surface area normalized resistance of 80 $\Omega \cdot cm^2$.

Fig. 7. Reduction of the lumen-negative PD of canine (A) and ovine (B) vocal fold epithelium induced by luminal application of acetylstrophanthidin. A: baseline electrophysiological properties for the canine membrane were PD = -5 mV, short-circuit current = 59 $\mu A/cm^2$, and membrane resistance = 85 $\Omega \cdot cm^2$. The time constant for the canine membrane was 500 s. B: for the ovine membrane, baseline PD = -8.5 mV, short-circuit current = 22 $\mu A/cm^2$, and membrane resistance = 390 $\Omega \cdot cm^2$. The time constant for this membrane was 1,400 s.

Fig. 8. Significant reductions of unidirectional water fluxes ($J$) across ovine vocal fold epithelia ($n = 7$) were caused by luminal application of acetylstrophanthidin. B to L, basal to luminal chamber; L to B, luminal to basal chamber.

demonstrates the ready luminal accessibility of the epithelial Na\textsuperscript{+}-K\textsuperscript{+}-ATPase to acetylstrophanthidin and the presence, on the basal side, of a barrier to its diffusion. We demonstrated that bidirectional water fluxes across vocal fold mucosa were reduced by acetylstrophanthidin. Together these data indicate that vocal fold hydration can be controlled by the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in the cells of squamous epithelium through the regulation of transepithelial ion and water fluxes.

The vocal folds are subjected to osmotic challenges. Superficial hydration of the vocal fold has been attributed to secretions of the tracheobronchial airways and laryngeal glands extrinsic to the vibratory margin of the vocal fold (10, 19, 29, 31). Secretions from the lower airways, however, amount only to 0.5 mg·kg body wt\textsuperscript{-1}·day\textsuperscript{-1} (34, 41). The secretions are transported up the trachea and converge to pass almost exclusively through the posterior commissure of the larynx (3).
during quiet respiration. Here we observed that ducts from mucosal glands open onto the transitional epithelium with some opening onto squamous epithelium of the vibratory vocal fold surface (Fig. 1, A and B). We showed that the electrically polarized, stratified epithelium of the vocal fold possesses an intrinsic Na\(^{+}\)-K\(^{+}\)-ATPase-dependent mechanism that regulates ion and water transport. In addition, the presence of the Na\(^{+}\)-K\(^{+}\)-ATPase provides the basis for the active volume regulation of vocal fold epithelium, important in this tissue’s role as a tunable baffle for mechanical stress of phonation. Shear and longitudinal stress of phonation provide an additional challenge to ion transport, as well as the barrier properties of the epithelium, as it has been shown in other tissues and cells (22, 37, 38). Both mechanical and osmotic challenges are implicated in numerous pathological states of vocal fold mucosa, most of which occur in the epithelium, basal lamina, or superficial layer of the lamina propria (e.g., region shown in Fig. 2B).

Albeit the ovine vocal fold and tracheal epithelial tissue both demonstrate a marked temporal response to acetylstrophanthidin of similar time course [time constant = 23.8 vs. 28.5 min (26)], these responses were obtained by application of acetylstrophanthidin to the luminal and basal surfaces, respectively. As the vocal fold generates a lumen-negative 9-mV PD, there must be an obligatory preponderance of the Na\(^{+}\)-K\(^{+}\)-ATPase or its activity in the basal direction of the cells in these tissues. The delayed and reduced action of acetylstrophanthidin when applied to the basal surface may be due to the magnitude of the unstirred layer on the basal side caused by some 2 mm of connective tissue vs. only <0.2 mm of superficial cells on the luminal side (Fig. 2, A and B).

We have considered the possibility that some of the PD measured was due to the epithelium on the superior and inferior margins of the vocal fold cover, where there is a transition between vocal fold epithelium and ciliated columnar epithelium covering the adjacent tracheal mucosa. The surface areas of the canine and ovine stratified, squamous vocal fold epithelia used for these electrophysiological studies were >2 cm\(^2\), thus minimizing any active transitional or columnar epithelia within the chamber. This was confirmed by the observations that lumen rather than basal acetylstrophanthidin more effectively inhibited the PD in the epithelia tested.

The Na\(^{+}\)-K\(^{+}\)-ATPase contributes to lumen-negative polarization of other wet epithelia, including those of the bronchial (16), tracheal (26), esophageal (23, 28), and buccal (24) mucosa. Similarly, vaginal mucosa is electrically polarized (7, 17), consistent with active ion transport. Similar to the vocal fold, esophageal, buccal, and vaginal epithelia are subjected to substantial mechanical stress in addition to other environmental insults. All of these epithelia are also characterized by a layer of basal cells that have numerous desmosomes and are anchored to the basal lamina via hemidesmosomes. Such epithelia also have numerous layers of suprabasal cells. The frog skin, however, provides an example in which the Na\(^{+}\)-K\(^{+}\)-ATPase can be preferentially localized to cell layers of a stratified, squamous epithelium, specifically the stratum spinosum and stratum germinativum [i.e., stratum basale (21)]. In comparison, the wet, stratified, squamous epithelia of the mammalian aerodigestive tract have no stratum spinosum per se, and all but the most superficial desquamating cells are viable. To our knowledge, the intense staining of the Na\(^{+}\)-K\(^{+}\)-ATPase with selected cell layers of a wet, stratified, squamous epithelium has not been demonstrated previously in humans or other mammals.

The stratified, squamous epithelium of the vocal fold sustains an electrophysiological PD and \(I_{sc}\). There are, however, obvious structural differences from surrounding pseudostratified, ciliated, columnar epithelium. The mean baseline PD (−9.3 ± 1.3 and −8.1 ± 2.8 mV for ovine and canine vocal folds, respectively) approximated that of ovine tracheal epithelium (lumen nega-
ative, \(-11.7 \pm 1.1 \text{ mV}\) when it was obtained with the use of the same apparatus (26). These estimates are lower than those of the trachea obtained in vivo and in vitro by using a Ringer-filled exploring bridge, where lumen-negative PD is approximately equal to \(-30\) and \(-37 \text{ mV}\) for canine and ovine tracheae, respectively (1, 2, 6, 20). It is possible that glandular and other secretory cells, numerous in the trachea but absent beneath the stratified squamous vocal fold epithelium (Figs. 1 and 2), could contribute to a greater PD for tracheal mucosa than for the vocal fold. On the other hand, these differences could be due to the “state” of the tissues.

Our membranes generated mean \(I_{sc}\) (ovine \(I_{sc} = 31 \pm 4 \mu \text{A/cm}^2\), canine \(I_{sc} = 41 \pm 10 \mu \text{A/cm}^2\)) much like those shown when the same apparatus is used for the ovine trachea [\(\text{mean} = 37 \pm 4 \mu \text{A/cm}^2\) (26)]. In this preparation, vocal fold membranes yielded electrical resistance values (ovine \(R_m = 342 \Omega \cdot \text{cm}^2\) and canine \(R_m = 254 \Omega \cdot \text{cm}^2\)) similar to the reported values for the trachea [ovine \(R_m = 362 \pm 33 \Omega \cdot \text{cm}^2\) (26)]. These values of resistance are notably lower than the buccal mucosa [canine \(R_m = 1,090 \pm 100 \Omega \cdot \text{cm}^2\) (24)] and vaginal mucosa [rat \(R_m = 827–2,366 \Omega\), depending on the stage of the oestrus cycle (7)]. The PD and \(I_{sc}\) of the tracheal epithelium are generated by a pseudostratified epithelial layer with tight junctions near the apical membrane, providing a high-resistance pericellular return path. The PD and \(I_{sc}\) of the vocal fold epithelium appear to be generated by multiple cells in series and a convoluted pericellular return path. It is not clear whether the PD is generated by the luminal squamous cells and/or the multiple basal and immediately adjacent cells in series together with a convoluted pericellular return path between these multiple layers of cells (Fig. 9). The maintenance of an electrochemical gradient and transmembrane resistance indicates the presence of a barrier to the diffusion of ions through the pericellular path. Some models of ion and water transport have incorporated cells in series (6, 30, 36). It could be that the luminal squamous cells are primarily responsible for the PD across the vocal fold, whereas the basal and immediately suprabasal cells are specialized for volume regulation. In the vocal fold epithelium, tight junctions or complete junctional complexes have not yet been identified in luminal cells, yet our physiological data and transmission electron microscopy (Fig. 9A) may suggest their presence. The mechanism(s) and structures underlying polarization of the vocal fold and its vectorial fluxes of water await further elucidation.

The bidirectional, transepithelial water fluxes of the ovine vocal folds \((J_{w B \rightarrow L} \text{ of } 5.1 \pm 0.3 \mu \text{L} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}\) and \(J_{w L \rightarrow B} \text{ of } 5.2 \pm 0.2 \mu \text{L} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}\)) approximated the magnitude found by using the same method in ovine tracheae \((J_{w B \rightarrow L} \text{ of } 4.5 \pm 0.3 \mu \text{L} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}\) and \(J_{w L \rightarrow B} \text{ of } 6.1 \pm 0.3 \mu \text{L} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}\) (25, 26)). As any pressure differential was \(<3 \text{ mmH}_2\text{O}\), its contribution to the measured water flux would be negligible. When tritiated water is used, Phipps and colleagues (27) have reported similar water flux values for the tracheal epithelium of sheep. The tracheal mucosa, however, favors a baseline net flux of absorption (25, 26), whereas this degree of asymmetry was not evident in the baseline water fluxes across the vocal fold mucosa. In this respect, we note that the method reported here is good for measuring unidirectional water fluxes. Subtraction of the fluxes propagates the experimental error, leading to uncertainty in the net flux, given the number of tissues studied. The present method, however, is improved over those using tritiated water, as the water fluxes in both directions are measured simultaneously in the same tissue.

The magnitude of acetylstrophanthidin-induced inhibition of water fluxes depended significantly on flux direction, with \(J_{w B \rightarrow L}\) reduced by 25% and \(J_{w L \rightarrow B}\) by 15%. This suggests that the water flux toward the mucosa involves active \(\text{Na}^+\) transport. Water flux toward the lumen may involve the transport of an indirectly regulated counterion such as \(\text{Cl}^-\) or \(\text{HCO}_3^-\). In the trachea also, acetylstrophanthidin decreased the \(J_{w L \rightarrow B}\) (26). When aerosolized acetylstrophanthidin is administered to the trachea in vivo, there is an increase in airway hydration (1, 2) and mucociliary transport (40). Superficial application of acetylstrophanthidin to the vocal folds can be predicted to induce swelling of epithelial cells as well as reduce fluid absorption, thereby increasing the hydration of the vocal fold epithelium as well as the surface fluid. Present findings suggest that topical pharmacological approaches may be able to exploit active ion transport to fine-tune the volume, rheological properties, and superficial hydration of vocal fold mucosa.

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