

Effects of Long-acting Muscarinic Antagonists on Promoting Ciliary Function in Airway Epithelium

Mineo Katsumata

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

Tomoyuki Fujisawa (✉ fujisawa@hama-med.ac.jp)

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

<https://orcid.org/0000-0002-8809-1781>

Yosuke Kamiya

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

Yuko Tanaka

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

Chiaki Kamiya

Department of Clinical Pharmacology and Therapeutics, Hamamatsu University School of Medicine

Yusuke Inoue

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

Hironao Hozumi

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

Masato Karayama

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

Yuzo Suzuki

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

Kazuki Furuhashi

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

Noriyuki Enomoto

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

Yutaro Nakamura

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

Naoki Inui

Department of Clinical Pharmacology and Therapeutics, Hamamatsu University School of Medicine

Masato Maekawa

Department of Laboratory Medicine, Hamamatsu University School of Medicine

Mitsutoshi Setou

Department of Cellular and Molecular Anatomy and International Mass Imaging Center, Hamamatsu University School of Medicine

Hiroshi Watanabe

Department of Clinical Pharmacology and Therapeutics, Hamamatsu University School of Medicine

Koji Ikegami

Department of Anatomy and Developmental Biology, Graduate School of Biomedical and Health Sciences, Hiroshima University

Takafumi Suda

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine

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Abstract

Background

Mucociliary clearance (MCC) is an essential defense mechanism in airway epithelia for removing pathogens from the respiratory tract. Impaired ciliary functions and MCC have been demonstrated in asthma and [chronic obstructive pulmonary disease \(COPD\)](#). Long-acting *muscarinic antagonists* (LAMAs) are a major class of inhaled bronchodilators, which are used for treating asthma and COPD; however, the effects of LAMAs on ciliary function remain unclear. This study aimed to identify the effects of LAMAs on airway ciliary functions.

Methods

Wild-type BALB/c mice were treated with daily intranasal administrations of glycopyrronium for 7 days, and tracheal samples were collected. Cilia-driven flow and ciliary activity, including ciliary beat frequency (CBF), ciliary beating amplitude, effective stroke velocity, recovery stroke velocity and the ratio of effective stroke velocity to recovery stroke velocity, were analyzed by imaging techniques. Using in vitro murine models, tracheal tissues were transiently cultured in media with/without LAMAs, glycopyrronium or tiotropium, for 60 min. Cilia-driven flow and ciliary activity were then analyzed. Well-differentiated normal human bronchial epithelial (NHBE) cells were treated with glycopyrronium, tiotropium, or vehicle for 60 min, and CBF was evaluated. Several mechanistic analyses were performed.

Results

Intranasal glycopyrronium administration for 7 days significantly increased cilia-driven flow and ciliary activity in murine airway epithelium. In the murine tracheal organ culture models, treatment with glycopyrronium or tiotropium for 60 min significantly increased cilia-driven flow and ciliary activity in airway epithelium. Further, we confirmed that 60-min treatment with glycopyrronium or tiotropium directly increased CBF in well-differentiated NHBE cells. In the mechanistic analyses, neither treatment with glycopyrronium nor tiotropium affected intracellular calcium ion concentrations in well-differentiated NHBE cells. Glycopyrronium did not increase protein kinase A activity in well-differentiated NHBE cells. Moreover, glycopyrronium had no effect on extracellular adenosine triphosphate concentration.

Conclusions

LAMAs exert a direct effect on airway epithelium to enhance ciliary function, which may improve impaired MCC in asthma and COPD. Further investigations are warranted to elucidate the underlying mechanisms of the effects of LAMAs on the promotion of airway ciliary function.

Background

The airway epithelium plays a pivotal role in the host's defense against inhaled foreign substances such as viruses, bacteria, and airway pollutants, serving as a physical barrier and regulator of innate and

adaptive immune responses [1–5]. Mucociliary clearance (MCC) is an innate frontline defense mechanism in the airway epithelium that removes inhaled pathogens from the airways [6, 7]. The functional components of MCC are the protective mucus layer, airway surface liquid layer, and cilia on the ciliated cells, which comprise a major fraction of airway epithelial cells. Adequate ciliary functions are essential for effective MCC in transporting foreign materials, such as viruses and bacteria, trapped in the mucous layer towards the mouth. Ciliary beat frequency (CBF) is a major determinant of ciliary function [8–10]. The importance of ciliary function in the host's airway defense is well documented in primary ciliary dyskinesia, in which genetic defects in cilia structure and motility impair MCC and are associated with recurrent airway infection [11]. We recently showed that influenza A virus infection in the airway epithelium stimulated ciliary function, which hastened the elimination of viruses from the respiratory tract as one of the host's initial defense responses [10].

Asthma and chronic obstructive pulmonary disease (COPD) are chronic airway diseases characterized by symptomatic airflow limitation [12–14], and both are known to have impaired ciliary function and MCC. Studies have demonstrated that CBF was decreased in patients with moderate to severe asthma and those with asthma with sputum neutrophilia [15, 16]. In patients with COPD, CBF reductions in ciliated cells were observed especially in moderate to severe COPD [17, 18]. Ciliary dysfunction in asthma and COPD causes impairment of mucus clearance, resulting in worsening of airway obstruction and susceptibility to infection. Restoring ciliary function is therefore an important therapeutic target for patients with asthma and COPD. Inhaled corticosteroids (ICS), long-acting β 2-agonists (LABAs), and long-acting muscarinic antagonists (LAMAs) have been widely used in real-world clinical practice to treat asthma and COPD [13, 14]. LABAs and LAMAs act as bronchodilators in patients with asthma and COPD, and β 2 agonists have been shown to modulate ciliary function and MCC [17, 19, 20]. A study demonstrated that salmeterol, one of the major LABAs, increased CBF in nasal epithelial cells [17].

In contrast to LABAs, there is scarce evidence for the effects of LAMAs on ciliary function in airway epithelium. LAMAs, such as tiotropium bromide (TIO), glycopyrronium bromide (GLY), and aclidinium bromide, inhibit the muscarinic receptors of the bronchioles and relax airway smooth muscle. The M3 receptor, a muscarinic receptor subtype, is highly expressed in smooth muscles cells, submucosal glands, and airway epithelial cells in the lungs [21, 22]. Given that LAMAs have strong selectivity for the M3 receptor, LAMAs might act on airway epithelium, and have modulatory actions on ciliary functions in the airway epithelium.

In the present study, we investigated the effects of LAMAs on airway ciliary functions using in-vivo mouse models, organ cultures of murine tracheal tissue, and primary culture of normal human bronchial epithelial (NHBE) cells. Here, we demonstrate that LAMAs have a direct effect on airway epithelium to enhance ciliary function to improve MCC.

Methods

Mice

BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan), and all experiments involving the mice followed protocols approved by the Animal Care and Use Committees of Hamamatsu University School of Medicine, Shizuoka, Japan (license number H31-047) and were performed in accordance with the relevant guidelines and regulations.

Chemicals

GLY and TIO were purchased from Sigma-Aldrich and dissolved in phosphate-buffered saline (PBS). GLY was used at 300 μM for the intranasal administration to mice and at 100 nM for the organ culture of murine tracheal tissue and primary NHBE cell culture under air-liquid interface (ALI) conditions. TIO was used at 100 nM for culture conditions. 2-Aminoethoxydiphenylborane (2-APB), an inhibitor of inositol-trisphosphate receptor, was purchased from Tocris Bioscience (Bristol, UK) and used at 40 μM . N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide, dihydrochloride (H89), a protein kinase inhibitor with a high specificity for protein kinase A (PKA), was purchased from Cayman Chemical (Ann Arbor, MI, USA) and used at 10 μM .

Isolation of murine trachea and organ culture of tracheal tissue

Tracheas were taken from 9 to 11-week-old female BALB/c mice and placed in cold collection medium solution (Dulbecco's Modified Eagle Medium with sodium pyruvate solution) kept in an ice bath. Excess fat and connective tissue debris were immediately removed from the trachea using forceps. The membranous portion of the trachea was then excised to expose the ciliated epithelium in the trachea. After the dissection, the tracheal tissue was cultured in 2 mL of culture medium in the presence or absence of GLY or TIO in 35-mm culture dishes at 37°C for 60 min as an organ culture. The tracheal epithelium was then observed and analyzed at room temperature (23–28°C) using a dedicated microscope.

Daily intranasal administration of glycopyrronium to mice

The 8-week-old female BALB/c mice were anesthetized with a 3% isoflurane oxygen mixture and administered 25 μL of GLY solution (300 μM) or PBS alone intranasally once daily for 7 days. Mice were sacrificed on day 8, after which tracheas were taken. After the organ culture of trachea was prepared as described above, the tracheal epithelium was immediately observed and analyzed at room temperature (23–28°C) using a dedicated microscope.

Cell culture

Primary NHBE cells were purchased from Lonza (catalog no. CC-2541; Basel, Switzerland). NHBE cells in a submerged condition were cultured on 6.5-mm Transwell (Corning, NT, USA) using commercially available bronchial epithelial growth medium (BEGM; Lonza) and incubated at 37°C in a humidified atmosphere with 5% carbon dioxide. When the NHBE cells reached full confluency in an immersed culture condition, the cells were transferred to an ALI culture condition using ALI culture medium (HBTEC Air-Liquid Interface Differentiation Medium; Lifeline Cell Technology, Frederick, MD, USA), as previously

described with slight modifications [3, 4, 23, 24]. The cells were then cultured for 4 weeks in ALI conditions to facilitate polarization and ciliary differentiation for subsequent experiments [25, 26].

Analysis of cilia-driven flow

Ciliary transport on the surface of the intact murine trachea was analyzed after transient organ culture, as previously described [10]. To visualize the cilia-driven flow, tracheal tissue was placed in a 35-mm culture dish with the luminal face down in 2 mL of culture medium containing 0.2- μ m-diameter polystyrene beads (0.2- μ m red fluorescent beads: Thermo Fisher Scientific, Waltham, MA, USA). Methylcellulose (M0512, Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium at a concentration of 0.5% to stabilize the movement of fluorescent beads by increasing the viscosity of the medium. The movement of the beads under the tracheal epithelium was observed from the bottom of the dish using an inverted fluorescence microscope (Eclipse TE2000-U, Nikon, Tokyo, Japan) equipped with a CFI Plan Fluor objective lens (Nikon) and a CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). The optimal configuration of the lens for tracking the movement of the beads, which were 0.5–2 mm from the chamber bottom, was $\times 20$ magnification, an NA of 0.5, and a long working distance (2.1 mm). The velocity of each bead was calculated by dividing the width of the field of view by the time each individual bead took to travel across the field. Three or more independent tracheal samples and at least 10 fields of view in each sample were analyzed (more than 30 fields for each condition, 5 or more beads in each field). The fluid movement velocity was calculated using the beads' migration distance and travel time by tracking individual fluorescent beads using Aquacosmos image analysis software (Hamamatsu Photonics). The rainbow trace was depicted using a macro for the free software, ImageJ, from Hiratsuka laboratory of JAIST (<https://www.jaist.ac.jp/ms/labs/hiratsuka/>, in Japanese only).

Analysis of the ciliary beating orientation

The analysis was performed as described previously [8–10] with slight modifications. After the murine tracheal tissue was transiently cultured in culture medium, the cilia tips of the ciliated cells were labeled with Indian ink diluted with culture medium (1:100) to analyze the intact cilia. The motility of the ink-labeled cilia tips was recorded using HAS-L1 and HAS-U1 high-speed cameras (DITECT Co. Ltd, Tokyo, Japan) at 300 fps to reflect ciliary motility. The recording was performed at 23–28°C. CBF was determined by subjecting the original traces to a fast Fourier transform using Microsoft Excel. The ciliary beating amplitude, effective stroke velocity, recovery stroke velocity, and the ratio of effective stroke velocity to recovery stroke velocity were calculated from the movement of the cilia tips. The CBF data are presented as the median (ranges). The ciliary beating amplitude, effective stroke velocity, recovery stroke velocity, and effective stroke velocity-to-recovery stroke velocity ratio are presented as the mean \pm SEM. Fully differentiated NHBE cells cultured under ALI conditions for 4 weeks were also used to measure CBF. Three independent tracheal samples or NHBE cell samples were examined with at least 10 ink-labeled cilia in each sample analyzed; i.e. more than 30 ink-labeled cilia for each condition were analyzed. Kymographs of ciliary beating were depicted with a macro embedded in ImageJ.

Monitoring of intracellular calcium ion concentrations

Intracellular calcium ion (Ca^{2+}) concentrations were measured using fura-2/AM, a fluorescent Ca^{2+} indicator. NHBE cells cultured under ALI conditions for 4 weeks were incubated with 2.5 μM fura 2-AM (Dojindo, Kumamoto, Japan) for 30 min at 37°C, and the cells were treated with GLY or TIO at each concentration. Fluorescent images of fura-2/AM were acquired and quantified every 30 s or 120 s from individual cells with a fluorescence analyzer (Aquacosmos, Hamamatsu Photonics, Hamamatsu, Japan) using an ultra-high sensitivity camera. Changes in the fluorescence ratio (F340/F380) of fura-2 were used to express changes in the intracellular Ca^{2+} concentrations. Thapsigargin (TG), an inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase pump, which causes a transient increase of intracellular Ca^{2+} concentrations [27], was used for positive controls.

Analysis of protein kinase A (PKA) activity

NHBE cells cultured under ALI conditions for 4 weeks were treated with GLY for 60 min. The cells were then harvested using RIPA buffer (ATTO, Tokyo, Japan) and PKA activity was determined using nonradioactive PKA activity assay kits (Enzo Life Sciences, Ann Arbor, MI, USA), according to the manufacturer's instructions. Briefly, the PKA substrate microtiter plates were soaked in kinase assay dilution buffer at room temperature. The cell lysates (1 μg of protein) were subsequently added, followed by the addition of adenosine triphosphate (ATP) to initiate the reaction. After incubation at 30°C for 90 min, the phosphor-specific substrate antibody was added, and the reaction was incubated at room temperature for 1 h. The HRP-conjugated secondary anti-rabbit IgG was subsequently added to each well, and incubated for an additional 30 min. The TMB substrate solution was added to each well, and further incubated for 30 min. Lastly, the stop solution was added, and the 96-well plate was read at 450 nm in a microplate reader.

Adenosine triphosphate (ATP) measurements

ATP concentrations were measured in culture supernatants using an ATP assay kit based on luminometric techniques (Lucifell 250 plus, Kikkoman Biochemifa, Tokyo, Japan) according to the manufacturer's protocol. In total, 100 μL of culture medium from murine tracheal tissue with or without GLY were used. Briefly, 100 μL of the ATP extraction reagent was added to each sample, and after 20 sec, luciferin-luciferase (100 μL) was added to each sample. The luminescence of each sample was measured using a Lumitester C-100 (Kikkoman Biochemifa).

Statistical analysis

The results are presented as means \pm SEM, unless otherwise noted. Comparisons between the groups were performed using Student's t-test, with $p \leq 0.05$ was considered statistically significant.

Results

Continuous intranasal administration of GLY promotes cilia-driven flow and ciliary activity in the murine tracheal epithelium

We initially investigated whether the daily administration of GLY promotes ciliary functions in the murine tracheal epithelium. Wild-type (WT) mice were treated with GLY or PBS intranasally for 7 days. After 7 days of treatment, we resected the tracheal samples and analyzed the cilia-driven flow over the tracheal surface using small polystyrene beads in each condition (Additional file 1–2). Representative bead trajectories and movies are shown in Fig. 1A. As shown in Fig. 1B and 1C, the intranasal administration of GLY significantly increased cilia-driven flow compared with the control (PBS alone).

Ciliary motility was recorded using a microscope equipped with a high-speed digital camera (Additional file 3–4). Kymographs of the movies under the conditions of GLY administration and the control are shown in Fig. 1D. An analysis of the ciliary beating revealed that CBF was significantly increased by the GLY administration compared with the control (Fig. 1E). The ciliary beating amplitude did not differ between the conditions (Fig. 1F). Both the effective stroke velocity and recovery stroke velocity were significantly greater with the GLY administration than with the control (Figs. 1G and H). The ratio of effective stroke velocity to recovery stroke velocity did not differ between the conditions (Fig. 1I).

Taken together, these results demonstrate that the 1-week continuous intranasal administration of GLY promotes the cilia-driven fluid flow and ciliary activity in murine airway epithelium.

Short-term treatment with GLY or TIO promotes ciliary functions in the murine tracheal epithelium in organ culture models

Continuous, i.e. long-term, administration of drugs might cause unknown changes in tracheal tissues. To rule out those uncertain indirect effects of LAMAs on ciliary functions in airway epithelium, we next investigated the effects of short-term treatment with GLY or TIO (1 h) in a tracheal organ culture model on cilia-driven flow and ciliary activity in murine tracheal epithelium. Figure 2A presents the representative bead trajectories with and without GLY treatment. As shown in Figs. 2B and 2C, GLY treatment for 1 h significantly increased cilia-driven flow compared with the control level. Figure 2D shows the kymographs of the movies in the presence or absence of GLY in the culture media. An analysis of the ciliary beating revealed that CBF was significantly increased by GLY treatment compared with the control findings (Fig. 2E). The ciliary beating amplitude did not differ between the conditions (Fig. 2F). Both the effective stroke velocity and recovery stroke velocity were significantly greater in the presence of GLY than in its absence (Figs. 2, G and H). The ratio of effective stroke velocity to recovery stroke velocity did not differ between the conditions (Fig. 2I).

We performed the same experiments using TIO, another LAMA, to observe the promoting effects on cilia-driven flow and ciliary activity in murine tracheal epithelium. Figure 3A presents the representative bead trajectories. As shown in Fig. 3B and 3C, TIO treatment significantly increased cilia-driven flow compared with the control level. Figure 3D shows the kymographs of the movies in the presence or absence of TIO. An analysis of the ciliary beating revealed that CBF was significantly increased by TIO treatment compared with the control findings (Fig. 3E). The ciliary beating amplitude did not differ between the conditions (Fig. 3F). The effective stroke velocity and recovery stroke velocity were significantly greater in

the presence of TIO than in the control (Figs. 3G and H). The ratio of effective stroke velocity to recovery stroke velocity did not differ between the conditions (Fig. 3I).

Collectively, these results indicate that short-term treatment with GLY or TIO have a potential to enhance cilia-driven flow and ciliary activity in murine airway epithelium.

LAMAs directly increase CBF in NHBE cells cultured on ALI condition

We further aimed to determine whether LAMAs have a direct promoting effect on airway ciliary functions on the human airway epithelium. To facilitate ciliated cell differentiation, NHBE cells were cultured for 4 weeks under ALI condition. Using well-differentiated NHBE cells cultured under ALI conditions for 4 weeks, we investigated whether short-term (1 h) treatments with GLY or TIO increased CBF. Figure 4A and 4C shows the ciliary motion kymographs. As shown in Fig. 4B, CBF was significantly increased by GLY treatment for 1 h compared with the control. Similarly, TIO treatment significantly increased CBF in NHBE cells under ALI conditions (Fig. 4D).

Taken together, all the results from the experiments using mouse models and well-differentiated NHBE cell cultures provide evidence that LAMAs directly enhance ciliary activity and promote ciliary clearance in the airway epithelium.

Search for the mechanism of LAMA-mediated promotion of ciliary functions

We investigated the underlying mechanisms of LAMA-mediated promotion of ciliary functions. The change of intracellular Ca^{2+} concentration is one of the mechanisms that modulate ciliary beating in airway epithelium [28, 29]. To assess whether the increase of intracellular Ca^{2+} concentration was involved in the LAMA-mediated promotion of ciliary functions, we used 2-APB, an inhibitor of inositol-trisphosphate receptor, to inhibit Ca^{2+} release from endoplasmic reticula [30]. The murine tracheal tissues were incubated with GLY or TIO with or without 2-APB for 1 h, and cilia-driven flow and CBF were evaluated. The 2-APB showed no inhibitory effects on GLY-mediated increases in cilia-driven flow (Fig. 5A) and CBF (Fig. 5B). Similarly, the 2-APB had no effect on TIO-mediated increases in cilia-driven flow (Fig. 5C) and CBF (Fig. 5D).

We also tested whether LAMAs treatment affected intracellular Ca^{2+} concentrations in NHBE cells using Fura-2/AM. As shown in Figs. 5E and 5F, neither treatment with GLY nor TIO changed the fluorescence ratio (F340/F380) of Fura-2 until 50 min, suggesting that LAMAs have no effect on intracellular Ca^{2+} concentration. We confirmed that Fura-2 properly worked as an indicator of intracellular Ca^{2+} concentration in NHBE cells; TG increased Fura-2 fluorescence in NHBE cells (Fig. S1).

A former study reported that PKA activation by isoproterenol increased CBF in bovine bronchial epithelial cells [31]. We thus assessed whether cAMP-dependent PKA activation was involved in GLY-mediated

increases in CBF. Well-differentiated NHBE cells cultured under ALI conditions for 4 weeks were treated with GLY with or without H89, a protein kinase inhibitor with a high specificity for PKA [28], for 1 h; CBF was then evaluated. The addition of H89 had no effect on the GLY-mediated increase in CBF (Fig. 6A). GLY treatment also had no effect on PKA activity (Fig. 6B), suggesting that PKA pathways are not involved in GLY-mediated increases in CBF.

Extracellular ATP is also known to increase CBF and hasten MCC in the airways by activating purinergic P2 receptor (P2R) signaling [10, 32]. Therefore, we next evaluated whether GLY induces release of ATP to extracellular milieu from murine tracheal tissues. ATP concentrations were measured in the culture supernatants with or without GLY treatment for 5–120 min. As shown in Figure S2, ATP concentrations did not differ between the GLY treatment and the control, indicating that GLY had no effect on extracellular ATP release.

Discussion

This study describes the novel effects of LAMAs on ciliary functions, with particular focusing on ciliary activity and cilia-driven flow, in airway epithelium. Our findings revealed that the intranasal administration of GLY increases ciliary activity and cilia-driven flow in murine airway epithelium. Using an organ culture of murine tracheal tissues and a primary culture of well-differentiated NHBE, we confirmed that both GLY and TIO directly increased CBF and cilia-driven flow in airway epithelium. Together, these results show that LAMAs, which are major bronchodilators for treating asthma and COPD, have the potential to enhance airway ciliary function and improve MCC.

The present study clearly indicated that LAMAs are multifunctional agents that not only relax airway smooth muscle as bronchodilators but also to enhance ciliary functions in airway epithelium, which provides effective MCC in the airway. We first conducted in-vivo experiments using the intranasal administration of GLY in mice, which represents an inhalation administration of LAMAs in the real clinical setting of asthma and COPD. The continuous intranasal administration of GLY significantly increased cilia-driven flow and ciliary activity; however, it remains unclear whether these are the direct effects of GLY on airway epithelial cells or indirect effects via diverse cellular and humoral factors. We therefore conducted in-vitro experiments using a murine tracheal organ culture to investigate the short-term effects of two types of LAMAs (GLY and TIO), on airway epithelium, and demonstrated that both GLY and TIO directly enhance ciliary functions in murine airway epithelium. Additionally, we further validated the LAMA-mediated promotion of airway ciliary function using human bronchial epithelial cells. Taken together, inhaled LAMAs in clinical practice might provide not only bronchodilation but also promotion of airway ciliary function, which relieves the clinical symptoms of airflow obstruction, and improves MCC and susceptibility to respiratory tract infections in patients with asthma and COPD.

In the present study, we clarified direct actions of LAMAs on promoting airway ciliary function using primary culture of well-differentiated NHBE cells. Primary culture of NHBE cells have the differentiation potential into ciliated cells under ALI culture conditions [25, 26]. Therefore, this culture system enabled us

to evaluate ciliary motion of ciliated cells without considering other cellular components. Using the well-differentiated NHBE cells under ALI conditions, we were able to evaluate ciliary motion by the imaging techniques and confirmed that LAMAs directly increased CBF in human airway epithelial cells.

Several studies have demonstrated that LAMAs exerted a variety of additive effects, including off-target effects [33–37], although the primary function of LAMAs is bronchodilation to block M3 receptor activity and relax smooth muscle in the airways. In the in vivo model, LAMAs showed anti-inflammatory activity, such as a decrease in cytokine levels in the bronchoalveolar lavage and inhibition of neutrophil chemotactic activity and eosinophil recruitment [33, 34], as well as inhibitory effects in airway remodeling [33]. In terms of LAMA actions on airway epithelial cells, Kistemaker *et al.* demonstrated that TIO inhibited IL-13-induced goblet cell metaplasia in human airway epithelial cells [36], which might be useful for protecting against mucus hypersecretion especially in patients with asthma. This study's findings elucidated the novel direct actions of LAMAs on airway epithelial cells: the promotion of airway ciliary functions. We also confirmed this promoting effect of LAMAs on airway ciliary function using two different types of LAMAs, GLY and TIO.

Although our findings reveal the potential of LAMAs to increase cilia-driven flow and ciliary activity in airway epithelium, the intracellular mechanisms of these stimuli are not fully understood. So far, a variety of mechanisms (e.g., intracellular Ca^{2+} concentration, PKA activation, and ATP-P2R pathway) have been reported to be involved in the regulation of CBF and ciliary activities in airway epithelium [10, 28, 31, 32, 38, 39]. In this study, we conducted several experiments to elucidate the underlying mechanism of LAMA-mediated promotion of ciliary function; however, we identified no specific mechanisms in these effects. TIO has been shown to act as an inverse agonist and as an antagonist for the M3 receptor [40]. Cassambai *et al.* recently showed that TIO increased intracellular Ca^{2+} concentration in cardiomyocytes [41]. In contrast to their findings, we observed no increase in Ca^{2+} concentration in the NHBE cells after the TIO or GLY treatment in this study. The difference in cell types might result in different LAMA actions. Interestingly, Birrell *et al.* reported the off-target effects of TIO in airway sensory nerves, in which inhaled TIO blocked capsaicin-induced coughing to inhibit the neuronal transient receptor potential V1, which was unrelated to anticholinergic activity [35]. Similar off-target effects for LAMAs might be involved in the promotion of ciliary function in the airway epithelium. Elucidating the underlying mechanisms of the LAMA-mediated promotion of airway ciliary function will be an important topic for exploration in future studies.

Conclusions

This study provides evidence that LAMAs, which are major bronchodilators for treating asthma and COPD, promote cilia-driven flow and ciliary activity in mouse and human airway epithelium. This study's findings imply that this direct LAMA action on airway epithelium might enhance MCC, in combination with its protective effects on airflow restriction, which prevents susceptibility to respiratory tract infections and exacerbation in patients with asthma and COPD. Further investigations are warranted to elucidate the underlying mechanisms of the direct effects of LAMAs on the promotion of airway ciliary function.

Abbreviations

ALI, air-liquid interface;

ATP, adenosine triphosphate;

CBF, ciliary beat frequency;

COPD, [chronic obstructive pulmonary disease](#);

GLY, glycopyrronium;

H89, N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide, dihydrochloride:

ICS, inhaled corticosteroids;

LABAs, long-acting b2-agonists;

LAMAs, long-acting muscarinic antagonists;

MCC, mucociliary clearance;

NHBE, normal human bronchial epithelial;

PBS, phosphate-buffered saline;

PKA, protein kinase A;

P2R, P2 receptors;

TG, Thapsigargin;

TIO, tiotropium;

WT, wild-type;

2-APB, 2-Aminoethoxydiphenylborane;

Declarations

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Authors' Contributions

T. F. designed and performed research, analyzed data, and wrote the paper; M. K. performed research, analyzed data, and wrote the paper; Y. K., Y. T, C. K. performed research; Y. I., H. H., M. K., Y. S, K. F., N. E., Y. N., and N. I. analyzed data; M. M., M. S., and H. W. designed research; K. I., T. S. designed research and analyzed data.

All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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References

1. Whitsett JA, Alenghat T: **Respiratory epithelial cells orchestrate pulmonary innate immunity.** *Nat Immunol* 2015, **16**:27-35.
2. Hiemstra PS, McCray PB, Jr., Bals R: **The innate immune function of airway epithelial cells in inflammatory lung disease.** *Eur Respir J* 2015, **45**:1150-1162.
3. Kusagaya H, Fujisawa T, Yamanaka K, Mori K, Hashimoto D, Enomoto N, Inui N, Nakamura Y, Wu R, Maekawa M, et al: **Toll-like receptor-mediated airway IL-17C enhances epithelial host defense in an autocrine/paracrine manner.** *Am J Respir Cell Mol Biol* 2014, **50**:30-39.
4. Niwa M, Fujisawa T, Mori K, Yamanaka K, Yasui H, Suzuki Y, Karayama M, Hozumi H, Furuhashi K, Enomoto N, et al: **IL-17A Attenuates IFN-lambda Expression by Inducing Suppressor of Cytokine Signaling Expression in Airway Epithelium.** *J Immunol* 2018, **201**:2392-2402.

5. Hewitt RJ, Lloyd CM: **Regulation of immune responses by the airway epithelial cell landscape.** *Nat Rev Immunol* 2021, **21**:347-362.
6. Bustamante-Marin XM, Ostrowski LE: **Cilia and Mucociliary Clearance.** *Cold Spring Harb Perspect Biol* 2017, **9**.
7. Whitsett JA: **Airway Epithelial Differentiation and Mucociliary Clearance.** *Ann Am Thorac Soc* 2018, **15**:S143-S148.
8. Ikegami K, Sato S, Nakamura K, Ostrowski LE, Setou M: **Tubulin polyglutamylation is essential for airway ciliary function through the regulation of beating asymmetry.** *Proc Natl Acad Sci U S A* 2010, **107**:10490-10495.
9. Ijaz F, Ikegami K: **Live cell imaging of dynamic behaviors of motile cilia and primary cilium.** *Microscopy (Oxf)* 2019, **68**:99-110.
10. Kamiya Y, Fujisawa T, Katsumata M, Yasui H, Suzuki Y, Karayama M, Hozumi H, Furuhashi K, Enomoto N, Nakamura Y, et al: **Influenza A virus enhances ciliary activity and mucociliary clearance via TLR3 in airway epithelium.** *Respir Res* 2020, **21**:282.
11. Lucas JS, Davis SD, Omran H, Shoemark A: **Primary ciliary dyskinesia in the genomics age.** *Lancet Respir Med* 2020, **8**:202-216.
12. Israel E, Reddel HK: **Severe and Difficult-to-Treat Asthma in Adults.** *N Engl J Med* 2017, **377**:965-976.
13. Rabe KF, Watz H: **Chronic obstructive pulmonary disease.** *Lancet* 2017, **389**:1931-1940.
14. Nakamura Y, Tamaoki J, Nagase H, Yamaguchi M, Horiguchi T, Hozawa S, Ichinose M, Iwanaga T, Kondo R, Nagata M, et al: **Japanese guidelines for adult asthma 2020.** *Allergol Int* 2020, **69**:519-548.
15. Thomas B, Rutman A, Hirst RA, Haldar P, Wardlaw AJ, Bankart J, Brightling CE, O'Callaghan C: **Ciliary dysfunction and ultrastructural abnormalities are features of severe asthma.** *J Allergy Clin Immunol* 2010, **126**:722-729 e722.
16. Wan WY, Hollins F, Haste L, Woodman L, Hirst RA, Bolton S, Gomez E, Sutcliffe A, Desai D, Chachi L, et al: **NADPH Oxidase-4 Overexpression Is Associated With Epithelial Ciliary Dysfunction in Neutrophilic Asthma.** *Chest* 2016, **149**:1445-1459.
17. Piatti G, Ambrosetti U, Santus P, Allegra L: **Effects of salmeterol on cilia and mucus in COPD and pneumonia patients.** *Pharmacol Res* 2005, **51**:165-168.
18. Yaghi A, Zaman A, Cox G, Dolovich MB: **Ciliary beating is depressed in nasal cilia from chronic obstructive pulmonary disease subjects.** *Respir Med* 2012, **106**:1139-1147.

19. Devalia JL, Sapsford RJ, Rusznak C, Toumbis MJ, Davies RJ: **The effects of salmeterol and salbutamol on ciliary beat frequency of cultured human bronchial epithelial cells, in vitro.** *Pulm Pharmacol* 1992, **5**:257-263.
20. Shiima-Kinoshita C, Min KY, Hanafusa T, Mori H, Nakahari T: **Beta 2-adrenergic regulation of ciliary beat frequency in rat bronchiolar epithelium: potentiation by isosmotic cell shrinkage.** *J Physiol* 2004, **554**:403-416.
21. Profita M, Bonanno A, Siena L, Ferraro M, Montalbano AM, Pompeo F, Riccobono L, Pieper MP, Gjomarkaj M: **Acetylcholine mediates the release of IL-8 in human bronchial epithelial cells by a NFkB/ERK-dependent mechanism.** *Eur J Pharmacol* 2008, **582**:145-153.
22. Meurs H, Dekkers BG, Maarsingh H, Halayko AJ, Zaagsma J, Gosens R: **Muscarinic receptors on airway mesenchymal cells: novel findings for an ancient target.** *Pulm Pharmacol Ther* 2013, **26**:145-155.
23. Fujisawa T, Velichko S, Thai P, Hung LY, Huang F, Wu R: **Regulation of airway MUC5AC expression by IL-1beta and IL-17A; the NF-kappaB paradigm.** *J Immunol* 2009, **183**:6236-6243.
24. Fujisawa T, Chang MM, Velichko S, Thai P, Hung LY, Huang F, Phuong N, Chen Y, Wu R: **NF-kappaB mediates IL-1beta- and IL-17A-induced MUC5B expression in airway epithelial cells.** *Am J Respir Cell Mol Biol* 2011, **45**:246-252.
25. Oltean A, Schaffer AJ, Bayly PV, Brody SL: **Quantifying Ciliary Dynamics during Assembly Reveals Stepwise Waveform Maturation in Airway Cells.** *Am J Respir Cell Mol Biol* 2018, **59**:511-522.
26. Lee DDH, Petris A, Hynds RE, O'Callaghan C: **Ciliated Epithelial Cell Differentiation at Air-Liquid Interface Using Commercially Available Culture Media.** *Methods Mol Biol* 2020, **2109**:275-291.
27. Sehgal P, Szalai P, Olesen C, Praetorius HA, Nissen P, Christensen SB, Engedal N, Moller JV: **Inhibition of the sarco/endoplasmic reticulum (ER) Ca(2+)-ATPase by thapsigargin analogs induces cell death via ER Ca(2+) depletion and the unfolded protein response.** *J Biol Chem* 2017, **292**:19656-19673.
28. Braiman A, Zagoory O, Priel Z: **PKA induces Ca2+ release and enhances ciliary beat frequency in a Ca2+-dependent and -independent manner.** *Am J Physiol* 1998, **275**:C790-797.
29. Schmid A, Salathe M: **Ciliary beat co-ordination by calcium.** *Biol Cell* 2011, **103**:159-169.
30. Muller S, Koch C, Weiterer S, Weigand MA, Sander M, Henrich M: **Caspofungin induces the release of Ca(2+) ions from internal stores by activating ryanodine receptor-dependent pathways in human tracheal epithelial cells.** *Sci Rep* 2020, **10**:11723.
31. Wyatt TA, Spurzem JR, May K, Sisson JH: **Regulation of ciliary beat frequency by both PKA and PKG in bovine airway epithelial cells.** *Am J Physiol* 1998, **275**:L827-835.

32. Idzko M, Ferrari D, Eltzschig HK: **Nucleotide signalling during inflammation.** *Nature* 2014, **509**:310-317.
33. Bos IS, Gosens R, Zuidhof AB, Schaafsma D, Halayko AJ, Meurs H, Zaagsma J: **Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison.** *Eur Respir J* 2007, **30**:653-661.
34. Wollin L, Pieper MP: **Tiotropium bromide exerts anti-inflammatory activity in a cigarette smoke mouse model of COPD.** *Pulm Pharmacol Ther* 2010, **23**:345-354.
35. Birrell MA, Bonvini SJ, Dubuis E, Maher SA, Wortley MA, Grace MS, Raemdonck K, Adcock JJ, Belvisi MG: **Tiotropium modulates transient receptor potential V1 (TRPV1) in airway sensory nerves: A beneficial off-target effect?** *J Allergy Clin Immunol* 2014, **133**:679-687 e679.
36. Kistemaker LE, Hiemstra PS, Bos IS, Bouwman S, van den Berge M, Hylkema MN, Meurs H, Kerstjens HA, Gosens R: **Tiotropium attenuates IL-13-induced goblet cell metaplasia of human airway epithelial cells.** *Thorax* 2015, **70**:668-676.
37. Koarai A, Ichinose M: **Possible involvement of acetylcholine-mediated inflammation in airway diseases.** *Allergol Int* 2018, **67**:460-466.
38. Zhao KQ, Cowan AT, Lee RJ, Goldstein N, Droguett K, Chen B, Zheng C, Villalon M, Palmer JN, Kreindler JL, Cohen NA: **Molecular modulation of airway epithelial ciliary response to sneezing.** *FASEB J* 2012, **26**:3178-3187.
39. Droguett K, Rios M, Carreno DV, Navarrete C, Fuentes C, Villalon M, Barrera NP: **An autocrine ATP release mechanism regulates basal ciliary activity in airway epithelium.** *J Physiol* 2017, **595**:4755-4767.
40. Kruse AC, Hu J, Pan AC, Arlow DH, Rosenbaum DM, Rosemond E, Green HF, Liu T, Chae PS, Dror RO, et al: **Structure and dynamics of the M3 muscarinic acetylcholine receptor.** *Nature* 2012, **482**:552-556.
41. Cassambai S, Mee CJ, Renshaw D, Hussain A: **Tiotropium bromide, a long acting muscarinic receptor antagonist triggers intracellular calcium signalling in the heart.** *Toxicol Appl Pharmacol* 2019, **384**:114778.

Figures

Figure 1

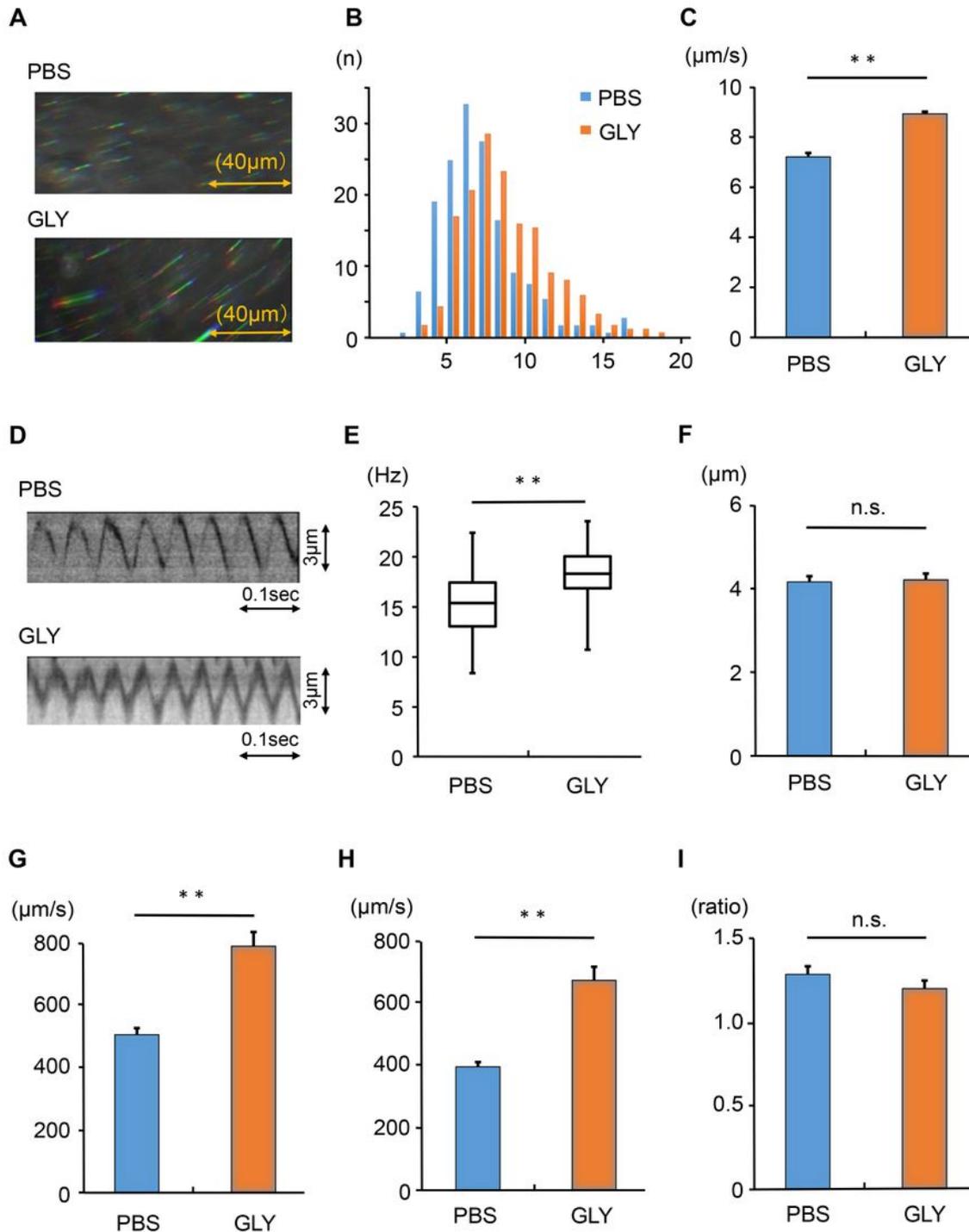


Figure 1

The effects of the intranasal administration of glycopyrronium on cilia-driven flow and ciliary motility Wild-type (WT) mice were treated with glycopyrronium (GLY) or phosphate-buffered saline (PBS) intranasally for 7 days, The trachea was then removed. Cilia-driven flow and ciliary motility were evaluated. A. Representative bead trajectories of cilia-driven flow (rainbow trace for 4.4 s). B and C. Both the histogram (B) and bar chart (C) of the cilia-driven flow demonstrated that 7-day GLY administration

significantly increased cilia-driven flow (PBS, $7.30 \pm 0.15 \mu\text{m/s}$; GLY, $9.04 \pm 0.08 \mu\text{m/s}$; $n = 150$ beads in each condition). D. Kymographs of the ciliary beating. E. GLY significantly increased ciliary beating frequency (CBF; PBS, $15.23 [8.20-22.27]$ Hz; GLY, $18.17 [10.55-23.44]$ Hz; $n = 30$ each). F. The ciliary beating amplitude did not differ between the conditions (PBS, $4.18 \pm 0.14 \mu\text{m}$; GLY, $4.23 \pm 0.15 \mu\text{m}$). G and H. The effective stroke velocity (G) (PBS, $503.7 \pm 21.1 \mu\text{m/s}$; GLY, $785.7 \pm 45.1 \mu\text{m/s}$) and recovery stroke velocity (H) (PBS, $396.8 \pm 15.3 \mu\text{m/s}$; GLY, $675.3 \pm 43.3 \mu\text{m/s}$) were significantly increased by GLY administration compared with the control. I. The ratio of effective stroke velocity to recovery stroke velocity did not differ between the two conditions (PBS, 1.29 ± 0.05 ; GLY, 1.20 ± 0.05). ** $p < 0.01$, n.s., not significant. CBF data are presented as the median (range).

Figure 2

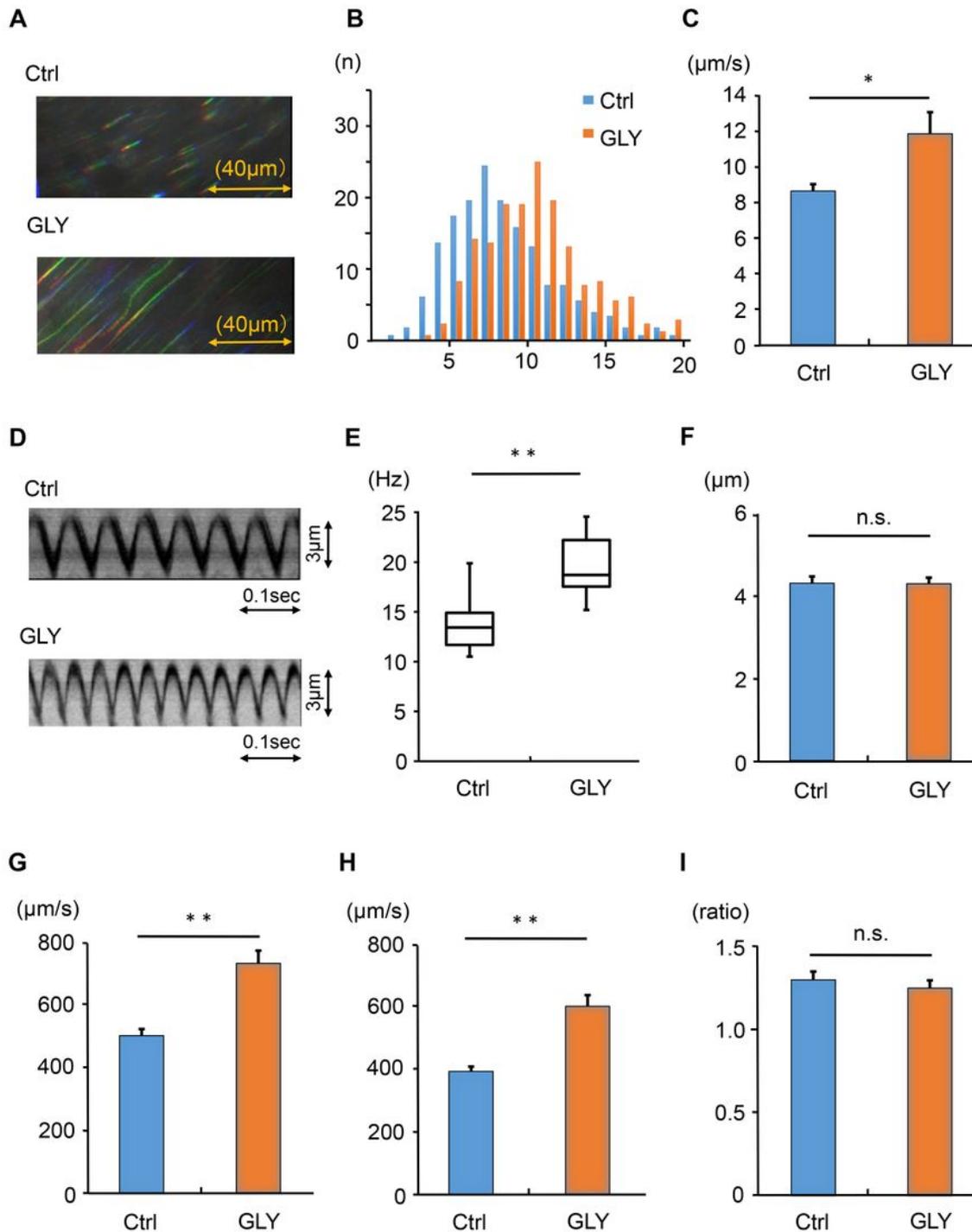


Figure 2

The short-term effects of glycopyrronium on cilia-driven flow and ciliary motility Murine tracheal epithelia were incubated for 1 h with/without glycopyrronium (GLY) in the culture medium. The cilia-driven flow and ciliary motility were then evaluated. A. Representative bead trajectories of cilia-driven flow (rainbow trace for 4.4 s). B and C. Both the histogram (B) and bar chart (C) of the cilia-driven flow demonstrated that GLY significantly increased cilia-driven flow (control, $8.63 \pm 0.39 \mu\text{m/s}$; GLY, $11.85 \pm 1.21 \mu\text{m/s}$; $n =$

150 beads in each condition). D. Kymographs of ciliary beating. E. GLY significantly increased ciliary beat frequency (CBF; control, 13.48 [10.55–19.92] Hz; GLY, 18.75 [15.23–24.61] Hz; n = 30 each). F. The ciliary beating amplitude did not differ between the conditions (control, $4.31 \pm 0.16 \mu\text{m}$; GLY, $4.29 \pm 0.15 \mu\text{m}$). G and H. The effective stroke velocity (G) (control, $504.4 \pm 20.7 \mu\text{m/s}$; GLY, $734.9 \pm 40.3 \mu\text{m/s}$) and recovery stroke velocity (H) (control, $393.9 \pm 15.1 \mu\text{m/s}$; GLY, $602.2 \pm 35.5 \mu\text{m/s}$) were significantly increased by GLY treatment compared with the control. I. The ratio of effective stroke velocity to recovery stroke velocity did not differ between the two conditions (control, 1.30 ± 0.05 ; GLY, 1.25 ± 0.05). *p < 0.05, **p < 0.01. Ctrl, control; n.s., not significant. CBF data are presented as the median (range).

Figure 3

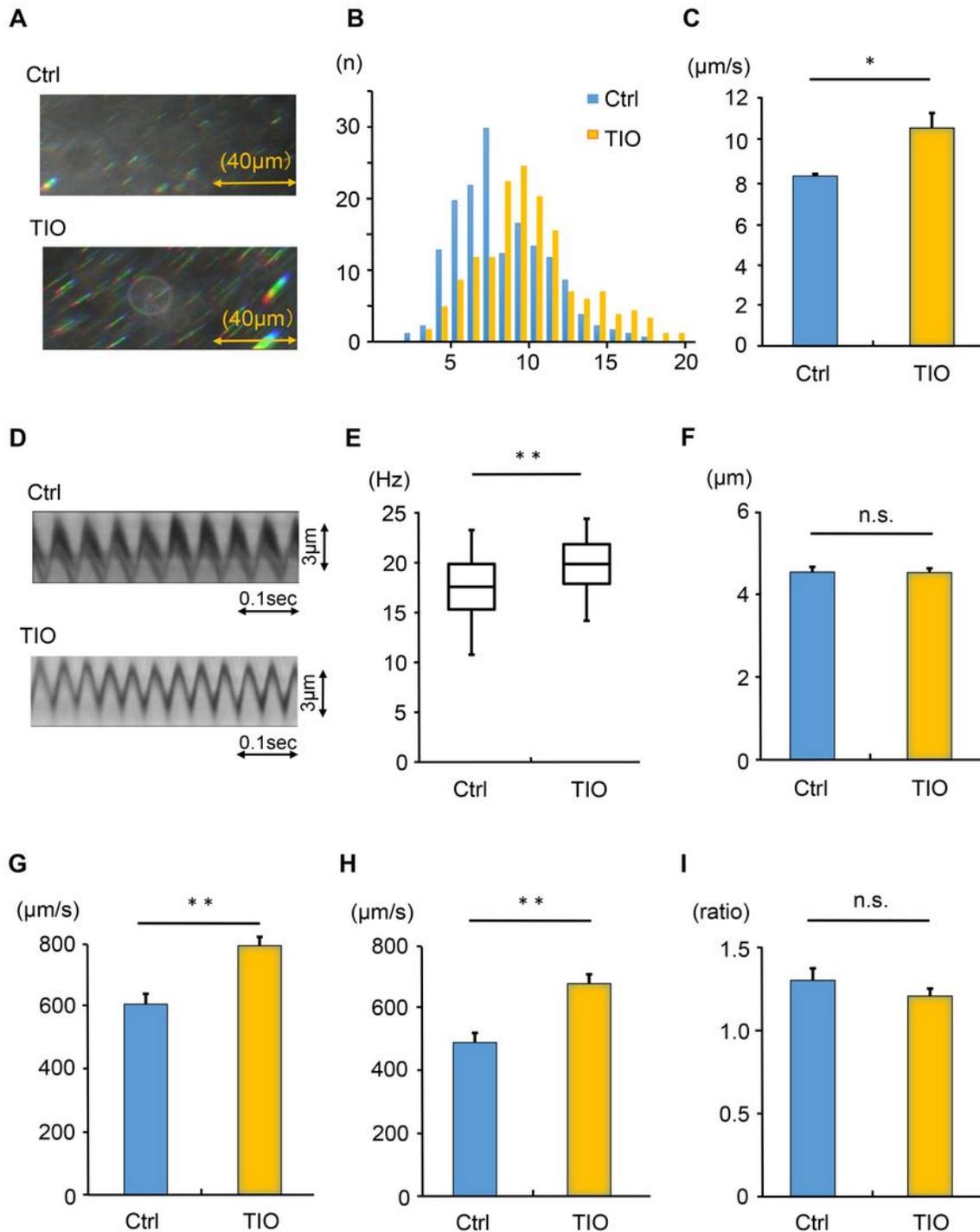


Figure 3

The short-term effects of tiotropium on cilia-driven flow and ciliary motility Murine tracheal epithelia were incubated for 1 h with/without tiotropium (TIO) in the culture medium. Cilia-driven flow and ciliary motility were then evaluated. A. Representative bead trajectories of cilia-driven flow (rainbow trace for 4.4 s). B and C. Both the histogram (B) and bar chart (C) of the cilia-driven flow demonstrated that TIO significantly increased cilia-driven flow (control, $8.26 \pm 0.11 \mu\text{m/s}$; GLY, $10.59 \pm 0.72 \mu\text{m/s}$; $n = 150$ beads

in each condition). D. Kymographs of ciliary beating. E. TIO significantly increased ciliary beat frequency (CBF; control, 17.58 [10.55–23.44] Hz; TIO, 19.92 [14.06–24.61] Hz; n = 30 each). F. The ciliary beating amplitude did not differ between the conditions (control, $4.56 \pm 0.13 \mu\text{m}$; TIO, $4.56 \pm 0.10 \mu\text{m}$). G and H. The effective stroke velocity (G) (control, $606.9 \pm 33.3 \mu\text{m/s}$; TIO, $794.0 \pm 27.9 \mu\text{m/s}$) and recovery stroke velocity (H) (control, $494.0.7 \pm 30.7 \mu\text{m/s}$; TIO, $683.1 \pm 29.7 \mu\text{m/s}$) were significantly increased by TIO treatment compared with the control. I. The ratio of effective stroke velocity to recovery stroke velocity did not differ between the two conditions (control, 1.29 ± 0.07 ; TIO, 1.20 ± 0.04). * $p < 0.05$, ** $p < 0.01$. Ctrl, control; n.s., not significant. CBF data were presented as the median (range).

Figure 4

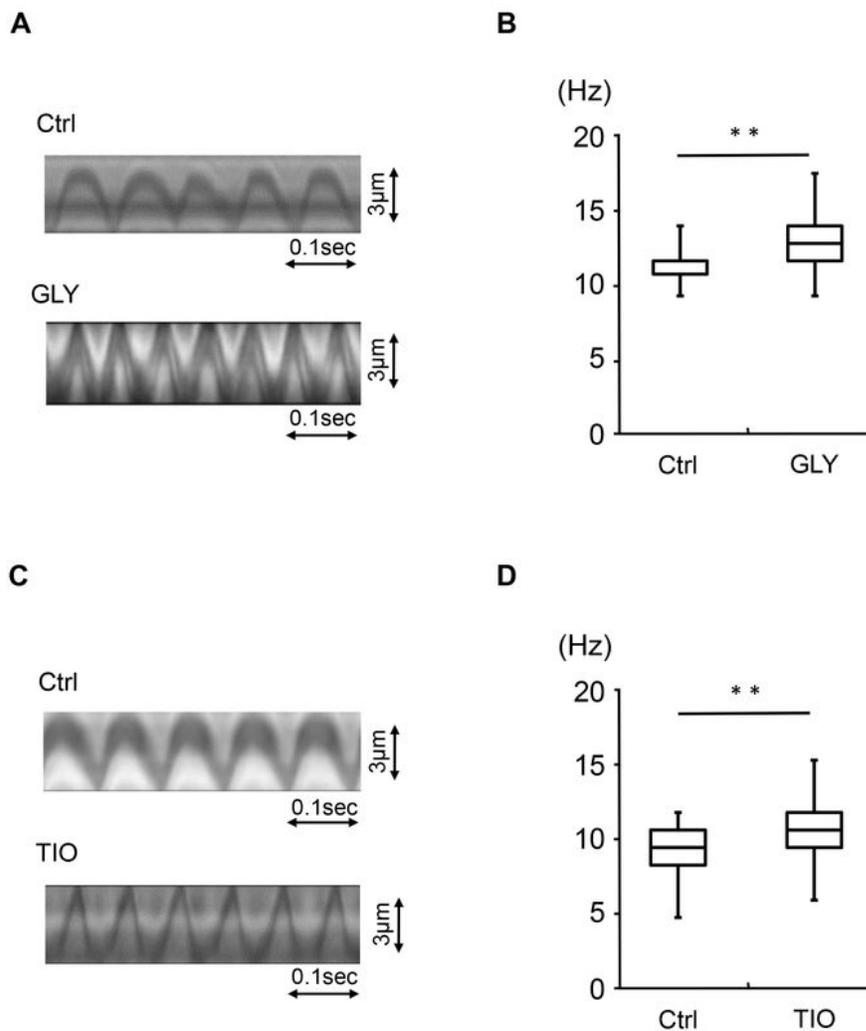


Figure 4

The direct effects of glycopyrronium or tiotropium on ciliary beat frequency in normal human bronchial epithelial cells Well-differentiated normal human bronchial epithelial (NHBE) cells cultured under air-liquid interface (ALI) conditions for 4 weeks were treated for 1 h with/without glycopyrronium (GLY) or tiotropium (TIO) in the culture medium. Ciliary beat frequency (CBF) was then evaluated. A. Kymographs of ciliary beating. B. GLY significantly increased CBF (CBF; control, 11.72 [9.38–14.06] Hz; GLY, 12.89

[9.38–17.58] Hz; n = 30 each). C. Kymographs of ciliary beating. D. TIO significantly increased CBF (control, 9.38 [4.69–11.72] Hz; TIO, 10.55 [5.86–15.23] Hz; n = 30 each). **p < 0.01. Ctrl, control; CBF data are presented as the median (range).

Figure 5

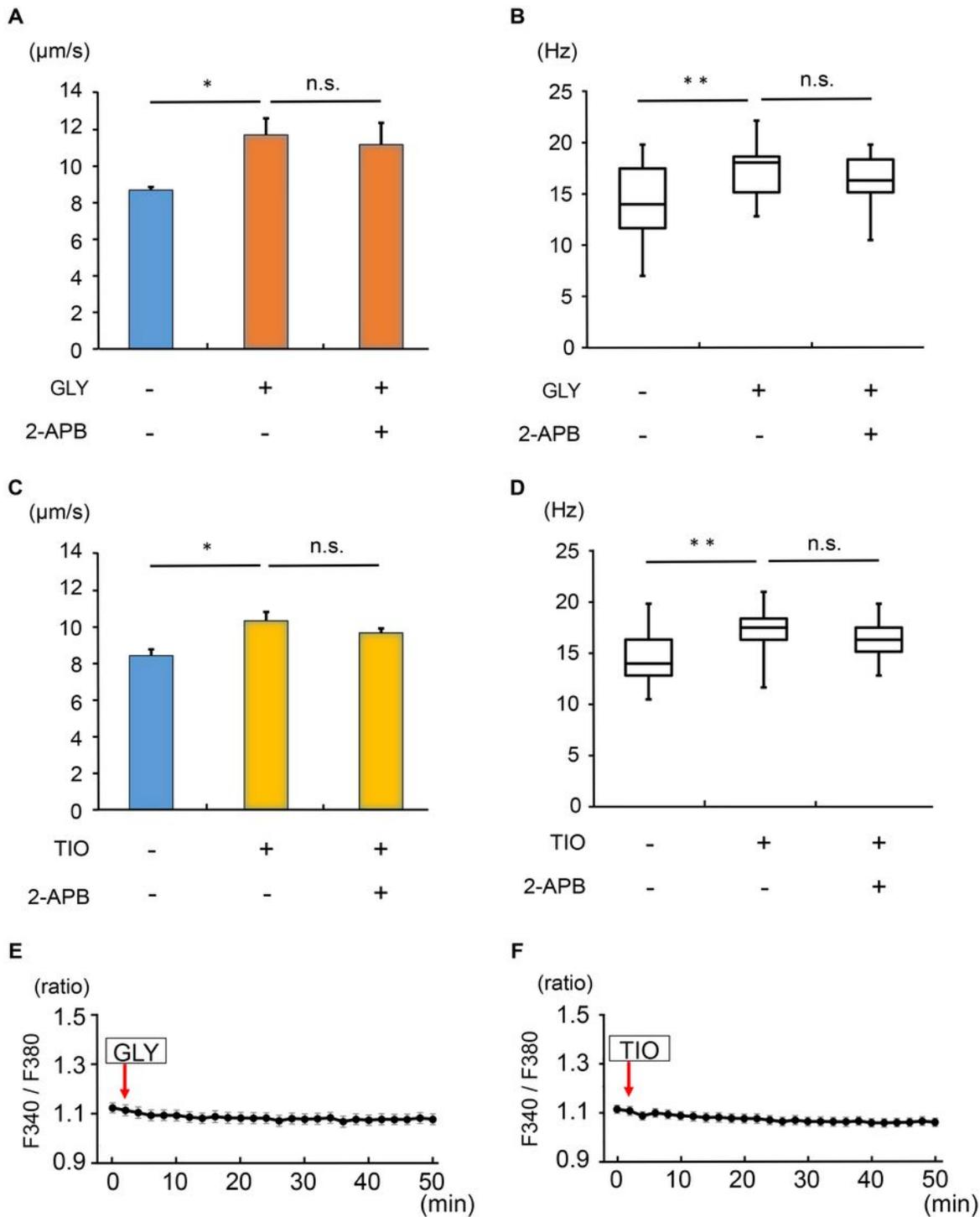


Figure 5

Examination of involvement of intracellular calcium ion in the glycopyrronium or tiotropium-mediated promotion of ciliary function The murine tracheal tissues were incubated with glycopyrronium (GLY) or

tiotropium (TIO) with/without 2-Aminoethoxydiphenylborane (2-APB) for 1 h. Cilia-driven flow and ciliary beat frequency (CBF) were then evaluated. A and B. 2-APB had few effects on GLY-mediated increases of cilia-driven flow (A) (control, $8.63 \pm 0.16 \mu\text{m/s}$; GLY, $11.63 \pm 0.90 \mu\text{m/s}$; GLY+2-APB, $11.10 \pm 1.18 \mu\text{m/s}$; $n = 150$ beads in each condition) and CBF (B) (control, $14.06 [7.03-19.92]$ Hz; GLY, $18.17 [12.89-22.27]$ Hz; GLY+2-APB, $16.40 [10.55-19.92]$ Hz; $n = 30$ each). C and D. 2-APB had few effects on TIO-mediated increases of cilia-driven flow (C) (control, $8.46 \pm 0.34 \mu\text{m/s}$; TIO, $10.37 \pm 0.48 \mu\text{m/s}$; TIO+2-APB, $9.54 \pm 0.38 \mu\text{m/s}$; $n = 150$ beads in each condition) and CBF (D) (control, $14.06 [10.55-19.92]$ Hz; TIO, $17.58 [11.72-21.09]$ Hz; TIO+2-APB, $16.40 [12.89-19.92]$ Hz; $n = 30$ each). E and F. Intracellular Ca^{2+} concentrations were measured using Fura-2/AM in normal human bronchial epithelial cells. Neither treatment with GLY (E) nor TIO (F) changed the fluorescence ratio (F340/F380) of Fura-2 until 50 min. * $p < 0.05$, ** $p < 0.01$, Ctrl, control; n.s., not significant. CBF data are presented as the median (range).

Figure 6

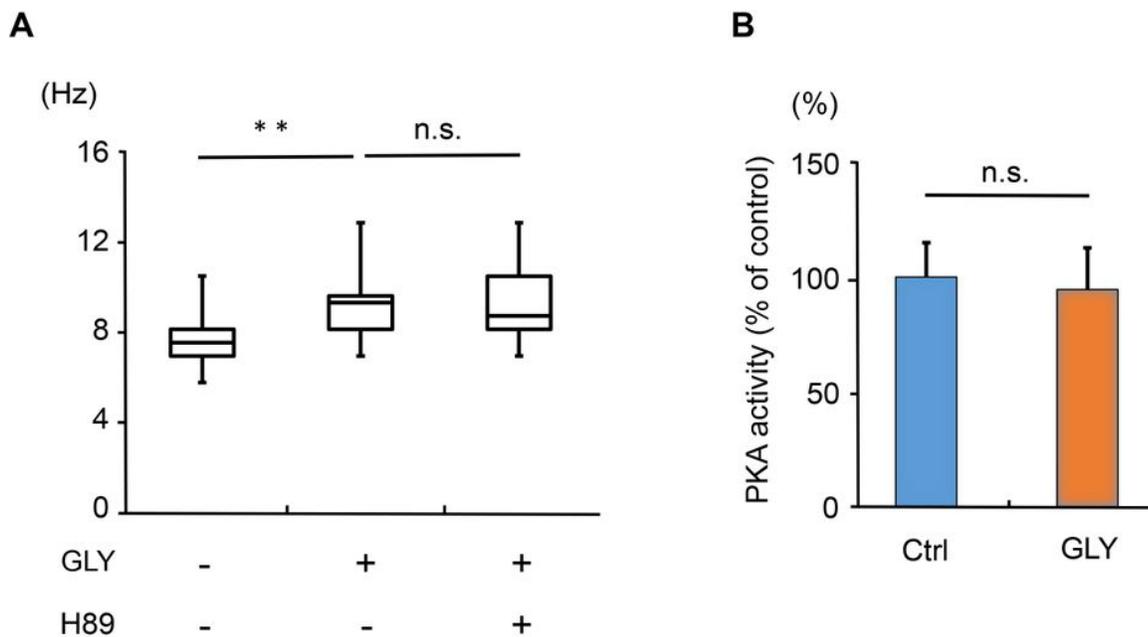


Figure 6

Examination of involvement of protein kinase A in the glycopyrronium-mediated increase of ciliary beat frequency Well-differentiated normal human bronchial epithelial (NHBE) cells were incubated with glycopyrronium (GLY) with or without H89, a protein kinase inhibitor with a high specificity for protein kinase A (PKA), for 1 h. Ciliary beat frequency (CBF) and PKA activity was then evaluated. A. H89 had few

effects on GLY-mediated increases in CBF (control, 7.62 [5.86–10.55] Hz; GLY, 9.38 [7.03–12.89] Hz; GLY+H89, 8.79 [7.03–12.89] Hz; n = 30 each). B. GLY treatment had few effects on PKA activity (control, 100 ± 14.95 %; GLY, 94.53 ± 18.14 %; n = 3). The results are presented as percent of control. **p < 0.01. Ctrl, control; n.s., not significant.

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