

Expression of T-Type Voltage-Gated Calcium Channel in the Cilia of Human Nasal Epithelial Cells

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Keywords

Nasal mucosa · Turbinate · Ciliary beat · T-type voltage-gated calcium channel

Abstract

Introduction: The mucociliary transport function of the airway epithelium is largely dependent on ciliary beating. The control signal of ciliary beating is thought to be intracellular Ca^{2+} . We herein investigated the expression of T-type voltage-gated calcium channel (VGCC), a generator of intracellular Ca^{2+} oscillation, in the human nasal mucosa. **Methods:** The inferior turbinate was collected from patients with chronic hypertrophic rhinitis. The expression of T-type VGCC $\alpha 1$ subunits was examined by immunohistochemistry, transmission immunoelectron microscopy, Western blot, and real-time reverse transcription-polymerase chain reaction (RT-PCR). Participation of T-type VGCC in the ciliary beat regulation was examined by pharmacological inhibition tests using specific blockers of T-type VGCC in ex vivo measurements of the ciliary beat frequency (CBF) and ATP release and in intracellular Ca^{2+} imaging of isolated ciliated cells. **Results:** Im-

munohistochemical staining showed the expressions of T-type VGCC $\alpha 1$ subunits, Cav3.1 and Cav3.3, on the surface of the epithelial cells. At the ultrastructural level, immunoreactivity for Cav3.1 was localized on the surface of the cilia, and that for Cav3.3 was localized in the cilia and at the base of the cilia. The existence of Cav3.1 and Cav3.3 was confirmed at the protein level by Western blot and at the transcriptional level by real-time RT-PCR. Specific blockers of T-type VGCC, mibefradil and NNC 55-0396, significantly inhibited CBF. These blockers also inhibited a CBF increase induced by 8-bromo-cAMP/8-bromo-cGMP and significantly lowered the intracellular Ca^{2+} level of isolated ciliated cells in a time-dependent manner. On the other hand, the ATP release from the nasal mucosa was not changed by mibefradil or NNC 55-0396. **Conclusion:** These results indicate that T-type VGCC $\alpha 1$ subunits, Cav3.1 and Cav3.3, exist at the cilia of the nasal epithelial cells and participate in the regulation of ciliary beating and that these channels act downstream of cAMP/cGMP.

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Introduction

Mucociliary transport function of the upper and lower airway epithelium is responsible for the clearance of foreign particles and pathogens on the epithelial surface and plays an important role in the mucosal defense of the airway tract. This function is mainly dependent on ciliary beating as well as on the viscosity of surface mucus. The cilia of the airway epithelial cells have the same ultrastructure as the flagella of the sperm and various protozoa. They have a common core skeleton called the axoneme. The axoneme is composed of a pair of central singlet microtubules surrounded by 9 outer doublet microtubules. To each doublet microtubule, inner and outer dynein arms are attached, and the outer dynein arm forms a bridge between the neighboring doublet microtubules. Dynein has an ATPase activity and changes its molecular configuration by consuming ATP. This reaction forces dynein to walk along the adjacent doublet microtubule, resulting in a bending motion of the axoneme [1].

The cilia and flagella also share the regulatory mechanism of beating in common. The regulatory pathway involves calmodulin and cAMP/cGMP [2]. Our recent study revealed that pannexin-1 and P2X7 purinergic receptor are coexpressed in the human nasal mucosa [3, 4] and involved in the regulatory pathway of ciliary beating [5]. Taking into account that pannexin-1 is reported to be an ATP-releasing channel and that P2X purinergic receptors are ATP-gated Ca^{2+} channels, it is likely that coexistence of these two molecules induces the continuous influx of extracellular Ca^{2+} into the cell. Change in the intracellular Ca^{2+} level is thought to directly control the activity of dynein and, thereby, ciliary beating [2]. These observations raise a question as to the identity of the generator of intracellular Ca^{2+} oscillation that is coupled to ciliary beating.

Previous studies reported the presence of voltage-gated calcium channels (VGCCs) in the flagella/cilia of algae and paramecia [6, 7]. Fujiu et al. [6] have documented that *Chlamydomonas* CAV2 gene, which is involved in the regulation of flagellar movement, encodes homologs of the α_1 subunit of VGCC, whose amino acid sequence is highly analogous to the human T-type VGCC. The T-type VGCC is well known as a self-pulsating calcium channel expressed in cardiac pacemaker cells of the sinoatrial node. This finding leads to an intriguing conjecture that T-type VGCC may be the generator of intracellular Ca^{2+} oscillation that regulates ciliary beating of the airway epithelium. In the present study, we explored the expression of the T-type VGCC in the human nasal mucosa.

Materials and Methods

Patients and Sample Collection

A total of 46 patients with chronic hypertrophic rhinitis were enrolled in this study. They were 34 males and 12 females, aged 15–83 years, with an average age of 48.2 years. Total and/or specific serum IgE levels were positive in 37 patients (80.4%). Specific serum IgE levels were measured for house dust mites, Japanese cedar pollen, cypress pollen, orchard grass pollen, short ragweed pollen, timothy grass pollen, and *Aspergillus*, which are major airborne allergens in Japan. There were 9 patients who had bronchial asthma. The inferior turbinate bone was resected together with the lateral mucosa of the turbinate under general anesthesia. The collected inferior turbinates were immediately soaked in O_2 -saturated Hank's balanced salt solution (HBSS; 8,000 [in mg/L] NaCl, 400 KCl, 350 NaHCO_3 , 140 CaCl_2 , 100 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 60 KH_2PO_4 , 47.8 Na_2HPO_4 , and 1,000 glucose) and thoroughly washed with HBSS to remove surface mucus. The lateral mucosa of the collected turbinate was separated from the underlying bone with surgical scissors, and subjected to the following process.

Chemicals

(1S,2S)-2-[2-[[3-(1H-Benzimidazol-2-yl)propyl]methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-(1-methylethyl)-2-naphthalenyl methoxyacetate dihydrochloride (mibefradil; a T-type VGCC blocker) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). (1S,2S)-2-[2-[[3-(1H-Benzimidazol-2-yl)propyl]methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-(1-methylethyl)-2-naphthalenyl cyclopropanecarboxylate dihydrochloride (NNC 55-0396; a highly selective T-type VGCC blocker), 8-bromo-cAMP (a cAMP analog), and 8-bromo-cGMP (a cGMP analog) were purchased from Tocris Bioscience (Bristol, UK). 1-Fluoro-2,4-dinitrobenzene (FDB; a creatine kinase inhibitor) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Fluo-8 acetoxymethyl ester (Fluo-8 AM) was bought from AAT Bioquest (Sunnyvale, CA, USA). Mibefradil and Fluo-8 AM were each dissolved in DMSO to make a $\times 1,000$ concentrated stock solution. NNC 55-0396, 8-bromo-cAMP, 8-bromo-cGMP, and FDB were each dissolved in distilled water to make a $\times 1,000$ concentrated stock solution. The final concentration of DMSO was 0–0.2%. Our previous investigation confirmed that 0.1–0.3% DMSO does not significantly change the baseline ciliary beat frequency (CBF) [4, 8].

Fluorescence Immunohistochemistry

The specimens were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 at 4°C overnight. The fixed samples were transferred into a solution of 20% sucrose in 0.1 M phosphate-buffered saline (PBS) at pH 7.4 and incubated at 4°C for 2 nights with 3–4 changes of the solution. The samples were then embedded while frozen in Tissue-Tek OCT Compound (Sakura Finetek, Tokyo, Japan) and stored at -80°C until sectioning. Seven-micrometer-thick sections were prepared using a cryostat, mounted on silane-coated glass slides (Superfrost; Matsunami Glass Industries, Osaka, Japan), and air-dried. The sections were hydrated in PBS with 0.3% Triton X-100 (PBST) for 20 min and treated with 1.5% normal goat serum in PBST for 1 h. They were then incubated with rabbit anti-human Cav3.1 (CACNA1G) polyclonal antibody (Cusabio, Houston, TX, USA), rabbit anti-human Cav3.2 (CACNA1H) polyclonal antibody (Novus Biologicals, Centennial, CO, USA), rabbit anti-human Cav3.3 (CACNA1I)

polyclonal antibody (Novus Biologicals), or rabbit anti-human Cav2.3 (CACNA1E) polyclonal antibody (Lifespan BioSciences, Seattle, WA, USA) at 4°C overnight. The primary antibodies were used at a dilution of 1:50 for Cav3.1, 1:100 for Cav3.2, and Cav2.3, or 1:200 for Cav3.3, in PBST containing 0.5% bovine serum albumin (BSA). As a negative control, the primary antibodies were omitted from the process. After a brief rinse with PBST, the sections were reacted at room temperature for 2 h with a secondary antibody, Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Eugene, OR, USA) diluted 1:1,000 in PBST containing 0.5% BSA. The sections were coverslipped with Prolong Gold antifade reagent containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) and examined under a Carl Zeiss Axioskop 2 Plus fluorescence microscope. The light source was an HBO 103 W/2 mercury vapor lamp. The light was let pass through a 475- to 495-nm bandpass filter for the excitation of Alexa Fluor 488 or through a 340- to 380-nm bandpass filter for DAPI. The emitted fluorescence was allowed to pass through a 515- to 565-nm bandpass filter for Alexa Fluor 488 or through a 435- to 485-nm bandpass filter for DAPI. Images were captured using a Carl Zeiss AxioCam digital camera attached to the microscope.

Quantitative Evaluation of Immunohistochemical Fluorescence Intensity

Immunohistochemical fluorescence intensity was quantitatively evaluated using AxioVision software (version 4.7.2.0; copyright 2006–2008; Car Zeiss Microscopy, White Plains, NY, USA). The fluorescence photomicrographs were displayed in a 16,384-step arbitrary scale of 0 (no fluorescence) to 16,383 (most intense fluorescence) for each pixel of the images. Areas in a representative positive staining slide were randomly chosen for each patient. The entire epithelial layer in each area was manually defined as the region of interest (ROI), and the mean pixel value of the ROI was calculated (fluorescence intensity value). Likewise, the fluorescence intensity value of the corresponding region in the negative control slide was calculated.

Transmission Immunoelectron Microscopy

The turbinates collected during surgery were fixed with 4% paraformaldehyde in 0.1 M PB at pH 7.4 at 4°C overnight. The fixed samples were transferred into 20% sucrose in 0.1 M PBS at pH 7.4 and incubated at 4°C for 2 nights with 3–4 changes of the solution. The samples were frozen and embedded in Tissue-Tek OCT Compound (Sakura Finetek) and then stored at –80°C until sectioning. Seven-micrometer-thick sections were prepared using a cryostat, mounted on silane-coated glass slides (Superfrost; Matsunami Glass Industries), and air-dried. The sections were then treated with 1% normal BSA in PBST for 1 h and incubated with rabbit anti-human Cav3.1 (CACNA1G) polyclonal antibody (Cusabio) or rabbit anti-human Cav3.3 (CACNA1I) polyclonal antibody (Novus Biologicals) at 4°C overnight. Both primary antibodies were used at a dilution of 1:50 in PBST containing 1% BSA. As a negative control, primary antibodies were omitted from the process. After thorough washing with PBST, the sections were reacted at 4°C overnight with HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:100 in PBST containing 1% BSA. The sections were washed with PBS, fixed with 1% glutaraldehyde in PB at room temperature for 5 min, and developed by incubating with 0.003% 3,3'-diaminobenzidine (DAB), 0.03% imidazole, and 0.014% H₂O₂ in 0.05 M Tris-HCl buffer for 10 min at room tem-

perature. The sections were then post-fixed with 1% OsO₄ in PB for 30 min at 4°C, dehydrated in serial concentrations of ethanol, and embedded in Epon 812. Seventy- to 100-nm-thick ultrathin sections were prepared using an ultramicrotome Leica EM UC7 (Leica Microsystems, Tokyo, Japan), stained with 4% lead nitrate, and examined under a JEOL JEM-1200EX transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Western Blot

The collected samples were minced, ground with a pestle, and soaked in the RIPA lysis buffer with protease inhibitor and phosphatase inhibitor (ATTO, Tokyo, Japan). The lysate was centrifuged at 14,000 g for 20 min at 4°C, and the soluble protein layer was transferred to another tube. The total protein concentration of the extract was measured by using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The sample was then mixed with an equivalent volume of EzApply (ATTO), containing 100 mM Tris-HCl (pH 8.8), 2% sodium dodecyl sulfate (SDS), 20% sucrose, 0.06% bromophenol blue, and 100 mM dithiothreitol, and the mixture was boiled for 5 min.

An aliquot of the mixture equivalent to 10 µg protein was loaded onto the well of an 8 × 9 cm, 1-mm thickness, 5–15% gradient polyacrylamide gel (ATTO), and electrophoresed at a constant current of 20 mA/gel for 80 min. The gel was then electrotransferred onto a nitrocellulose membrane (10600004; Amersham, Buckinghamshire, UK) at a constant current of 180 mA/gel for 60 min. After a brief rinse with 25 mM Tris-HCl with 0.1% Tween 20 at pH 7.5 (TBST), the blotted membrane was incubated at room temperature with rabbit anti-human Cav3.1 (CACNA1G) polyclonal antibody (Cusabio) or rabbit anti-human Cav3.3 (CACNA1I) polyclonal antibody (Novus Biologicals) overnight or mouse anti-human β-actin monoclonal antibody (A5411; Sigma-Aldrich) for 30 min. The membrane was washed with PBST, reacted with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Thermo Fisher Scientific) at room temperature for 1 h, and soaked in Luminescence solutions A and B (Wako Pure Chemical Industries, Osaka, Japan) for chemiluminescence detection. Images were captured by using a LumiCube CMOS digital camera (Canon, Tokyo, Japan).

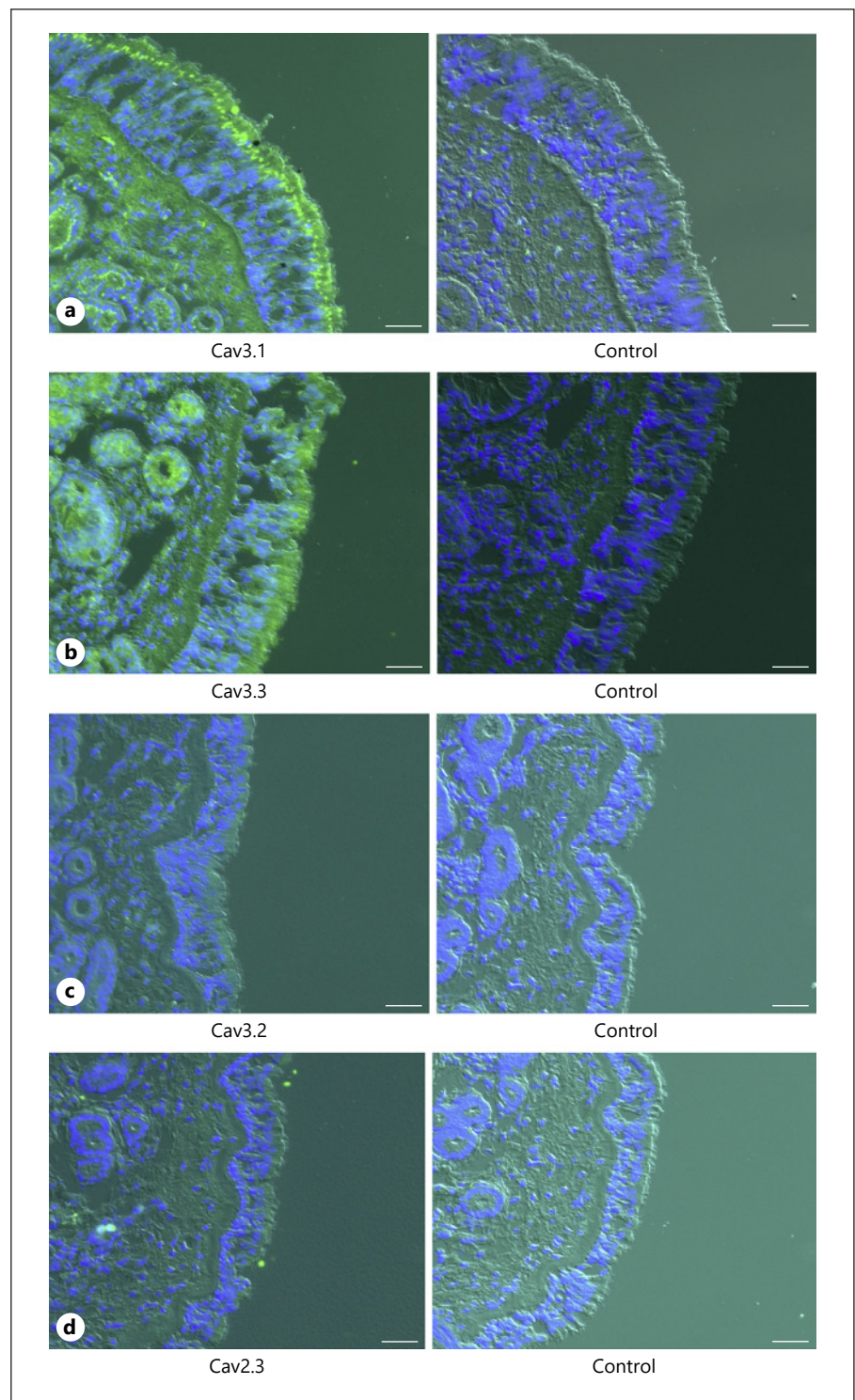
Preparation of Total RNA

The collected tissues were minced with surgical scissors, soaked in 1 mL TRIzol reagent (Invitrogen), and sonicated by using an ultrasonic homogenizer (Taitec, Saitama, Japan). Two hundred microliters of chloroform was added, and after thorough shaking, the mixture was centrifuged at 22,000 g for 15 min at 4°C. The aqueous layer was transferred to another tube, and total RNA was extracted by the acid guanidiniumthiocyanate-phenol-chloroform method and cleaned up with a BioRobot EZ1 system (QIAGEN, Hilden, Germany), which enables fully automated extraction and purification of nucleic acids by magnetic bead technology. The purity of RNA was assessed by determining the ratio of light absorption at 260 nm (*A*₂₆₀) to that at 280 nm (*A*₂₈₀). An *A*₂₆₀/*A*₂₈₀ ratio in the 1.9–2.1 range was considered acceptable. The RNA concentration was determined from *A*₂₆₀.

Real-Time RT-PCR

The total RNA was reverse-transcribed to cDNA with a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA), which uses random primers. The real-time reverse

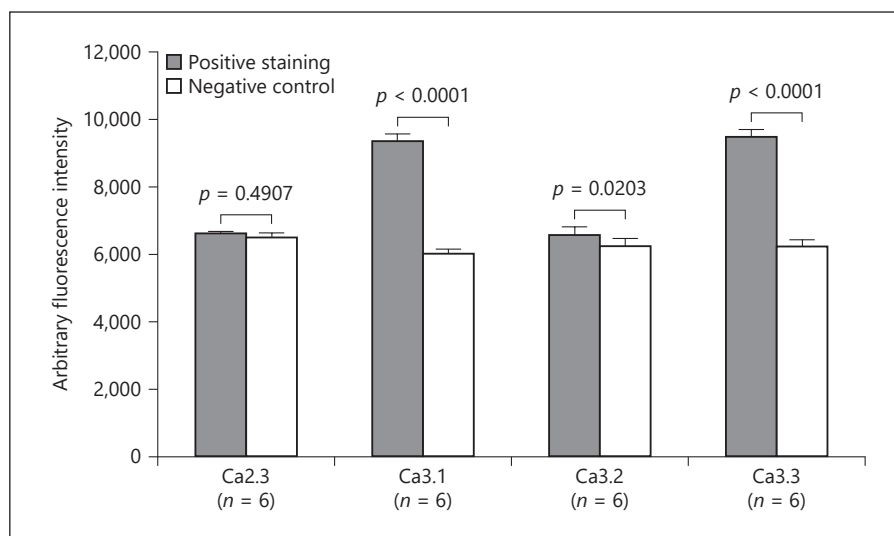
Fig. 1. Photomicrographs of fluorescence immunohistochemical staining. The nasal turbinate specimens were fixed with 4% paraformaldehyde and embedded while frozen in Tissue-Tek OCT Compound. Seven-micrometer-thick sections were prepared and mounted on silane-coated glass slides. The sections were incubated with rabbit anti-human Cav3.1 (CACNA1G) polyclonal antibody, rabbit anti-human Cav3.2 (CACNA1H) polyclonal antibody, rabbit anti-human Cav3.3 (CACNA1I) polyclonal antibody, or rabbit anti-human Cav2.3 (CACNA1E) polyclonal antibody. As a negative control, the primary antibodies were omitted from the process. The sections were then reacted with a secondary antibody, Alexa Fluor 488-conjugated goat anti-rabbit IgG, coverslipped with Prolong Gold antifade reagent containing DAPI, and examined under a Carl Zeiss Axioskop 2 Plus fluorescence microscope. Images were captured using a Carl Zeiss AxioCam digital camera attached to the microscope. Green and blue colors express the fluorescence of Alexa Fluor 488 and DAPI, respectively. Moderate fluorescence for Cav3.1 and Cav3.3 is observed on the surface of the epithelial cells and nasal gland cells (**a**, **b**), whereas there is no fluorescence for Cav3.2 or Cav2.3 (**c**, **d**). Scale bar, 20 μ m.



transcription-polymerase chain reaction (RT-PCR) analysis was performed with an Applied Biosystems StepOnePlus real-time PCR system using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) with *glyceraldehyde-3-phosphate dehydrogenase*

(*GAPDH*) as a housekeeping gene according to the manufacturer's instructions. The TaqMan Gene Expression Assays for *CACNA1G* (assay identification number: Hs00367969_m1), *CACNA1I* (assay identification number: Hs01096207_m1), and *GAPDH* (assay

Fig. 2. Quantitative evaluation of immunohistochemical fluorescence intensity. The fluorescence photomicrographs were displayed in a 16,384-step arbitrary scale of 0 (no fluorescence) to 16,383 (most intense fluorescence) for each pixel of the images. Areas in a positive staining slide were randomly chosen for each patient. The entire epithelial layer in the area was manually defined as the ROI, and the mean pixel value of the ROI was calculated (fluorescence intensity value) using AxioVision software. Likewise, the fluorescence intensity value of the corresponding region in the negative control slide was calculated.



identification number Hs99999905_m1) were purchased from Applied Biosystems. Ten nanograms of cDNA in 1 μ L was mixed with TaqMan Universal PCR Master Mix with AmpErase (uracil N-glycosylase) and the primer/probe set of the TaqMan Gene Expression Assays, and the mixture was subjected to PCR amplification with real-time detection. The thermal cycler conditions were as follows: holding at 95°C for 2 min, followed by two-step polymerase chain reaction of 40 cycles of 95°C for 1 s and 60°C for 20 s. Each sample was assayed in duplicate. The measured threshold cycle (C_T) was normalized by subtracting the C_T for *GAPDH* of each sample from those for *CACNA1G* and *CACNA1I*. From the obtained ΔC_T , the ratio of the target mRNA to *GAPDH* mRNA was calculated by the following formula: target mRNA/*GAPDH* mRNA ratio = $2^{-\Delta C_T}$.

Preparation of Mucosal Pieces from the Turbinate Sample for Video Recording

The turbinate mucosa was cut into thin strips at right angles to the mucosal surface using a razor blade. The mucosal strips were immediately immersed in O_2 -saturated HBSS and transferred into another tube filled with O_2 -saturated HBSS containing the chemical(s) to be tested. The sample was then put in a 20 $\times$ 6 $\times$ 1 mm test chamber filled with the same solution containing the chemical(s), and mucociliary movement was observed under a Nikon Eclipse 80i phase-contrast light microscope (Nikon, Tokyo, Japan) equipped with a high-speed digital video camera. All procedures were performed at room temperature (approximately 24°C) and completed within 3 h after the sample collection.

Measurement of Ciliary Beat Frequency

Four ciliary beat recordings of 2–3 s each were made every 60 s at a speed of 200 frames/s using the high-speed digital imaging system HAS-U1 (DITECT, Tokyo, Japan) and analyzed by using HAS-XViewer Camera Memory ver. 1.2.12 (DITECT). The number of ciliary beats was counted manually by checking the video in a slow replay mode. CBF was measured at three different portions of a mucosal strip. The CBF value in each experiment was determined by averaging the 12 measurements (4 times $\times$ 3 portions).

Intracellular Ca^{2+} Imaging of Isolated Ciliated Cells

Nasal epithelial cells were isolated by gently brushing the surface of the turbinate mucosa in O_2 -saturated HBSS. Cell suspension was transferred to a thin-bottomed petri dish (Matsunami Glass Industries) coated with adhesive spray (Tack Spray; Nitto Nitoms, Tokyo, Japan) and incubated with 5 μ M Fluo-8 AM for 20 min. Equal volume of 20 μ M mibefradil or NNC 55-0396 in HBSS containing 5 μ M Fluo-8 AM was then gently added (the final concentration of the VGCC blockers was 10 μ M). The fluorescence of isolated ciliated cells was observed under a Carl Zeiss Axioskop 2 Plus fluorescence microscope. The light source was an HBO 103 W/2 mercury vapor lamp. The light was let pass through a 475- to 495-nm bandpass filter for the excitation, and the emitted fluorescence was allowed to pass through a 515- to 565-nm bandpass filter. Fluorescence images were recorded just before and 10/15 min after the addition of the VGCC blockers, using the high-speed high-sensitivity digital imaging system HAS-D71 (DITECT) attached to the microscope at a speed of 100 frames/s. The fluorescence intensity was quantitatively analyzed by using HAS-XViewer Camera Memory ver. 1.3.0.13 (DITECT). The fluorescence images were displayed in a 256-step arbitrary scale of 0 (no fluorescence) to 255 (most intense fluorescence) for each pixel of the images. ROI that included a target cell was manually defined, and the mean pixel value of the ROI was calculated. The shape and size of the ROI were fixed for each target cell. The % fluorescence intensity was calculated for each cell by dividing the fluorescence intensity of interest by that just before the addition of the VGCC blockers.

Measurement of ATP Release from the Nasal Mucosa

Ex vivo ATP release from the nasal mucosa was measured as described by Koizumi et al. [9]. Round pieces measuring 4 mm in diameter were cut out from the turbinate mucosa using a metal circular punch. The cutout mucosal pieces were preincubated in O_2 -saturated HBSS for 30 min. After a brief wash with HBSS, the samples were incubated in a 12-well culture plate containing 4 mL of HBSS in each well with or without a VGCC blocker. The incubation time was 15 min for mibefradil and 10 min for NNC 55-

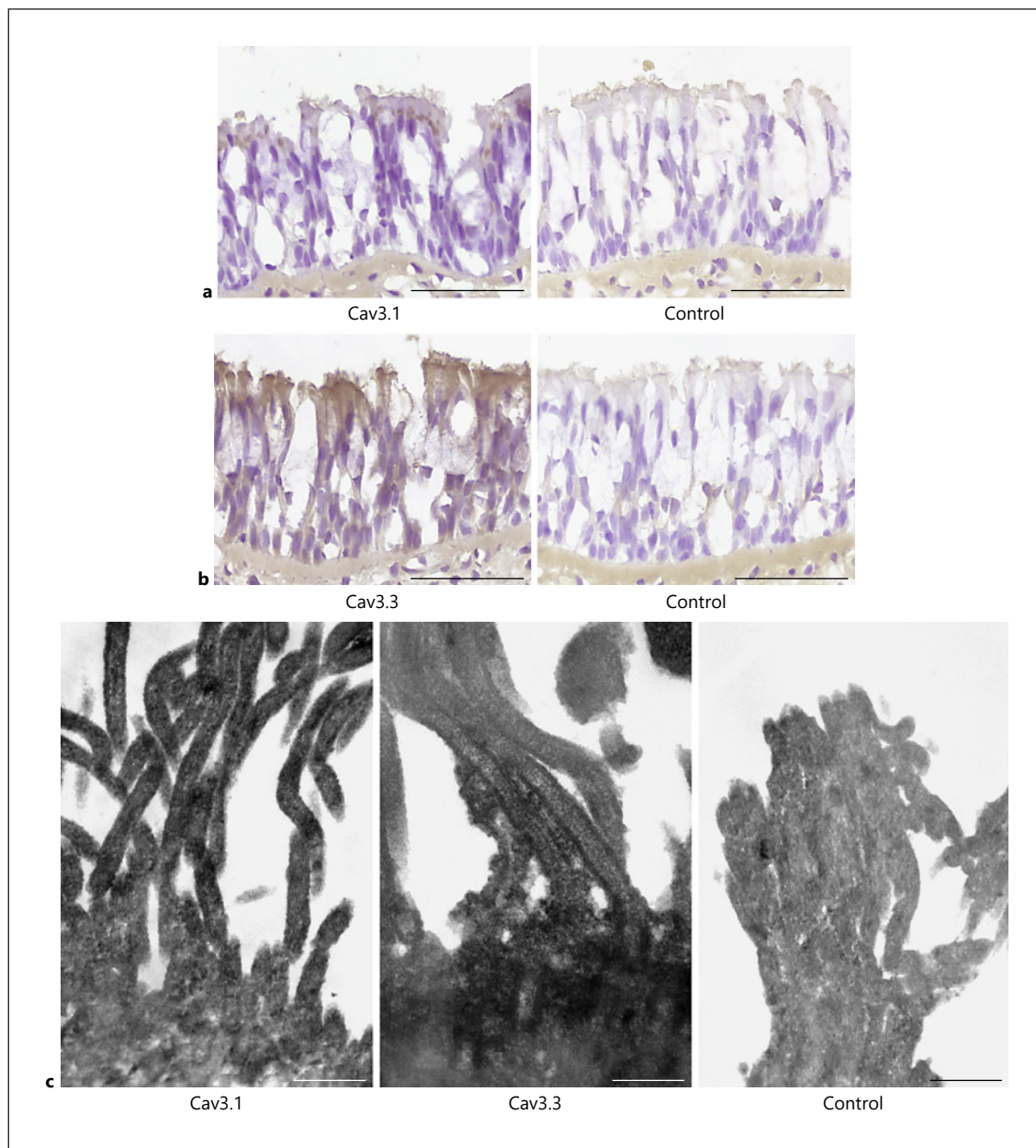


Fig. 3. Immunoelectron microscopy. The nasal turbinate specimens were fixed with 4% paraformaldehyde and embedded while frozen in Tissue-Tek OCT Compound. Seven-micrometer-thick sections were prepared using a cryostat and mounted on silane-coated glass slides. The sections were incubated with rabbit anti-human Cav3.1 (CACNA1G) polyclonal antibody or rabbit anti-human Cav3.3 (CACNA1I) polyclonal antibody, fixed with 1% glutaraldehyde, and developed with DAB. As a negative control, the primary antibodies were omitted from the process. The sections were then post-fixed with 1% OsO₄ and embedded in Epon

812. Seventy- to 100-nm-thick ultrathin sections were prepared using an ultramicrotome, stained with 4% lead nitrate, and examined under a JEOL JEM-1200EX transmission electron microscope. **a, b** Photomicrographs of DAB immunohistochemical staining. Immunoreactivities for both Cav3.1 (**a**) and Cav3.3 (**b**) are observed on the surface of the epithelial cells. Scale bar, 50 μm. **c** Immunoelectron micrographs for Cav3.1 and Cav3.3. Immunoreactivity for Cav3.1 was localized on the surface of the cilia, and that for Cav3.3 was localized in the cilia and at the base of the cilia. Scale bar, 0.5 μm.

0396. One hundred microliters of medium was then collected by using an ATP water-testing device, AquaSnap Total (Hygiena, Camarillo, CA, USA), and the ATP concentration was measured by a luciferin-luciferase assay using a SystemSURE luminometer (Hygiena). A calibration curve was made by measuring the ATP levels of standard ATP solutions (10^{-11} to 10^{-8} M). All procedures were performed at room temperature (approximately 24°C) and completed within 1 h after the sample collection.

Statistical Analysis

Data were expressed as the means $\pm$ SEM. Statistical analysis was performed with the BellCurve for Excel Statistics (Social Survey Research Information Co., Tokyo, Japan). Differences between two groups were analyzed by a two-tailed paired *t* test. *p* values <0.05 were considered significant.

Results

Figure 1 shows representative photomicrographs of fluorescence immunohistochemical staining of the turbinate mucosa for three isoforms of a T-type VGCC α_1 subunit, Cav3.1, Cav3.2, and Cav3.3, and an R-type VGCC α_1 subunit, Cav2.3. Moderate fluorescence for Cav3.1 and Cav3.3 was observed on the surface of the epithelial cells and nasal gland cells (Fig. 1a, b). On the other hand, there was almost no fluorescence for Cav3.2 or Cav2.3 (Fig. 1c, d). Figure 2 is the result of the quantitative evaluation of immunohistochemical fluorescence intensity. The fluorescence intensity was significantly different between positive staining and negative control for Cav3.1 ($p < 0.0001$), Cav3.2 ($p = 0.0203$), and Cav3.3 ($p < 0.0001$), but not for Cav2.3 ($p = 0.4907$). The difference was large and clear for Cav3.1 and Cav3.3 with fluorescence intensity ratios (positive/negative) of 1.555 and 1.521, respectively. On the other hand, the difference was small for Cav2.3 and Cav3.2 with fluorescence intensity ratios of 1.019 and 1.053, respectively. Therefore, we further explored for Cav3.1 and Cav3.3.

Immunoreactivities for Cav3.1 and Cav3.3 were confirmed in DAB immunohistochemical staining (Fig. 3a, b). At the ultrastructural level, immunoreactivity for Cav3.1 was localized on the surface of the cilia, and that for Cav3.3 was localized in the cilia and at the base of the cilia (Fig. 3c). The existence of Cav3.1 and Cav3.3 was confirmed by Western blot analysis (Fig. 4).

Results of real-time RT-PCR are presented in Figure 5. The exponential rise of the trace of amplification plots (Fig. 5a) proved the presence of *CACNA1G* mRNA and *CACNA1I* mRNA in the human nasal mucosa. The average ratios of *CACNA1G* mRNA/*GAPDH* mRNA and *CACNA1I* mRNA/*GAPDH* mRNA were 0.0088 ± 0.0075

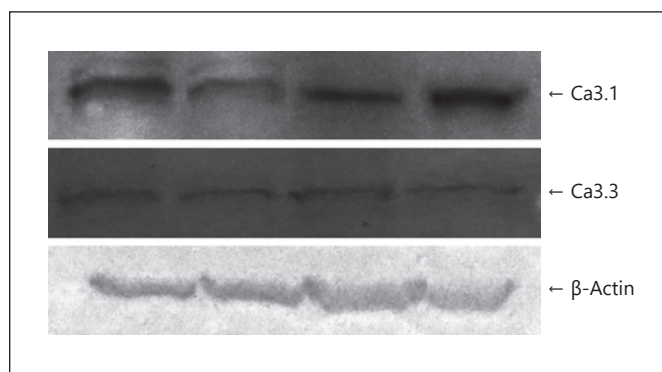


Fig. 4. Western blot analysis. The collected turbinate mucosa was minced/ground and soaked in the RIPA lysis buffer with protease inhibitor and phosphatase inhibitor. The lysate was centrifuged, and the supernatant was mixed with EzApply and boiled. An aliquot of the mixture equivalent to 10 μ g protein was loaded onto the well of a 5–15% gradient polyacrylamide gel and electrophoresed at a constant current of 20 mA/gel for 80 min. The gel was then electrotransferred onto a nitrocellulose membrane at a constant current of 180 mA/gel for 60 min. The blotted membrane was incubated with rabbit anti-human Cav3.1 (CACNA1G) polyclonal antibody, rabbit anti-human Cav3.3 (CACNA1I) polyclonal antibody, or mouse anti-human β -actin monoclonal antibody. The membrane was reacted with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG and soaked in Luminescence solutions for chemiluminescence detection. Images were captured by a LumiCube CMOS digital camera.

and 0.0110 ± 0.0072 , respectively ($n = 8$; Fig. 5b). The expression level of *CACNA1I* mRNA tended to be higher than that of *CACNA1G* mRNA ($p = 0.0544$).

Figure 6a illustrates the effects of T-type VGCC blockers on ciliary beats. The preincubation time was 15 min for mibefradil and 10 min for NNC 55-0396. The baseline CBF was 7.35 ± 0.15 Hz ($n = 22$). The CBF was inhibited by the addition of 1–10 μ M mibefradil, a T-type VGCC blocker, in a dose-dependent manner (7.20 ± 0.20 Hz at 1 μ M [$n = 7$], $p = 0.0002$; 6.76 ± 0.23 Hz at 10 μ M [$n = 7$], $p < 0.0001$). A highly specific T-type VGCC blocker, NNC 55-0396 (10 μ M), also significantly inhibited the CBF (7.96 ± 0.20 Hz vs. 6.41 ± 0.27 Hz [$n = 7$], $p < 0.0001$). cAMP and cGMP are well-known activators of ciliary beating [2]. The CBF was significantly increased by the addition of cAMP/cGMP analogs, 8-bromo-cAMP (100 μ M) and 8-bromo-cGMP (100 μ M), together (Fig. 6b) and separately (Fig. 6c). This increase was inhibited by 10 μ M mibefradil and 10 μ M NNC 55-0396 (Fig. 6b, c), but not changed by 1 μ M FDB, a creatine kinase inhibitor (Fig. 6d).

Figure 7 represents changes in Fluo-8 fluorescence of isolated ciliated cells after the addition of the VGCC blockers. Ten micromolar mibefradil and 10 μ M NNC 55-

0396 significantly lowered the % fluorescence intensity in a time-dependent manner. On the other hand, the ATP release from the nasal mucosa was not changed by mibefradil or NNC 55-0396 (Fig. 8).

These results indicate that T-type VGCC α_1 subunits, Cav3.1 and Cav3.3, exist at the cilia of the nasal epithelial cells and participate in the regulation of ciliary beats and that these channels act downstream of cAMP/cGMP. The results also suggest that regulatory signals of cAMP/cGMP are not transmitted to creatine kinase, which plays an essential role in the intraciliary energy shuttle system [4].

Discussion

The present study is the first report to provide the evidence for the existence of T-type VGCC in the human airway mucosa. We also showed using specific blockers that T-type VGCC participates in the regulation of ciliary beating in this tissue.

The cilia on the surface of the airway mucosa continuously beat in an orderly pattern and generate coordinated metachronal waves, which is essential for effective mucociliary clearance. The axoneme of the cilia has a calcium sensor inside; i.e., the light chain of outer arm dynein has

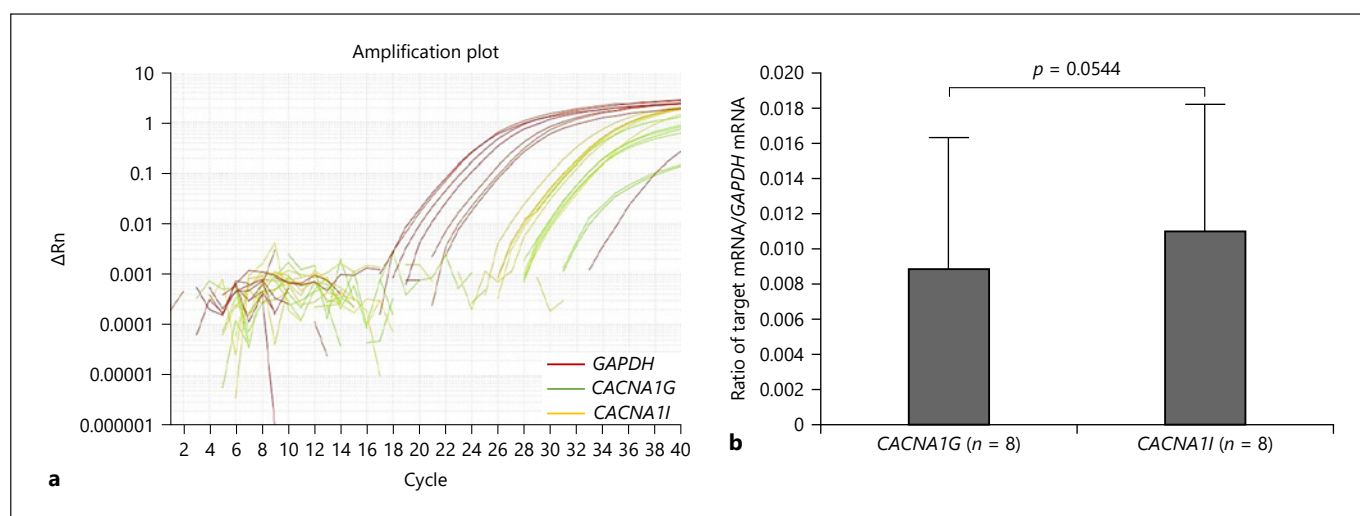


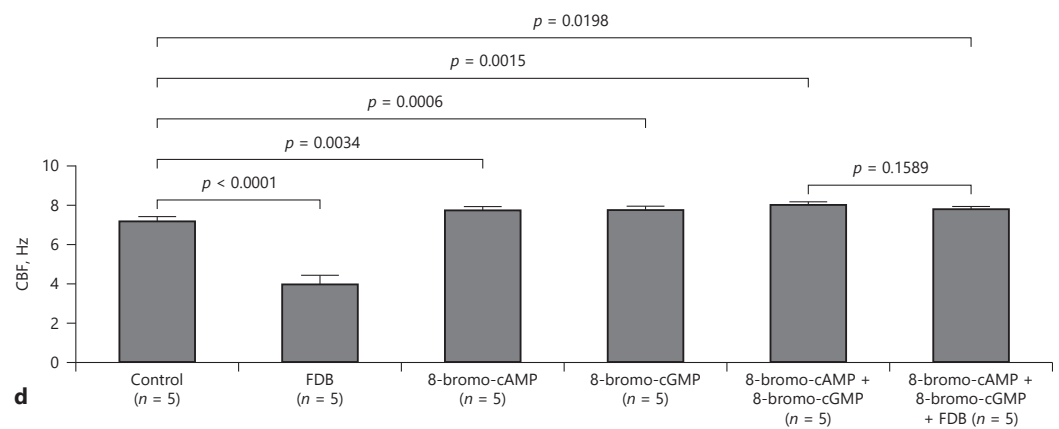
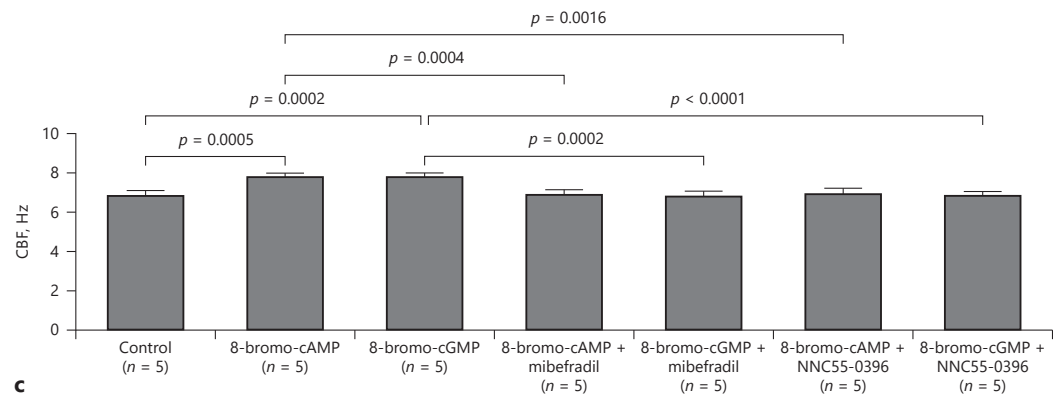
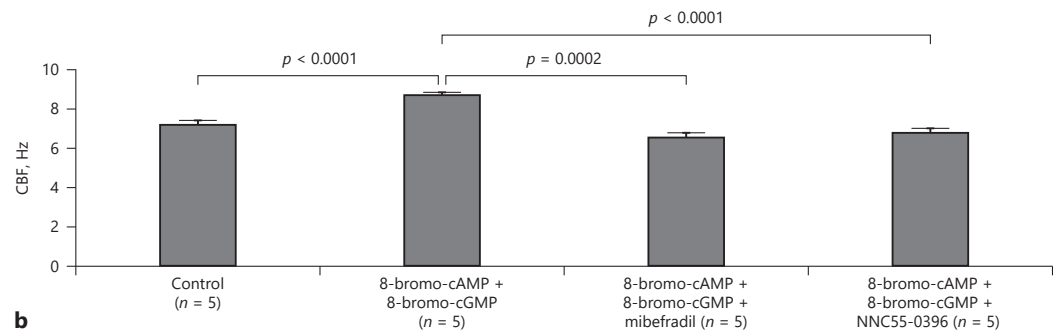
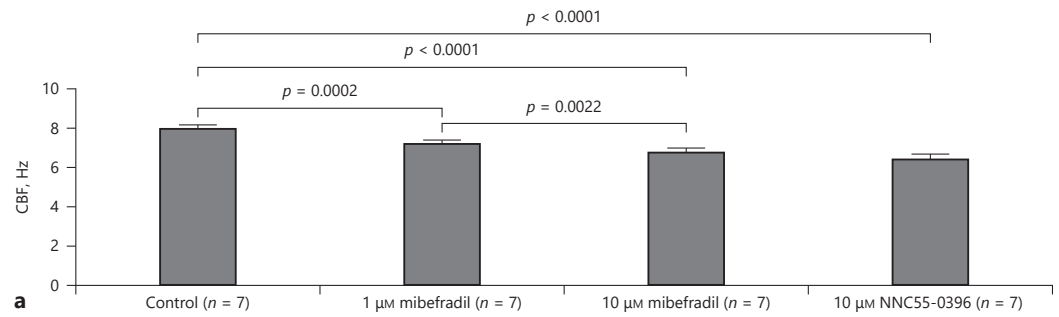
Fig. 5. Expressions of *CACNA1G* mRNA and *CACNA1I* mRNA. The collected turbinate mucosa was minced and soaked in TRIzol reagent. Total RNA was extracted by the acid guanidiniumthiocyanate-phenol-chloroform method, cleaned up with a BioRobot EZ1 system, and reverse-transcribed to cDNA using a High-Capacity RNA-to-cDNA Kit. The real-time reverse transcription-polymerase chain reaction analysis was performed with an Applied Biosystems StepOnePlus real-time PCR system using TaqMan Fast Universal PCR Master Mix with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as a housekeeping gene. The thermal cycle conditions were as follows: holding at 95°C for 2 min, followed

by two-step polymerase chain reaction of 40 cycles of 95°C for 1 s and 60°C for 20 s. The measured threshold cycle (C_T) was normalized by subtracting the C_T for *GAPDH* of each sample from those for *CACNA1G* and *CACNA1I*. From the obtained ΔC_T , the ratio of the target mRNA to *GAPDH* mRNA was calculated by the following formula: target mRNA/*GAPDH* mRNA ratio = $2^{-\Delta C_T}$. **a** Representative trace of amplification plots of real-time RT-PCR. **b** Expression levels of *CACNA1G* mRNA and *CACNA1I* mRNA. The average ratios of *CACNA1G* mRNA/*GAPDH* mRNA and *CACNA1I* mRNA/*GAPDH* mRNA are 0.0088 ± 0.0075 and 0.0110 ± 0.0072 , respectively ($n = 8$).

Fig. 6. Inhibition of CBF by T-type calcium channel blockers. The turbinate mucosa was cut into thin strips at right angles to the mucosal surface. The mucosal strip was put in a test chamber filled with O_2 -saturated HBSS containing the chemical(s) to be tested, and mucociliary movement was observed under a phase-contrast light microscope equipped with a high-speed digital video camera. Four ciliary beat recordings of 2–3 s each were made every 60 s at a speed of 200 frames/s. The number of ciliary beats was counted manually by checking the video in a slow replay mode. CBF was measured at three different portions of a mucosal strip. The CBF value in each experiment was determined by averaging the 12 mea-

surements (4 times $\times$ 3 portions). CBF, ciliary beat frequency. **a** The CBF was inhibited by the addition of mibefradil, a T-type calcium channel blocker, in a dose-dependent manner. A highly specific T-type calcium channel blocker, NNC 55-0396, also significantly inhibited the CBF. **b, c** The CBF was significantly increased by the addition of cAMP/cGMP analogs, 8-bromo-cAMP (100 μ M) and 8-bromo-cGMP (100 μ M), together (**b**) and separately (**c**). This increase was inhibited by 10 μ M mibefradil and 10 μ M NNC 55-0396. **d** The CBF increase elicited by 8-bromo-cAMP/8-bromo-cGMP was not changed by the addition of 1 μ M FDB, a creatine kinase inhibitor.

(For figure see next page.)



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Fig. 7. Effects of T-type calcium channel blockers on the intracellular Ca^{2+} level of isolated ciliated cells. Nasal epithelial cells were isolated by gently brushing the surface of the turbinate mucosa in O_2 -saturated HBSS. Cell suspension was transferred to a thin-bottomed petri dish coated with adhesive spray and incubated with $5 \mu\text{M}$ Fluo-8 AM for 20 min. Mibefradil or NNC 55-0396 was then gently added (the final concentration of the blockers was $10 \mu\text{M}$). The fluorescence of isolated ciliated cells was observed under a Carl Zeiss Axioskop 2 Plus fluorescence microscope. Fluorescence images were recorded just before and 10/15 min after the addition of the VGCC blockers, using the high-speed high-sensitivity digital imaging system attached to the microscope at a speed of 100 frames/s. The fluorescence images were displayed in a 256-step arbitrary scale of 0 (no fluorescence) to 255 (most intense fluorescence) for each pixel of the images. An ROI that included a target cell was manually defined, and the mean pixel value of the ROI was calculated. The % fluorescence intensity was calculated for each cell by dividing the fluorescence intensity of interest by that just before the addition of the blockers.

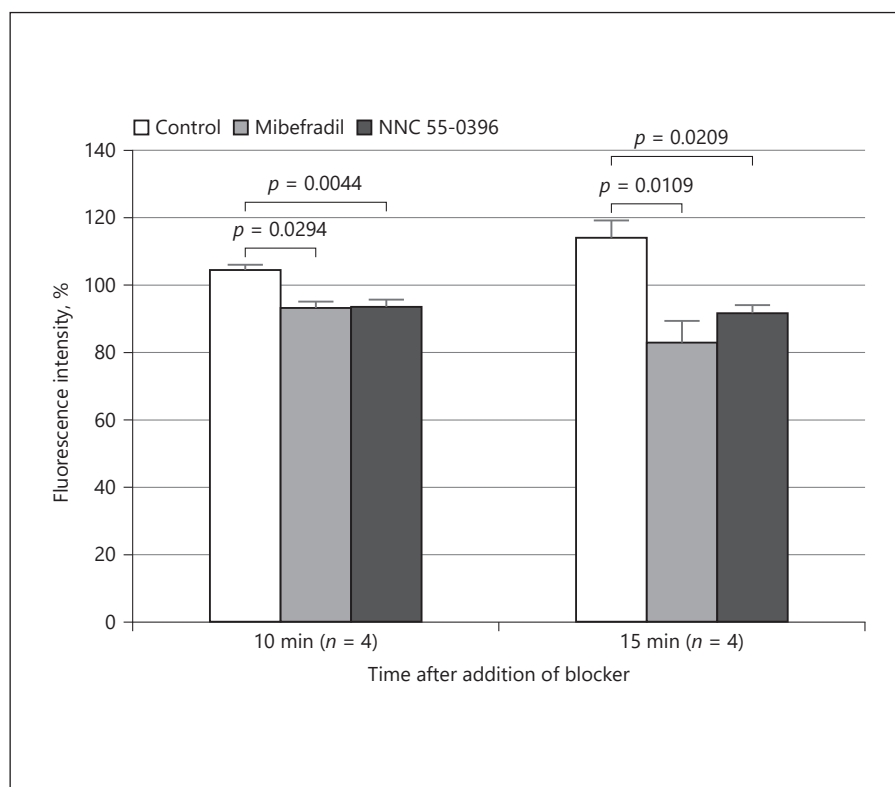
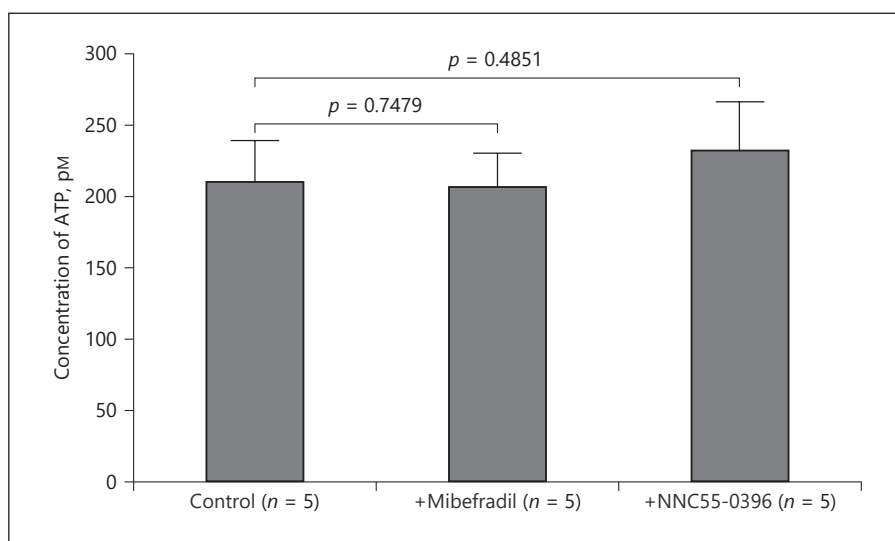


Fig. 8. ATP release from the nasal mucosa. Round pieces measuring 4 mm in diameter were cut out from the turbinate mucosa using a metal circular punch. The cutout mucosal pieces were preincubated in O_2 -saturated HBSS for 30 min. After a brief wash with HBSS, the samples were incubated in a 12-well culture plate containing 4 mL of HBSS in each well with or without a VGCC blocker. The incubation time was 15 min for mibefradil and 10 min for NNC 55-0396. The ATP concentration was measured by a luciferin-luciferase assay.



a Ca^{2+} -sensing domain and controls the dynein heavy chain, a force-generating ATPase [10, 11]. Therefore, intracellular Ca^{2+} is a probable candidate for an intracellular signal that directly controls the change in molecular configuration of dynein and thereby the change in configuration of the axoneme [2].

VGCCs exist in the membrane of a variety of cells throughout the body and play important roles in signal transduction related to muscle contraction, chemotaxis, gene expression, synaptic plasticity, and secretion of hormones and neurotransmitters [12]. Compared to L-type VGCC, the most abundant and well-characterized VGCC, T-type VGCC is activated at lower voltages of the mem-

brane potential and shows rapid voltage-dependent inactivation and slow deactivation [13]. T-type VGCC is expressed in the brain, peripheral nerves, heart, smooth/skeletal muscles, endocrine glands, endothelium, sperm, and lung [14]. The membrane potential ranges of activation and inactivation of this channel overlap each other, and this property leads to the window current, generating repetitive bursts of Ca^{2+} influx [14]. This phenomenon has well been investigated in the cerebellar Purkinje cells, thalamic/hypothalamic neurons, adrenal glomerulosa/fasciculata cells, and sinoatrial node pacemaker cells [12, 13].

Involvement of VGCCs in the regulation of the flagellar/ciliary movement has been reported in algae and paramecia [6, 7]. Fujiu et al. [6] found that the deduced amino acid sequence of CAV2, a VGCC homolog expressed in the flagella of *Chlamydomonas*, has high similarity to human Cav3.1 (T-type VGCC $\alpha 1$ subunit) and human Cav2.3 (R-type VGCC $\alpha 1$ subunit). CAV2 has been shown to be responsible for the flagellar movement: A mutation in CAV2 causes a defect in backward swimming and photophobic/mechanoshock responses of this protozoan [6].

The present results provided strong evidence for the existence of T-type VGCC in the cilia of the human upper airway mucosa and showed that this channel participates in the regulation of ciliary beating. We also revealed that T-type VGCC blockers induce a decrease in the intracellular Ca^{2+} level of isolated ciliated cells, supporting the participation of T-type VGCC in the regulation of intracellular Ca^{2+} of these cells. It has been known that ciliary beating in the airway mucosa is activated by cAMP/cGMP and protein kinases A/G [2, 8]. Interestingly, the similar pharmacological properties of T-type VGCC have been reported: T-type VGCC is upregulated by 8-bromo-cAMP and forskolin (an adenylate cyclase activator) and downregulated by Rp-cAMPS (a protein kinase A inhibitor) [12]. The present study clearly showed the interrelation between cyclic nucleotides and T-type VGCC in the regulatory pathway of ciliary beating: a CBF increase elicited by cAMP/cGMP analogs was inhibited by the specific blockers of T-type VGCC, indicating that this channel is downstream of cAMP/cGMP in the regulatory pathway. As in the brain and heart, where T-type VGCC acts as an autogenerator of intracellular Ca^{2+} oscillation, this channel is likely to act in the same way in the cilia of the airway epithelial cells, generating direct control signals for ciliary beating.

On the other hand, we found no significant change in the level of ATP released from the nasal mucosa after the

addition of the VGCC blockers (Fig. 8). Our previous study on the regulatory pathway of ciliary beating demonstrated that extracellular ATP release is mediated by pannexin-1 channel, which acts upstream of the calmodulin-adenylate/guanylate cyclases-cAMP/cGMP cascade [4, 5, 8]. Considering the present results showing that T-type VGCC acts downstream of cAMP/cGMP, it is reasonable that the blockers of VGCC did not change the ATP release.

One of the limitations of the present study is that the property of T-type VGCC in the epithelial cells was not examined electrophysiologically. Electrophysiological means such as the patch-clamp technique would be a powerful tool to clarify the electrophysiological properties of ion channels. However, ciliated cells continuously shake as the cilia beat, making the patch-clamp technique infeasible. Another limitation is that we failed to detect intracellular Ca^{2+} oscillation of the isolated ciliated cells in the experiment using Fluo-8. The reason for this is unclear. Intracellular Ca^{2+} oscillation may be small in amplitude and confined only within the cilia, and therefore oscillation of Fluo-8 fluorescence may have been masked by fluorescence fluctuation caused by ciliary motion. These issues remain to be addressed in future studies.

Conclusions

We investigated the expression of T-type VGCC as a candidate for the generator of calcium signals that regulate ciliary beating in the human nasal epithelial cells. The results showed that 2 subtypes of the T-type VGCC $\alpha 1$ subunit, Cav3.1 and Cav3.3, were expressed at the transcriptional and protein levels. At the ultrastructural level, these channels were localized at the cilia. Pharmacological inhibition tests using specific blockers revealed the participation of T-type VGCC in the regulation of ciliary beating and intracellular Ca^{2+} . A better understanding of the regulatory mechanism of airway ciliary beating will be helpful in developing a new therapeutic strategy for intractable airway diseases.

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Statement of Ethics

Written informed consent was obtained from all patients. The study was approved by the Institutional Review Board of the University of Occupational and Environmental Health (UOEHC19-014) and conducted in accordance with the World Medical Association Declaration of Helsinki.

Conflict of Interest Statement

The authors declare no conflicts of interest to disclose.

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Author Contributions

H.S. and T.K. planned and designed the study. J.O. and T.K. collected nasal samples. T.N.N. and R.B. performed immunohistochemistry and immunoelectron microscopy. T.N.N. and Y.Y. performed Western blot and real-time RT-PCR. T.N.N., J.O., and T.W. measured the ciliary beat frequency. Intracellular Ca^{2+} imaging and ATP measurement were conducted by T.N.N., H.S., J.O., and T.W. T.N.N., H.S., R.B., Y.Y., and T.W. analyzed data. T.N.N., H.S., and T.K. wrote the manuscript.

Data Availability Statement

Data that support the findings of this study are available on request to the corresponding author.

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