Peripheral opioidergic regulation of the tracheobronchial mucociliary transport system

Lian Wang, Ruslan L. Tiniakov, and Donovan B. Yeates

Departments of Medicine and Bioengineering University of Illinois at Chicago, and Veterans Affairs Health Care System, West Side, Chicago, Illinois 60612

Submitted 12 August 2002; accepted in final form 22 February 2003

Wang, Lian, Ruslan L. Tiniakov, and Donovan B. Yeates. Peripheral opioidergic regulation of the tracheobronchial mucociliary transport system. J Appl Physiol 94: 2375–2383, 2003. First published February 28, 2003; 10.1152/japplphysiol.00741.2002.—We hypothesized that, in the airway mucosa, opioids are inhibitory neural modulators that cause an increase in net water absorption in the airway mucosa (as in the gut). Changes in bidirectional water fluxes across ovine tracheal mucosa in response to basolateral application of the opioid peptides β-endorphin, dynorphin A-(1–8), and [d-Ala², d-Leu⁵]-enkephalin (DADLE) were measured. β-Endorphin and dynorphin A-(1–8) decreased luminal-to-basolateral water fluxes, and dynorphin A-(1–8) and DADLE increased basolateral-to-luminal water flux. These responses were electroneutral. In seven beagle dogs, administration of aerosolized β-endorphin (1 mg) to the tracheobronchial airways decreased the clearance of radiotagged particles from the bronchi in 1 h from 34.7 to 22.0% (P < 0.001). Naloxone abrogated the β-endorphin-induced changes in vitro and in vivo. Contrary to our hypothesis, the opioid-induced changes in water fluxes would all lead to a predictable increase in airway surface fluid. The β-endorphin-induced increases in airway fluid together with reduced bronchial mucociliary clearance may produce procongestive responses when opioids are administered as antitussives.

opioidergic regulation; transepithelial water transport; β-endorphin; naloxone

IN THE CONDUCTING AIRWAYS of the lungs, the mucociliary transport system is arguably primarily responsible for protecting the lungs and the body from insults due to inhaled toxins, air pollutants, pathogens, and antigens while maintaining patent airways to facilitate the passage of air into and out of the alveoli. To fulfill this role, the mucociliary transport system should operate at a very low level when demands to its capacity are likely low, as during sleep (4, 13), yet, on challenge, be capable of dramatic increases in its ability to rid the airways of a respiratory and bodily threat, such as may be caused by inhaled allergens that can induce anaphylactic reactions (43). Whereas neural, humoral, and cellular mechanisms that stimulate the mucociliary transport system have been described (36, 44), the documentation of inhibitory mechanisms has been much more elusive. Inhibitory neural control of the mucociliary transport has been demonstrated in the nasopharynx (6, 7) and has been postulated in the intrathoracic airways by Yeates and colleagues (39, 43). Inhibitory neural regulation could sustain a low level of mucociliary transport either by suppressing ciliary beat frequency (CBF) or mucus secretion or by reducing the volume of the periciliary fluid. Clearly, such a system could maintain an efficient low level of mucociliary activity when the capacity for rapid cleansing of the airways is not required. The inhibition of such inhibitory mechanisms could enable excitatory neural or cellular pathways to facilitate a rapid increase in mucociliary clearance. Thus, together with the excitatory neural pathways, the inhibitory neural pathways could provide an efficient, robust, yet highly responsive system to defend the airways and the body against inhaled insults.

In the gut, opioid agonists have been shown to increase Na⁺ and Cl⁻ absorption and inhibit Cl⁻ secretion (31), actions that are consistent with the observed opioid-induced reduction in transport of water, Na⁺, and Cl⁻ into the ileum lumen (2) as well as the presence of opioid receptors in intestinal cells (19). Thus, in the gut, the observed decrease in water absorption associated with surgical denervation of a segment (17) could be due to a decrease in opioidergic action. Opioidergic nerves have been identified in the airway mucosa, and the presence of opioid binding sites in respiratory system has been observed (2, 4). The net absorption of fluid from the airway surface liquid, as it is transported up the converging bronchial airways, maintains the mucus overlying the cilia in close proximity to the propelling tips of the cilia. Albeit the propensity to absorb fluid in the gut is likely to exceed that of the conducting airways, given the embryonic relationships of the gut and the lungs, their exposure to the external environment, and their need to both absorb water in the basal state yet secrete water on exposure to a noxious challenge, we questioned whether a similar inhibitory neural regulation causes an increased absorption of fluid in the airway. Thus we hypothesized that opioid peptides are inhibitory modulators that cause increased net water absorption in the airway.

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Address for reprint requests and other correspondence: D. B. Yeates, Univ. of Illinois at Chicago, M/C 788, 1940 W. Taylor St. RM#212, Chicago, IL 60612 (E-mail: yeates-d@uic.edu).
To determine the role of opioids in the regulation of airway mucosal function, we investigated the action of the naturally occurring opioid peptides β-endorphin and dynorphin A-(1–8) and the synthetic opioid peptide [d-Ala², D-Leu⁵]-enkephalin (DADLE) on the transepithelial water fluxes across the ovine epithelial membranes in vitro. Our in vitro data revealed that administration of β-endorphin resulted in an electroneutral decrease in luminal-to-basolateral water flux ($J_{w-L-B}$) that persisted for at least 1 h; DADLE caused an increase of basolateral-to-luminal water flux ($J_{w-B-L}$) of short duration whereas dynorphin A-(1–8) both decreased $J_{w-L-B}$ and increased $J_{w-B-L}$, also of short duration. These data suggest that opioids may have potential therapeutic value in increasing the clearance of secretions from the lungs. To date, agents that increase net flux of water across the mucosa toward the lumen in vivo increase bronchial mucociliary transport in vivo (8, 37), and perturbations, which predictably dehydrate the airways, decrease bronchial mucociliary transport (38). These observations are consistent with theoretical models of mucociliary transport, which predict large changes in the rate of mucus transport for small changes in the periciliary fluid depth (41). However, codeine, an opioid agonist, has been shown to decrease CBF (21). We, therefore, chose to determine how β-endorphin delivered by aerosol to the airways in vivo would alter basal bronchial mucociliary clearance (BMC) in unanesthetized dogs. β-Endorphin decreased BMC. To evaluate whether the β-endorphin-induced changes in water fluxes and in bronchial mucociliary clearance were specific to the activation of opioid receptors in the mucosa, we investigated whether the opioid antagonist naloxone would abolish these responses.

**METHODS AND MATERIALS**

**Epithelium Membrane Study**

Water and ion-transport measurement system. A pair of identical measurement systems was used to simultaneously measure the unidirectional water fluxes across a biological membrane as well as monitor the electrophysiological parameters (26). Briefly, the measurement of the vectorial water fluxes exploits the properties of the membrane-impermeable fluorescent probe molecule, 8-aminonaphthalene-1,3,6-trisulfonate (ANTS; Molecular Probes, Eugene, OR), which has a threefold higher quantum yield in D₂O-based (99.9 atom% D, Sigma-Aldrich, St. Louis, MO) over H₂O-based Hanks’ balanced salt solutions (HBSS; in mM: 136.8 NaCl, 5.6 dextrose, 5.4 KCl, 4.2 NaHCO₃, 1.3 CaCl₂, 0.8 MgSO₄, 0.4 KH₂PO₄, 0.3 Na₂HPO₄; ICN, Costa Mesa, CA). Each membrane was mounted between the half-chambers. Underlying connective tissue, smooth muscle, and cartilage. These membranes were mounted between the half-chambers. Initially, each membrane underwent a 0.5-h equilibration with 4 mL H₂O-HBSS as bathing solution on each side. The H₂O-HBSS bathing solutions in the luminal and basolateral sides, these solutions were again replaced with H₂O-HBSS-ANTS solution. The volume of each half-chamber and its associated circulation loop totaled 4 mL. These solutions were circulated at a flow rate of 20 mL/min past the membrane and through a quartz tube (33458, Heraeus Amersil, Buford, GA) incorporated in each circulation loop. Violet light of 394-nm wavelength was used to excite the ANTS in each quartz tube. The emitted fluorescence from the ANTS in each quartz tube was collected by a photon-counting photomultiplier tube (Hamamatsu, Bridgewater, NJ) at a wavelength of 515 nm. The $J_{w-L-B}$ was proportional to the gradient of the increase in fluorescence detected on the luminal side, and the $J_{w-B-L}$ was proportional to the gradient of the decrease in fluorescence detected on the basolateral side. Two electrodes (no. 3435, NaviCyte, Reno, NV) were placed on each side of each membrane to enable the measurement of the potential difference (PD) via a current-voltage clamp (DVC1000, WPI, Sarasota, FL). The bathing solutions were maintained at 37°C using Peltier heater/coolers (D12–8, Marlow Industries, Dallas, TX) in conjunction with type J thermocouples incorporated into the circulation loops and their respective PD (proportional-integral-derivative) temperature controller (CN8500, Omega, Stamford, CT). The media were oxygenated by 95% O₂ and 5% CO₂ flowing at 25 ml/min through thin-walled gas-permeable silicone tubes (STSH-T-012, Sani-Tech, Lafayette, NJ).

The fluorescent photon counts were acquired in sequential 10-min intervals. These raw photon counts were analyzed off-line. A running median filter (10) was employed to remove any outliers (spikes in the data) caused by the environmental interference on the photomultiplier tubes. After background subtraction and normalization, the relative water fluxes, i.e., the temporal changes of the fluorescent photon counts in the basolateral and luminal solutions caused by the fluxes of H₂O and D₂O across the membrane, respectively, were determined by linear regression performed at 10-min intervals. The absolute water flux during each 10-min period could be derived from the corresponding slope by a simple multiplication, with a factor estimated by system calibration. The water flux data were acquired under open-circuit conditions. The PD across the membrane was monitored throughout each experiment.

**Experimental procedure and protocol.** Before the measurement of water fluxes, the system was filled with HBSS without a membrane inserted and was allowed to equilibrate at 37°C. Any PD between the voltage-sensing electrodes was nulled, and the series resistance compensation circuitry was adjusted to compensate for the resistance of the fluid. The system was drained. Ovine tracheae, transported from the local abattoir in 4°C HBSS, were cut longitudinally through the anterior aspect to expose the posterior epithelium. Posterior epithelia (1.3 × 1.3 cm) were dissected free of the underlying connective tissue, smooth muscle, and cartilage. These membranes were mounted between the half-chambers. Initially, each membrane underwent a 0.5-h equilibration with 4 mL H₂O-HBSS as bathing solution on each side. The H₂O-HBSS bathing solutions in the luminal and basolateral sides were then replaced by 4 mL H₂O-HBSS-ANTS and 4 mL D₂O-HBSS-ANTS, respectively. Immediately after the solutions were changed, measurements of the fluorescent counts from both the luminal and basolateral media were recorded over a 1-h period as a prechallenge control (Fig. 1, A and B). After another 0.5-h equilibration with H₂O-HBSS on both sides, these solutions were again replaced with H₂O-HBSS-ANTS in luminal bath and D₂O-HBSS-ANTS in basolateral bath. Because endogenous opioids would be expected to have greater access to the submucosa than to the apical surface of the epithelial cells, the test agents were added to the basolateral bath. This was done immediately after the solution change. The fluorescent photon counts were counted for 1 h (Fig. 1, C and D). Relative water fluxes, derived by averaging the slopes of the fluorescent counts for three consecutive 10-min intervals, were determined over the two sequential 30-min
periods. Relative changes (%) in water fluxes were estimated by comparing the prechallenge slope to the postchallenge slope on the same membrane (Fig. 1, E and F). If, during an experiment, the PD fell below 4 mV, those data were excluded from further processing. The value of a PD recorded in the midpoint of prechallenge period was considered the basal PD value.

In separate experiments, the electrophysiological responses of the tissues to the agents tested were also obtained in an Ussing chamber system (34) with voltage-current clamp (DVC1000, WPI, Sarasota, FL). The agents were added to the basolateral bath. The baseline PD and short-circuit current \( (I_{sc}) \) were measured before the addition of each agent(s). The system was then set to either open circuit or short circuit to measure the responses of either PD or \( I_{sc} \), respectively. After the 10-min equilibration period, the test agent(s) was administered.

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Measurement of lung retention. The dog, in the standing position, was comfortably immobilized in the sling of a restraint system in front of a PHOGAMMA LPOV scintillation gamma camera (model 6413, Searle Radiographics, Des Plaines, IL) such that the dog’s left lateral chest abutted the face of the gamma camera (27). The gamma camera was coupled to a personal computer equipped with Nuclear MAX Plus V3.1 program (MEDX, Arlington Heights, IL). This

**Data analysis.** The relative changes (%) in \( J_{\text{fl}}^{w} \) and \( J_{\text{fl}}^{b} \), as well as the PD and \( I_{sc} \), were presented as means \( \pm SE \). Paired-difference, two-tailed, Student’s \( t \)-tests were performed to determine whether the changes in vectorial water flux values before and after the challenge in each group were significant. One-tailed Student’s \( t \)-tests (assuming equal variances) were used to examine the difference between treatment groups.

**Animal Study**

**Measurement of BMR.** Bronchial mucociliary retention (BMR) in beagle dogs was measured by using radioaerosol techniques combined with nuclear imaging similar to that described by Piel et al. (27).

**Radioaerosol generation and delivery.** Iron oxide colloid \((1.45\%)\) (BioTechPlex, Chicago, IL) was labeled with \( ^{99m}\)Tc (Mallinckrodt, Chicago, IL), as previously described (35), by using an Amicon stirring cell (model 52) fitted with a PM 10 Diallo ultrafiltration membrane (Amicon, Beverly, MA) under a nitrogen atmosphere. The system used to generate and deliver the radioaerosol particles to the dog has been described in detail elsewhere (28). Briefly, the radiolabeled iron oxide colloid was injected via an infusion pump (Harvard 600–900VDCM, Cover, MA) at the rate of 0.51 ml/min into a jet nebulizer (model CSL, Turbotak, Waterloo, Ontario, Canada) that was operated at an airflow of \(-8\) l/min. Heated air \((-100\) l/min) was added to dry the aerosol. This diluted aerosol was concentrated by virtual impaction. In this concentrator, the aerosol particles were directed into the large end of seven cones arranged in parallel. A plate containing seven holes (3.9 mm in diameter) aligned with the cones was positioned 4.2 mm away from the outlet ends (3.0 mm in diameter) of the cones. The particles were propelled through the holes in the plate because of their high inertia relative to air, whereas the most (80%) of the air was extracted from the space between the cones and the plate. The concentrated radioaerosol particles were contained in the output air. The aerosol generation system and the delivery system were maintained at a positive pressure of 20–25 cmH\(_2\)O. Inhalation and exhalation were regulated by the electronically controlled temporal sequencing of two high-flow straight-through solenoid valves (model EF80171, Automatic Switch, Florham Park, NJ). The inhalation valve (normally closed) was actuated for 2 s and then closed for a 1.5-s breath hold, after which the exhalation valve was opened for 2 s. There was a 4.5-s delay before the beginning of the next breath. This resulted in aerosol being delivered to the dog at 6 breaths/min.

**Measurement of lung retention.** The dog, in the standing position, was comfortably immobilized in the sling of a restraint system in front of a PHOGAMMA LPOV scintillation gamma camera (model 6413, Searle Radiographics, Des Plaines, IL) such that the dog’s left lateral chest abutted the face of the gamma camera (27). The gamma camera was coupled to a personal computer equipped with Nuclear MAX Plus V3.1 program (MEDX, Arlington Heights, IL). This
program enabled the collection, display, and processing of the scintigrams. To prevent regions of high activity outside the lung regions from obscuring the lung image, a 6-mm-thick lead shield with a cutout coinciding with the region of the lateral projection of the lungs of the largest dog, was placed on the face of the gamma camera’s detector head. The legs of the restrained dog passed through holes in the sling. This sling was suspended from a stainless steel frame mounted on a stainless steel tray. The dog’s legs were immobilized by using gauze hobbles that were tied to the frame. The dog was also secured by two leather straps, one of which fitted snugly over the dog’s lumbar area and the other loosely over the chest. The frame design allowed for horizontal and vertical positioning of the sling such that the image of the dog’s lungs could be centered within the cutout of the lead shield. To prevent the dog from moving its body forward, a U-shaped, slightly concave stainless steel plate was positioned with slight pressure on the tissues anterior to and including the cranial border of the scapula while avoiding pressure on the trachea. When necessary, the dog’s head was immobilized by a combination of a chin rest and slight forward tension on a two-tined fork attached into the anatomical depression behind the dog’s head formed by the atlantocipital joint. A vertical sliding plate, which fitted onto the chin rest, acted as a blinder. The plate, chin rest, and fork were secured to the frame and were all adjustable.

Animal preparation and animal protocol. Seven male beagle dogs (Covance) between 4 and 5 yr old, weighing between 11 and 16 kg, were studied. All dogs were housed at the Veterans Affairs Chicago Health Care System, West Side, animal facility. The National Research Council’s Guide for the Care and Use of Laboratory Animals was followed throughout this study. The studies were approved by the Institutional Animal Care Committees.

Twelve hours before a study, each dog was fasted but allowed free access to water. The dog was placed in the restraint system. The dog was anesthetized with intravenous bolus of propofol (7 mg/kg, AstraZeneca Pharmaceuticals, Wilmington, DE) and immediately intubated. The endotracheal tube (7.5–8.5 mm ID, Mallinckrodt Medical, Argyle, NY) was positioned in the proximal trachea with the inflated cuff placed just beyond the vocal cleft. While dogs were under anesthesia, the radioaerosol was delivered through the endotracheal tube for 3 min or until 100 μCi of 99mTc was deposited in the dog’s lungs, whichever was reached first. The test drug(s) were then administered. The dog’s position was adjusted such that the image of the activity in the lungs was centered within an outline scribed onto a persistence oscilloscope of the border of the cutout in the lead shield. About 6 min after the intubation, the laryngeal reflex returned and the dog was extubated. When the dog’s normal breathing pattern resumed (2–6 min after the extubation), 60 sequential 1-min numerical lung images (64 × 64 pixels) were collected. During the first 5 min of data acquisition, the dog was fed ~100 g of moisturized dog chow to clear the mouth and esophagus of any radioactive particles. One of the investigators was present to attend to the physical needs of the animal so as to minimize any animal movement. After completion of this experimental period, the dog was placed in radiation isolation quarters, with food and water provided ad libitum. Each dog was again placed in front of the gamma camera ~24 h after aerosol deposition, and 10 sequential 1-min images were made of the activity remaining in the lungs. This represents an index of the aerosol particles deposited in the alveolar regions of the lungs (40). It was assumed that particles in the tracheobronchial airways were all removed within this 24-h time period and that the particles in the alveoli clear with a much longer half-time. No anesthesia or intubation was needed for the repeat measurement. In addition, 10 images of background radioactivity were recorded, each for 1 min, before each radioaerosol inhalation and each 24-h measurement in each dog. After a preview of the collected 60 sequential images, a region of interest was selected such that the activity in all 60 images was included. The number of counts in this region was obtained for each lung image as well as for background radiation. The measurements of retained radioactivity within the lung were corrected for background and radioactive decay. The resultant counts represent the retention of the iron oxide particles in the lungs. The activity of particles deposited in the alveoli (24-h measurement) was subtracted from the activities measured in the first hour. To obtain the BMR curve, these values were normalized to the initial value. The bronchial mucociliary clearance in 1 h (BMC0) was the difference between the initial and the ending time points of BMR curve. The 24-h retention (R24(%)) was used as an indicator to evaluate the aerosol delivery pattern. It was defined as the ratio (%) of the activity deposited in the alveoli (24-h measurement) to the initial whole lung activity.

Experimental protocols were conducted using a randomized block design. The BMR in each dog was measured under four conditions: 1) administration of aerosolized β-endorphin; 2) intramuscular administration of naloxone; 3) coadministration of naloxone and β-endorphin; and 4) control. To determine whether β-endorphin decreases BMC, 1 mg β-endorphin, dissolved in 250 μl saline, was administered by aerosol to the dog tracheobronchial airway. This dose was chosen as in preliminary experiments when, administered by aerosol, it appeared to decrease bronchial clearance whereas, when administered intravenously, no measurable effect on bronchial clearance could be discerned. To determine whether basal mucociliary transport is under tonic opioidergic control, 2 mg naloxone prepared in 2 ml saline were injected to the dog intramuscularly (im). To determine whether naloxone abolishes the hypothesized β-endorphin-induced inhibition of mucociliary clearance, naloxone (im) was administered before the β-endorphin delivery. In the control study, 250 μl saline aerosol was administered to tracheobronchial airways. All chemicals were purchased from Sigma-Aldrich. The aerosols were delivered to the tracheobronchial airways through a MicroSprayer catheter (Penn-Century, Philadelphia, PA), 40 cm in length and 1 mm in diameter, which was inserted through the lumen of the endotracheal tube such that the atomizing nozzle at the end of the catheter protruded ~5 mm past the distal tip of the endotracheal tube (8). A stainless steel syringe (Penn-Century) attached to the Micro Sprayer catheter was used to pressurize the challenging agent through the catheter into the tracheal lumen. This device gives particle sizes of volume median diameter of 22 μm.

Data analysis. The R24(%) and BMC0 from the studies were summarized as means ± SE. R24(%) and BMC0 were examined for differences among treatment groups by single-factor ANOVA.

RESULTS

In Vitro Water and Ion Fluxes Studies in Ovine Trachea

The PDs monitored in 87 ovine tracheal epithelial membranes, mounted in the water flux measurement system, demonstrated that the viability of tissues was maintained throughout the experimental procedure. The mean PD at the end of the first equilibration...
period was 13.9 ± 0.6 mV. At the midpoint of the prechallenge period the PD was 11.9 ± 0.5 mV, and at the midpoint of the perturbation period it was 10.6 ± 0.6 mV. The mean basal PD of −11.9 ± 0.5 mV (lumen negative) in these tissues was similar to the mean basal PD of −12.4 ± 0.4 mV, measured in the tissues (n = 87) mounted in the Ussing chamber. For tissues mounted in the Ussing chamber, the mean basal I_sc was 51 ± 1 μA/cm². The mean resistance of these tissues, calculated by dividing the open-circuit PD by the I_sc, was therefore 221 ± 6 Ω·cm².

The bioelectric responses of ovine epithelium to the vehicle and treatments are shown in Fig. 2. No changes in PD could be attributed to the action of any of the test agents (Fig. 2A). A minor transient (10 min) increase in I_sc was observed immediately after the application of β-endorphin (Fig. 2B). No changes in I_sc could be attributed to the action of any of the other agents tested. Notably, whereas naloxone itself did not affect the I_sc, it abolished the small transient increase of I_sc induced by β-endorphin.

The mean percent changes in unidirectional trans-epithelial water fluxes induced by the vehicle and the three opioid peptides tested are shown in Fig. 3. No significant changes in water fluxes were observed in the sham experiments (n = 14). In the first 30-min period after the application of β-endorphin (n = 14), the J_L decreased by 7.1 ± 2.6%, significantly different from zero (P = 0.016) and from sham (P = 0.009). This β-endorphin-induced response persisted for 30 more min with the decrease being 6.7 ± 2.4% (P = 0.015 vs. zero and P = 0.02 vs. sham). Rather than decrease J_L, DADLE (n = 15) caused an increase in J_L by 6.3 ± 2.0% (P = 0.008 vs. zero, and P = 0.006 vs. sham). Dynorphin A-(1–8) (n = 15) not only caused the suppression of J_L by 6.2 ± 1.9% (P = 0.006 vs. zero and P = 0.006 vs. sham) but also caused J_B to increase by 4.2 ± 1.8% (P = 0.03 vs. zero and P = 0.02 vs. sham). The significant changes in water fluxes induced by DADLE and dynorphin A-(1–8) were limited to the 30 min after their application. Thus β-endorphin induced a more sustained effect on the water fluxes across the ovine tracheal epithelial membrane. None of the responses to these opioids was proabsorptive, as hypothesized.

The effects of naloxone, the opioid antagonist, on the basal water fluxes and the β-endorphin-induced changes in water fluxes are illustrated in Fig. 4. Naloxone alone (n = 14) did not cause significant changes in the basal J_L or J_B. Also, the basal water fluxes were not significantly changed by coadministration of...
Fig. 3. Changes in unidirectional water fluxes (expressed as a percentage of the respective prechallenge water flux) induced by sham, β-endorphin, DADLE, and dynorphin A-(1–8) (n = 14–15) for the first (A) and second (B) 30-min periods after administration of the test agents. Error bars are SE. *P < 0.05, **P < 0.01 vs. zero; †P < 0.05, ††P < 0.01 vs. sham group.

β-endorphin and naloxone (n = 15). Naloxone abolished the β-endorphin-induced decrease in $J_{w-L}^B$ during both the first ($P = 0.03$) and the second 30-min periods ($P = 0.01$).

The water flux responses of the ovine tissues to DADLE and dynorphin A-(1–8) were electrically silent. The 1-h-long increases in $J_{w-L}^B$ in response to β-endorphin were accompanied by only a minor and transient (<10 min) increase in the $I_{sc}$, whereas there were no changes in PD that could be attributed to β-endorphin administration. This perturbation of $I_{sc}$ induced by β-endorphin was also mediated through opioid receptors, as naloxone abolished this response. On the basis of the discordant timing of the β-endorphin-induced inhibition of the $J_{L-B}^w$ and minor temporal increase in the $I_{sc}$, it is likely that these responses are two independent events that are both mediated through the opioid receptors.

In Vivo Bronchial Mucociliary Clearance Studies in Beagle Dogs

Examples of the bronchial retention curves for one representative dog under all four conditions (control, β-endorphin aerosol, naloxone im, and the combination of β-endorphin aerosol and naloxone im) are shown in Fig. 5. It can be seen in this dog that particles were cleared from the tracheobronchial airways slower after the treatment with β-endorphin than in the control experiment, whereas administration of naloxone or naloxone before β-endorphin resulted in retention curves that were indistinguishable from the control retention curve.

The averaged BMR curves under each of four experimental conditions are shown in Fig. 6. The mean BMC$_{60}$ in dogs treated with aerosolized β-endorphin was $22.0 \pm 2.1\%$, significantly less than that of the control experiments ($34.7 \pm 2.8\%; P < 0.001$). After the im naloxone administration, the mean BMC$_{60}$ was $36.3 \pm 3.9\%$. This did not differ from the control value ($P = 0.8$). Naloxone, as expected, abolished the inhibitory action of β-endorphin on the BMC$_{60}$ with the mean BMC$_{60}$ in the group being $35.2 \pm 4.3\% (P < 0.001$ vs. β-endorphin alone and $P = 0.6$ vs. control).

The mean $R_{24}(%)$ values were $6.3 \pm 0.7\%$, $7.9 \pm 0.9\%$, $7.7 \pm 0.9\%$, and $6.5 \pm 0.9\%$ for the control, aerosolized β-endorphin, naloxone im + aerosolized β-endorphin, and naloxone im groups, respectively. No statistical difference was found in the $R_{24}(%)$ values among the groups. These data suggest that the aerosol deposition patterns in the lungs for each experimental condition were similar.

DISCUSSION

We demonstrated herein that the opioid peptides β-endorphin, dynorphin A-(1–8), and DADLE caused agent-specific changes in vectorial fluid fluxes across the airway mucosa in vitro that were electrogenically silent. In each case, these changes in fluxes would lead to a predictable increase in airway surface fluid. β-Endorphin, primarily, caused a decrease in $J_{L-B}^w$, whereas DADLE, primarily, caused an increase of $J_{L-B}^w$. However, dynorphin A-(1–8) did both decrease $J_{L-B}^w$ and increase $J_{B-L}^w$. These observed changes in fluid transport were at variance with our hypothesis that opioids would enhance fluid absorption. β-Endorphin did not result in increase in mucociliary transport; rather, the delivery of β-endorphin by aerosol to the tracheobronchial airways decreased bronchial mucociliary clear-

Fig. 4. Changes in unidirectional water fluxes (expressed as a percentage of the respective prechallenge water flux) induced by sham, β-endorphin, naloxone, and β-endorphin + naloxone (n = 14–15) for the first (A) and second (B) 30-min periods after administration of the test agents. Error bars are SE. *P < 0.05 vs. zero; †P < 0.05, ††P < 0.01 vs. sham group; #P < 0.05 vs. β-endorphin group.
In conscious dogs. That the β-endorphin-induced changes in water fluxes and the decrease in bronchial mucociliary clearance were abolished by naloxone indicates that both of these responses were mediated via opioid receptors.

Dynorphin A-(1–8) predominantly binds to the κ-receptor with some δ-binding capacity, whereas DADLE is a δ-selective ligand and β-endorphin shows a higher binding affinity to μ- and δ-receptors than to the κ-receptor (12, 15). Thus differential activation of the opioid receptors and their respective intracellular pathways may be involved in the opioidergic regulation of water transport across the airway mucosa.

Studies have identified opioidergic nerves in the airway mucosa and have shown the presence of opioid binding sites in respiratory system (3, 5). Thus opioid peptides may affect airway function after local release of these peptides from either the nerves innervating the airways (1, 32) or the pulmonary endocrine cells (9). Alternatively, respiratory responses may be induced after the release of opioids from the anterior pituitary into the blood (24). Opioids have been shown to be involved in the inhibitory regulation of airway muscle tone, mucus secretion, and basal CBF. These responses were predominantly mediated through μ-opioid receptors (12, 21). These findings indicate that opioids, acting as either neuromodulators or hormones, play an important physiological role in the regulation of the functions of the bronchial mucosa.

Although changes in PD and $I_{sc}$ have often been used to predict changes in water transport, it is clear that changes in water fluxes across epithelia can be independent of predictable changes in transepithelial electrical parameters (25, 30). Also, electrogenically silent changes in water fluxes have been reported in the gut (14), the kidney (22), and now the lung. There are three possible underlying mechanisms for the observed electrosilent water transport herein: 1) Water fluxes may be coupled to separate cation and anion ion-transport mechanisms that were balanced so there was no net change in charge across the membrane. 2) Opioids may have altered the water fluxes by affecting electroneutral cotransporters, for example, Na$^+$/K$^+$/2Cl$^-$ co-transporter. Such cotransporters may transport water in and/or out of the cell (44, 45). 3) Albeit there are no reports on opioidergic regulation of aquaporins, it is possible that opioids may decrease the permeability of water channels in the epithelium (23). The elucidation of mechanisms by which the water fluxes across biological membranes are regulated remains a major challenge.

In this study, the relative changes of postchallenge water fluxes rather than the absolute values of water fluxes were derived, to avoid the frequent time-consuming calibration procedure. The magnitudes of the water flux values were similar to those of Phillips and colleagues (25, 26).

It is instructive to speculate as to the interpretation of the mechanisms involved with the observed in vitro and in vivo responses. Mucociliary clearance could decrease if β-endorphin caused the periciliary layer to become too deep. In experiments to date, perturbations that caused a predictable increase in airway hydration increased bronchial clearance (37), and those that predictably decreased airway hydration decreased mucociliary clearance (38). Impaired clearance through an opioid-induced increase in airway surface fluid is considered unlikely, especially considering the moderate changes in the β-endorphin-induced water fluxes observed. It is considered unlikely that there was a marked increase in mucus secretion induced by the awakening from the short-term propofol-induced anesthesia that, together with an increase in airway surface liquid, would cause impaired clearance. Codeine, an exogenous μ-opioid agonist, has been shown to suppress CBF in vitro dose dependently (21). The effective

![Fig. 5. Bronchial mucociliary retention (BMR) curves for dog 9256 after administration of either aerosolized saline, aerosolized β-endorphin, intramuscular naloxone, or the combination of intramuscular naloxone and aerosolized β-endorphin (Nal-Endo). The aerosolized challenge agents were delivered to the tracheobronchial airways.](image)

![Fig. 6. Mean BMR curves for 7 dogs after administration of either aerosolized saline, aerosolized β-endorphin, intramuscular naloxone, or Nal-Endo. The aerosolized challenge agents were delivered to the tracheobronchial airways. ***$P < 0.001$ vs. control group; ###$P < 0.001$ vs. β-endorphin group.](image)
concentration of codeine (10^{-7} g/ml) is comparable to our β-endorphin concentration (0.1 μM) used in the in vitro study given that the molecular weight of codeine is ~590 Da. Another study reported no change in CBF when 10 mg/kg codeine was administered intravenously to guinea pigs but did report slight decreases in CBF at higher doses of codeine (15 mg/kg) (16). However, in these experiments, the potent μ-opioid analgesic fentanyl administered to the animal may have masked a codeine-induced decrease in CBF in the excised tissues. Alternatively, decreased mucociliary clearance could also be expected if the β-endorphin inhibited the basal level of mucus secretion from the submucosal glands and goblet cells. Although the signal-transduction mechanisms regulating macromolecular secretions differ from those regulating transepithelial water fluxes, both mechanisms may be initiated through opioid receptors. Some in vitro studies showed that opioids diminished a challenge-induced mucus secretion in airway mucosa (18, 29), whereas another has shown opioid-induced stimulation of basal mucus secretion (20). However, in this latter study, β-endorphin had no effect on basal rate of mucus secretion. It is notable that the β-endorphin-induced decrease in mucociliary clearance was totally abrogated by the administration of intramuscularly administered naloxone, indicating that these agents acted on the same site in the airway mucosa. Although the influence of species specificity and the differences between in vivo and in vitro conditions (11) cannot be excluded, these data suggest that this observed decrease in mucociliary clearance was more likely caused by an opioid-induced reduction of CBF rather than due to decrease in mucus production or increase in periciliary fluid.

The effects of anesthetic agents on the mucociliary transport system may suppress the responses of the mucociliary transport system to the administration of β-endorphin. To avoid this potential problem, we chose to conduct our experiments with minimal use of non-opioidergic anesthetics. A temporary state of anesthesia was induced with a short-acting hypnotic, propofol, to facilitate intubation and the controlled delivery of radioactive aerosol to the lungs, as well as the administration of β-endorphin to the tracheobronchial airways through the MicroSprayer catheter. To minimize any possible effects caused by anesthetic, we commenced data acquisition when the dog regained consciousness and reassumed its normal breathing pattern.

We chose to administer β-endorphin topically, by aerosol, to deliver a relatively high effective concentration on the surface of the trachea and proximal bronchial airways while minimizing any possible adverse effects that might occur if the peptide was administered intravenously. Although β-endorphin is not able to cross the blood-brain barrier, there are a number of peripheral targets. For example, μ-opioid agonists have been shown to reduce both heart rate and blood pressure (15, 24). The opioid-induced respiratory suppression is also a well-known phenomenon (15) that we chose to avoid.

Opioids are the most powerful cough suppressors used in clinical practice. Their role in the inhibition of mucociliary clearance is not consistent with the need to maintain bronchial hygiene, especially in the absence of cough. This may be exacerbated by their induced changes in water fluxes, which are predicted to cause an increase in the volume of airway secretions (33). However, such an increase in airway surface fluid would be expected to aid the clearance of mucus by cough, if it occurs.

The β-endorphin-induced procongestive effect, as indicated by an increase in airway fluid, decrease in ciliary activity, and mucociliary clearance, may have little significance under the resting physiological conditions, when basal activity of endogenous opioid system is minimal. It may be of importance, however, in many physiological and especially pathological conditions associated with an increase in activity of the endogenous opioid system. This may explain the decrease in mucus transport known to occur in humans during a night sleep (4) and the apparent increase of mucus clearance on awakening. Opioids are potential mediators for a proposed model of inhibitory neural regulation of tracheobronchial mucociliary clearance (42, 43). Also, stress-induced elevation of the level of endogenous opioids may play a role in the pathogenesis of some congestive lung diseases. An opioid-induced increase in airway fluid together with impairment of mucus transport induced by endogenously released β-endorphin or exogenous opiates may also contribute to the development of the chest congestion often observed in patients with severe traumas. Thus a more complete understanding of the role of the opioids in regulation of the mucociliary transport system under both normal and pathological conditions could produce considerable improvement in the treatment of congestive lung diseases.

These studies are contained in L. Wang’s thesis for a Master of Science degree in bioengineering. The studies were supported by the National Institute of Environmental Health Sciences Grant ES-08982. The canine experiments were performed at Veterans Affairs Chicago Health Care System, Westside Division.

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J Appl Physiol • VOL 94 • JUNE 2003 • www.jap.org


