Molecular and functional expression of anion exchangers in normal human nasal epithelial cells

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Molecular and functional expression of anion exchangers in normal human nasal epithelial cells

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The Master's Thesis submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirement for the degree of Master of Medical Science

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I look forward to the day when I become able to write a better thesis and end my acknowlegement

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Abstract

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Anions play an important role in the regulation of airway surface liquid volume, viscosity, and pH. However, functional localization of anion exchanger is poorly understood in human nasal epithelium, and the effect of other anion transporters on anion exchanger activity have not been clearly reported. The aim of this study was to investigate the regulation of mRNA expression level of the anion exchangers in accordance with mucociliary differentiation and the functional expression of the anion exchanger in both luminal and basolateral membranes in normal human nasal epithelial cells. In order to do this, we first induced mucociliary differentiation of the normal human nasal epithelial cells and then confirmed it through histological and molecular

characterization. Second, we identified the types anion exchanger isoforms present in normal human nasal epithelial cells, and determined if they were affected by mucociliary differentiation. Finally, we examined the functional activity of anion exchanger in the luminal and basolateral membranes of the polarized monolayer normal human nasal epithelial cells. In this study, we showed that epithelial cells differentiated into mucociliary epithelium through histological and molecular characterization and that while various anion exchanger isoforms are expressed, only some of them were affected by mucociliary differentiation. In addition, we found that anion exchange activity was affected by intracellular cyclic adenosine monophosphate(cAMP)-mediated and calcium signaling, and that diphenylamine-2,2'-dicarboxylic acid(DPC) and 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate(DIDS)-sensitive anion exchange activity existed in both the luminal and basolateral membranes of normal human nasal epithelial cells. Our findings through molecular and functional studies using normal human nasal epithelial cells suggest that anion exchanger may play an important role in the regulation of pH*i* and airway surface liquid pH.

Key words : anion exchanger, pH, mucociliary differentiation, human nasal epithelial cells.

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Ⅰ. INTRODUCTION

Anions play an important role in the regulation of airway surface liquid (ASL) volume, viscosity, and pH. Both the HCO_3^- and Cl^- are major constituents of secreted fluids and have an effect on ASL pH. Low ASL pH induces an increase in mucin viscosity, which facilitates bacterial infection. Therefore, adequate regulation of ASL pH is essential for the prevention of airway infection and obstruction[1]. In addition, anion secretion contributes to the regulation of intracellular pH (pH_i) and cell volume in human airway epithelium[2]. Various anion transporters, such as the anion exchanger (AE), cystic fibrosis transmembrane conductance

regulator (CFTR), sodium bicarbonate cotransporter (NBC), and calcium activated chloride channel (CaCC) are expressed in the human airway[3-5]. Among them, AE plays its role by translocating monovalent anions, such as Cl^- and HCO_3^-, across the plasma membranes.

Recent studies have identified various families of AE such as solute carrier(SLC)4 and SLC26 gene family[6, 7]. The 10 kinds of SLC4 gene and protein family contain three types of HCO_3^- transporters: AE, NBC and Na^+-driven Cl^-–HCO_3^- exchangers(NDCBE). Some of them, SLC4A1,-A2,-A3 and SLC4A9 were reported as Cl^-–HCO_3^- exchanger type and these isoforms are named AE1, AE2, AE3 (bAE3 and cAE3)and AE4 respectively[8]. Moreover, in present study, 11 kinds of SLC26 gene families were reported as AE. Each isoform of SLC26 exchangers participates in transport of various anions such as SO_4^{2-}, Cl^-, I^-, OH^- and HCO_3^-. Individual transport of anions was controlled by separate isoforms, which have specificity. However, expression of many SLC4 and SLC26 isoforms has not been reported in human nasal epithelium. Moreover, effect of mucociliary differentiation on the expression of AE isoforms has not been reported in human airway epithelial cells. Only AE2, bAE3 and SLC26A9 were reported to be expressed in human tracheobronchial tree and lung cancer cell line by reverse transcriptase

polymerase chain reaction(RT-PCR) [9, 10].

Recently, functional characterization of various AEs have been investigated by phamacological studies in various kinds of human cells and tissue including distal colon[11], pancreatic duct cell line[12]. However, there are only a few reports on the function of AEs in human airway [3, 5, 13]. Moreover, functional localization of AE was poorly understood in human nasal epithelium, and effect of other anion transporters on the AE activity were not clearly reported so far.

Therefore, the aim of this study was to investigate the regulation of mRNA expression level of the AEs in accordance with mucociliary differentiation and the functional expression of the AE in both luminal(LM) and basolateral(BLM) membranes in normal human nasal epithelial (NHNE) cells. In order to do this, we first induced mucociliary differentiation of the NHNE cells and then confirmed it through histological and molecular characterization. Second, we identified the types AE isoforms present in NHNE cells, and determined if they were affected by mucociliary differentiation. Finally, we examined the functional activity of AE in the LM and BLM of the polarized monolayer NHNE cells.

Ⅱ. MATERIALS AND METHODS

Cell culture

Passage-2 NHNE cells ($1x10^5$ cells/culture) were seeded in 0.5 ml of culture medium onto 24.5 mm, 0.45μm pore, Transwell-clear (Costar Co., Cambridge, MA, USA) culture inserts. The procedure was approved by the Institutional Review Board. The cells were cultured using a 1:1 mixture of bronchial epithelial growth medium (BEGM)and Dulbecco's modified Eagle's medium (DMEM) containing all the supplements[14].

The cultures were grown submerged for the first 9 days, during which the culture medium was changed daily. An air-liquid interface (ALI) was created on the 9^{th} day by removing the apical medium and feeding the cultures only from the basal compartment. The culture medium was changed daily after the ALI was applied. In order to determine the time duration effects, RNA was collected on the day of confluence and on the 7^{th}, 14^{th}, and 28^{th} days after confluence.

Morphologic examination

For the examination of cell differentiation, cultured cells on the semi-permeable membrane were fixed in 10% buffered neutral formalin, embedded in paraffin, and then sectioned. They were stained with hematoxylin and eosin, and then observed with a light microscope (Olympus Light Microscope, Vanox-S type, Japan) on the day of confluence and on the 7^{th}, 14^{th}, and 28^{th} day after confluence.

Periodic Acid-Schiff (PAS) Staining

Serial sections were collected on coated slides. The slide sections were treated with 3% glacial acetic acid for 3 minutes. Then they were incubated with 0.5% periodic acid (Fisher Scientific, Pittsburgh, PA, USA) solution for 5 minutes. The slides were rinsed with phosphate-buffered saline. Then the sections were reacted with Schiff solution (Sigma Chemical Company, St. Louis, MO, USA) for 5 minutes, washed in tap water, and counterstained with Harris hematoxylin.

RT-PCR

Gene-specific PCR primer sets for human AE2, bAE3, AE4, SLC26A3-A11 except SLC26A5 and SLC26A10 were designed to detect the isoform-specific mRNA in human airways. MUC5AC and Cornifin-alpha(α) primer sets were used to confirm the differentiation of NHNE cells according to culture period. The sequence information of some isoforms was retrieved from GenBank. The oligonucleotide amplimers for β2 microglobulin, which generated a 266 base pair(bp) PCR fragment, were used as the control gene for RT-PCR (Table 1).

Table I. *PCR primer sequences specific to the target genes and annealing temperature*

Primer	Sequence	Annealing temperature (℃)	Size (bp)
AE2	**Sense:** 5'-GAA GAT TCC TGA GAA TGC CT-3' **Antisense:** 5'-GTC CAT GTT GGC AGT AGT CG-3'	55.5	**181**
bAE3	**Sense:** 5'-ATC TGA GGC AGA ACC TGT GG-3' **Antisense:** 5'-TTT CAC TAA GTG TCG CCG C-3'	60	**418**
AE4	**Sense:** 5'-AGC GCT TGG ACT GCC TTG GTA TGT-3' **Antisense:** 5'-AGG GGG AAG ATG ATG GCT GCA GGG GTA GAC-3'	57	**431**
SLC26A3	**Sense:** 5'- TGC CAC AGC CAA CAG AAA AAT CAA A -3' **Antisense:** 5'- GGG GGA ATG TCG ACC AGC AGA G -3'	58	**330**
SLC26A4	**Sense:** 5'-GTT TAC TAG CTG GCC TTA TAT TTG GAC TGT-3' **Antisense:** 5'-AGG CTA TGG ATT GGC ACT TTG GGA ACG-3'	55	**484**
SLC26A6	**Sense:** 5'- TAG GGG AGG TTG GGC CAG GGA TGC -3' **Antisense:** 5'- TGC CGG GAA GTG CCA AAC AGG AAG AAG TAG AT -3'	60	**456**
SLC26A7	**Sense:** 5'- CAC TGT GTC TGG GAT AAT GTT GG -3' **Antisense:** 5'-CCA GTT GCA GCA CAA ACA TG-3'	65	**353**
SLC26A8	**Sense:** 5'-CCA AGA CCC AGA CCG AGA TG-3' **Antisense:** 5'-GAG TCT GAG ACT GGG TGG AAG C-3'	58	**150**
SLC26A9	**Sense:** 5'-TCC AGG TCT TCA ACA ATG CCA C-3' **Antisense:** 5'-CGA GTC TTG TGC ATG TAG CGA G-3'	58	**400**
SLC26A11	**Sense:** 5'- ATC CCG CCC TTC TCA GTG AC -3' **Antisense:** 5'- TAG TCC AGA GAC AGC AGC ACC AG -3'	65	**329**
MUC5AC	**Sense:** 5'- TCCGGCTCCATCTTCTCC -3' **Antisense:** 5'- ACTTGGGCACTGGTGCTG -3'	60	**680**
cornifin-α	**Sense:** 5'- CATTCTGTCTCCCCCAAAAA -3' **Antisense:** 5'- ATGGGGGTATAAGGGAGCTG -3'	60	**172**
β2M	**Sense:** 5'-CTC GCG CTA CTC TCT CTT TCT GG -3' **Antisense**: 5'-GCT TAC ATC TCT CCA TCC CAC TTA A-3'	55	**266**

The total RNAs were collected from NHNE cells using Trizol solution (Gibco BRL, Rockville, MA, USA), and PCR was performed. The RT-PCR products were separated by electrophoresis on a 2% agarose gel containing 50 ng/ml ethidium bromide. Bands of the expected sizes were visualized under ultraviolet light and photographed with Polaroid Type 55 film. Negative controls were performed by omitting reverse transcriptase from the RT reactions to verify that the amplified products were from the mRNA and did not originate from genomic deoxyribonucleic acid(DNA) contamination.

No PCR products were observed in the absence of reverse transcriptase. Specific amplification of all target genes was confirmed by sequencing (ABI PRISM 3100) the PCR fragments (double stranded(ds) DNA Cycle Sequencing System, Gibco BRL, Rockville, MA, USA).

Measurement of pHi

On the day of confluence, the NHNE cells were washed twice with a N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid(HEPES) solution and incubated in the same solution containing 1 μM 2', 7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF/AM). For BCECF

loading, the cells were incubated for 15-20 min at room temperature. The cells were then mounted in a miniature Ussing chamber attached to the stage of an inverted microscope. The Ussing chamber consisted of top (mucosal) and bottom (serosal) half-chambers (volume = 250 μM), each made from light-absorbing polyacetal. The Transwell wafer containing the polarized epithelial monolayer was mounted between the two half-chambers with the mucosal surface facing upwards. Effective sealing was achieved using rubber O-rings embedded in the grooves of the two half-chambers, which were screwed tightly together. A glass cover slip was affixed to the bottom of the serosal chamber with dental sticking wax (model Deiberit-502; Ludwig Bohme). The mucosal chamber was open to the atmosphere. Both half-chambers had inlet and outlet ports to allow solution flow. The serosal and mucosal perfusates were heated to 37°C and delivered to the chamber by gravity flow (rate = 3~5 ml/min). The BCECF/AM fluorescence ratio was recorded (Photon Technology International Delta Ram, NJ, USA) from an area in the center of the epithelium. The BCECF/AM fluorescence ratio was recorded at excitation wavelengths of 440 and 490 nm and the results are expressed as the 490/440 nm fluorescence ratio.

Solutions and chemicals

The HEPES-buffered solution contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, and 10 HEPES (pH 7.4 with NaOH). The HCO_3^-- buffered Cl^- free solution contained (in mM) 115 Na-gluconate, 5 K-gluconate, 1 $MgSO_4$, 1 Ca cyclamate, 10 D-glucose, 5 HEPES, and 25 $NaHCO_3$ (pH 7.4 with NaOH). The HCO_3^--buffered NaCl solution contained (in mM) 120 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, 5 HEPES, and 25 $NaHCO_3$ (pH 7.4 with NaOH). The HCO_3^--buffered high K^+ (100 mM K^+) content solution contained 25 mM NaCl, 100 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Glucose, 5 mM HEPES, and 25 mM $NaHC0_3$. All solutions were adjusted to pH 7.4 with NaOH and HCO_3^-. All HCO_3^--buffered solutions were continuously gassed with 95% O_2 and 5% CO_2 to maintain solution pH. The osmolarity of all solutions was adjusted to 310 mOsm with the major salt prior to use. BCECF-AM was purchased from Molecular Probes (Eugene, OR, USA), Forskolin was purchased from Calbiochem and ATP, DPC and DIDS were purchased from Sigma.

Statistical analyses

Data are presented as original recordings and as mean values ± SD from n observations. Student's t test was used to determine statistical significance. Differences were considered significant for $P < 0.05$.

Ⅲ. Results

Induction of mucociliary differentiation of human nasal epithelial cells according to time

NHNE cells were analyzed histologically as a function of time on the 0, 7^{th}, 14^{th} and 28^{th} day after confluence. NHNE cells differentiated into mucociliary epithelia with time in the presence of 10^{-7} M retinoic acid. At the time of confluence, there was only a monolayer of cells (Fig. 1A). On the 7^{th} day after confluence, the cells grew to form several layers (Fig. 1B). On the 14^{th} day after confluence, ciliated cells could occasionally be seen (Fig. 1C). On the 28^{th} day after confluence, the amount of ciliated cells was increased and the cells themselves became more cuboidal (Fig. 1D). To see whether these cells were mucous cells, they were stained with PAS solution. Many cells containing mucus could be observed (Fig. 1E).

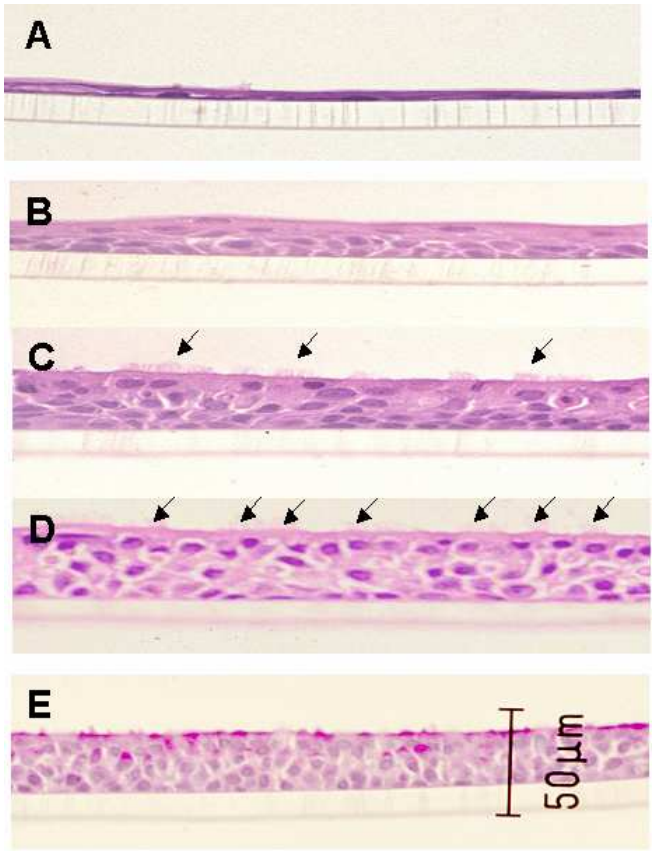

Fig. 1. Histological appearance of mucociliary differentiation of human nasal epithelial cells according to time. At the time of confluence, there was only a monolayer of cells (A). On the 7th day after confluence, the cells grew to form several layers (B). On the 14th day after confluence, ciliated cells could occasionally be seen (C). On the 28th day after confluence, the amount of ciliated cells was increased and the cells themselves became more cuboidal (D). To see whether these cells were mucous cells, they were stained with PAS solution. Many cells

containing mucus could be observed (E). Arrows indicate the ciliated cells.

To confirm such mucociliary differentiation on a molecular level, we performed RT-PCR. The gene expression of cornifin-α, a marker of squamous cell differentiation, decreased progressively on the 14^{th} and 28^{th} day after confluence. In contrast, the expression of MUC5AC, a marker of mucous differentiation, increased as time passed by. β2-microglobulin, used as a control, was not altered (Fig. 2A). To show the reliability of this study, this experiment was performed three separate times. Semi-quantitation of the three experiments was performed for each target gene (Fig. 2B, 2C).

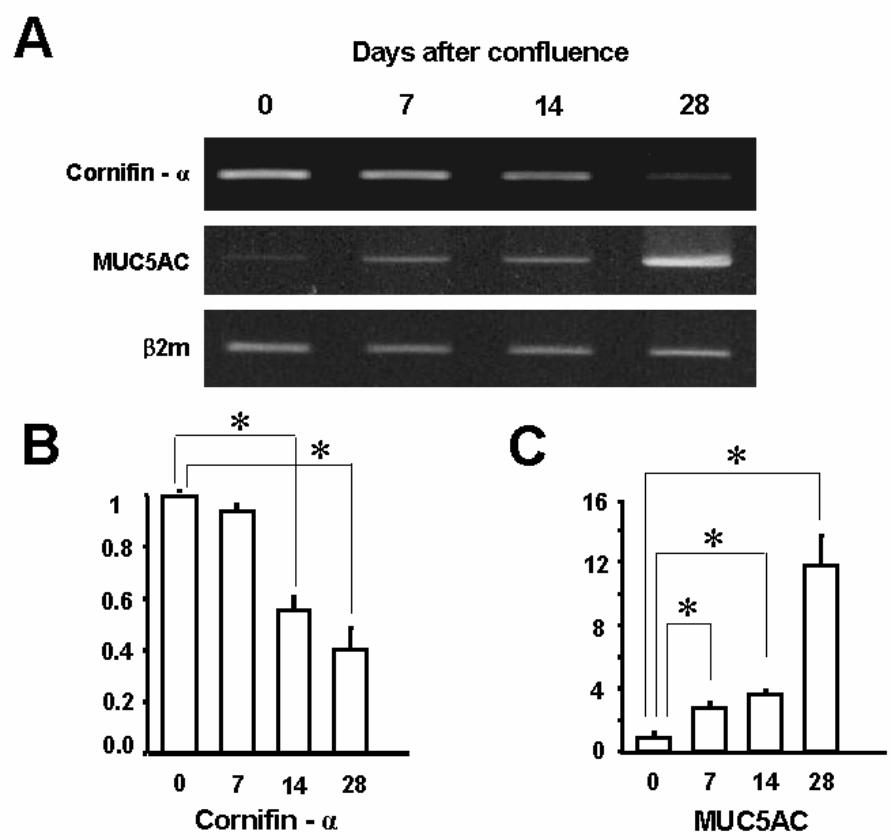

Fig. 2. Molecular characterization of mucociliary differentiation of human nasal epithelial cells according to time. The gene expression of cornifin-α, a marker of squamous cell differentiation, decreased progressively on the 14th and 28th day after confluence. In contrast, the expression of MUC5AC, a marker of mucous differentiation, increased as time passed by. β2-microglobulin, used as a control, was not altered (A). The figure shows the mean ± S.D. of separate experiments performed under each condition (B, C). Asterisk indicates statistical significance ($p < 0.05$).

Expression of AE isoforms as a function of mucociliary differentiation in NHNE cells

We examined the presence of 10 AE isoforms in this study. The mRNAs of AE2, bAE3, AE4, SLC26A4, SLC26A6, SLC26A7, SLC26A8 and SLC26A11 were expressed in NHNE cells (Fig. 3A). However, the mRNAs of SLC26A3 and SLC26A9 were not expressed. In previous studies, AE1 and cAE3 were not expressed in the human airway [9,15], and thus were excluded from this study. To confirm that SLC26A3 and SLC26A9 are not expressed in NHNE cells, we used T-84 cells, a human colonic cell line, as a positive control for SLC26A3 (Fig. 3B), and Capan-1 cells, a metastatic human pancreatic cancer cell line, for SLC26A9 (Fig. 3C).

The mocociliary differentiation did not affect the mRNA expression levels of AE3, SLC26A6, and SLC26A11. However, AE2 and SLC26A4 mRNA expression levels decreased, and AE4, SLC26A7 and SLC26A8 mRNA expression levels progressively increased on the 14^{th} and 28^{th} day after confluence.

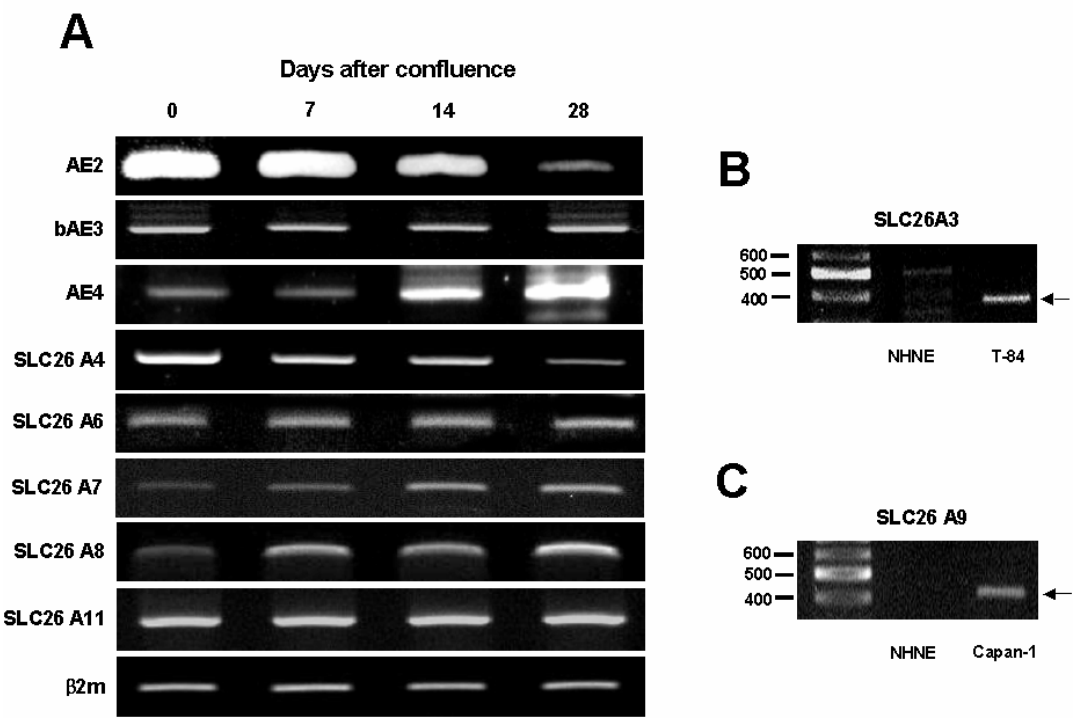

Fig. 3. Expression of AE isoforms as a function of mucociliary differentiation in NHNE cells. The mRNAs of AE2, bAE3, AE4, SLC26A4, SLC26A6, SLC26A7, SLC26A8 and SLC26A11 were expressed in NHNE cells (A). However, the mRNAs of SLC26A3 and SLC26A9 were not expressed. To confirm that SLC26A3 and SLC26A9 are not expressed in NHNE cells, we used T-84 cells, a human colonic cell line, as a positive control for SLC26A3 (B), and Capan-1 cells, a metastatic human pancreatic cancer cell line, for SLC26A9 (C).

Selection of potassium concentration for functional anion exchange activity experiments

We used a standard protocol for the removal and addition of chloride to the incubation medium buffered with bicarbonate to follow anion exchange activity. In brief, removal of Cl^- from the incubation medium of NHNE cells resulted in a slow and modest increase in pH*i* by AE activation, which was completely reversed upon the re-addition of Cl^- to the medium. Initial rates of pH*i* changes were obtained from the first derivative of the traces using a single exponential fit for anion exchange activity. To better observe luminal-specific anion exchange activity, the basolateral side of the monolayer was perfused with Cl^--free solutions, and vice versa. To examine the effect of other electrogenic anion movement on anion exchange activity, we measured anion exchange activity in both high (100 mM K^+) and normal (5 mM K^+) extracellular potassium conditions. In the LM, the basal Cl^-/ HCO_3^- exchange activity was 0.039 ± 0.002 ΔpH unit/min in 5 mM K^+, which increased to 0.074 ± 0.010 ΔpH unit/min in 100 mM K^+ (Fig. 4A). In the BLM, the basal Cl^-/ HCO_3^- exchange activity was 0.030 ± 0.007 ΔpH unit/min in 5 mM K^+, which then increased to 0.077 ± 0.012 ΔpH unit/min in 100 mM K^+ (Fig. 4B).

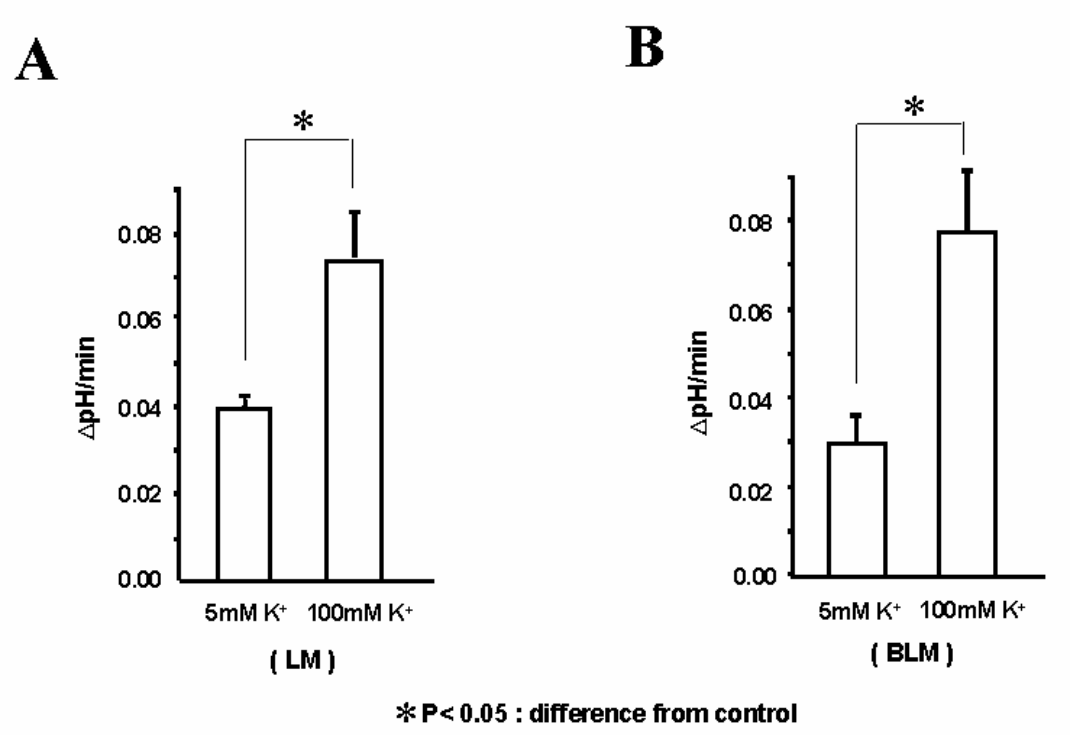

Fig. 4. Selection of potassium concentration for functional anion exchange activity experiments. The luminal (A) and basolateral (B) side was exposed to Cl^- free solutions in the presence of 5 or 100 mM K^+. The figure shows the mean ± standard deviation(S.D.) of five separate experiments. Asterisk indicates statistical significance ($p < 0.05$).

Consequently, it could be seen that high concentration of potassium increased the rate of anion exchange activity approximately 2-fold in both sides of the membrane. These results indicate that membrane

depolarization by high K^+ reduced other electrogenic anion movement such as CFTR- dependent efflux of HCO_3^- and outward Cl^- movement through K^+/ Cl^- cotransporter. Thus, 100 mM K+ conditions were used in all the subsequent experiments.

The effect of forskolin on AE functional activity

The resting pH*i* of the monolayered NHNE cells was 7.35 ± 0.11 in the presence of HCO_3^-. The effect of forskolin, a cAMP-elevating agent, on anion exchange activity was determined from the extent of pH*i* changes caused by Cl- removal and addition before and after 5 μM forskolin stimulation on each side of the membrane. The luminal AE activity was 0.070±0.008 ΔpH unit/min, which increased to 0.283±0.027 ΔpH unit/min in the presence of 5 μM forskolin (Fig. 5A, 5B) and the basolateral AE activity was 0.054±0.006 ΔpH unit/min, which also increased to 0.208±0.038 ΔpH unit/min in 5 μM forskolin (Fig.5C, 5D).

These results imply that the AE activation pathway is related with intracellular cAMP signaling and anion exchange activity is affected by cAMP-mediated mechanisms such as CFTR-dependent HCO_3^- transport.

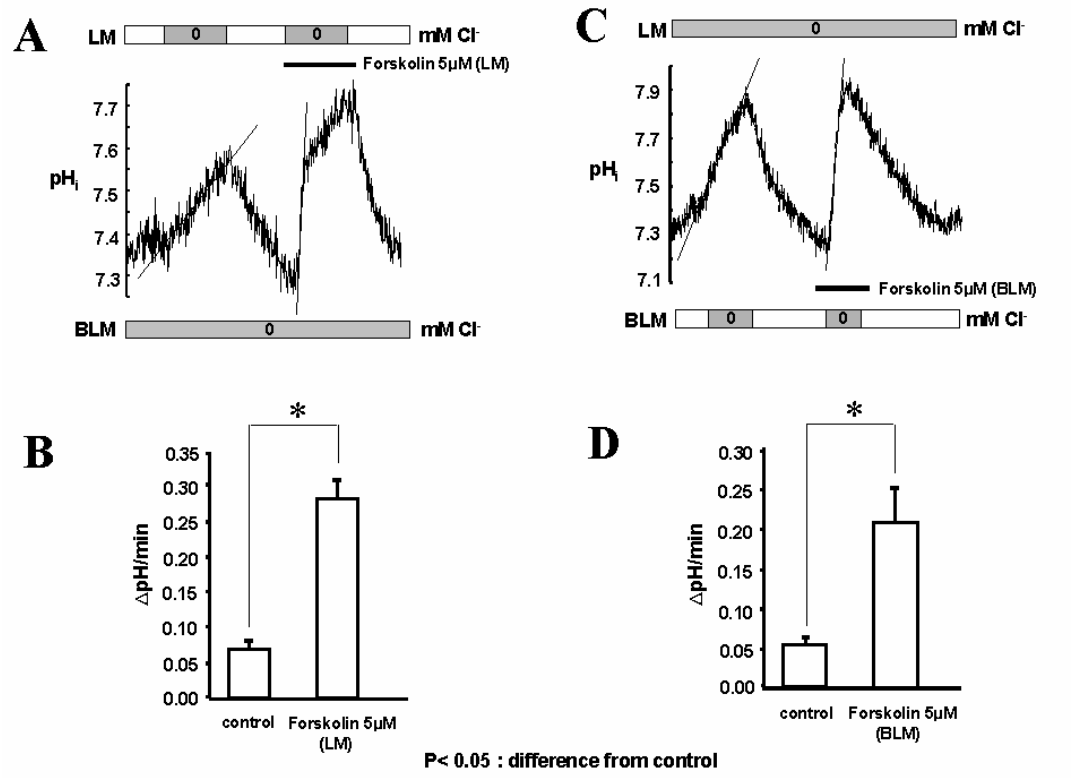

Fig. 5. Effect of forskolin on AE functional activity. The effect of forskolin, a cAMP-elevating agent, on AE activity was determined from the extent of pH*i* changes caused by Cl- removal and addition before and after 5 μM forskolin stimulation on the luminal (A, B) and the basolateral (C, D) side of the epithelial cells. The figure shows the mean ± S.D. of six separate experiments. Asterisk indicates statistical significance ($p < 0.05$).

The effect of ATP on AE functional activity

The relationship between AE activity and purinergic receptors was examined by testing the NHNE cells with 100 μM ATP. The basal AE activity was 0.071±0.012 ΔpH unit/min, which increased to 0.257± 0.039 ΔpH unit/min in 100 μM ATP in the LM (Fig. 6A, 6B). However, the basolateral anion exchange activity was 0.077±0.018 ΔpH unit/min, which decreased to 0.075±0.010 ΔpH unit/min in 100 μM ATP (Fig. 6C, 6D).

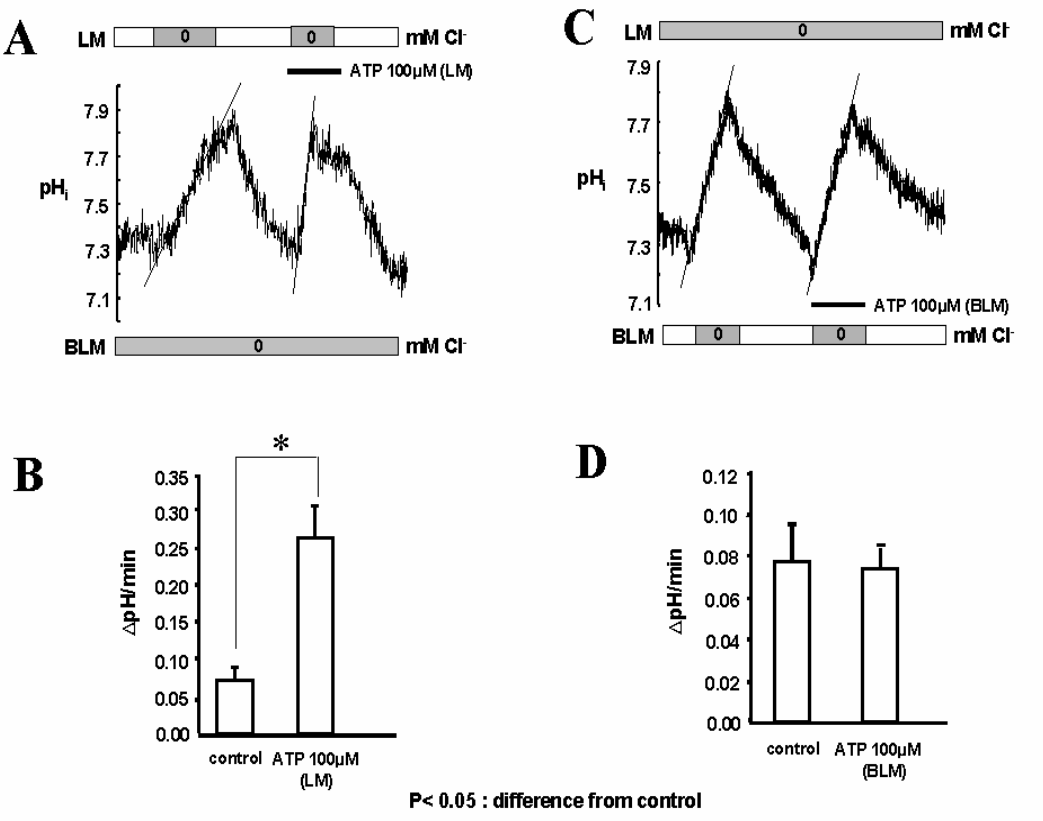

Fig. 6. Effect of ATP on AE functional activity. The relationship between AE activity and purinergic receptors was examined by testing the NHNE cells with 100 μM ATP on the LM (A, B) and BLM (C, D) of the cells. The figure shows the mean ± S.D. of six separate experiments. Asterisk indicates statistical significance ($p < 0.05$).

These results suggest that the luminal anion exchange activity is affected by activation of luminal purinergic receptors.

The effect of DPC and DIDS on AE functional activity

To examine the effect of an anion antagonist on AE activity, we used DPC, a Cl^- channel inhibitor, and DIDS, an AE inhibitor, on the both sides of the membrane. The basal AE activity of the LM was 0.080±0.005 ΔpH unit/min, which was serially decreased to 0.044±0.003 ΔpH unit/min and 0.015±0.003 ΔpH unit/min in 100 μM and 1 mM DPC (Fig. 7A, 7B). The BLM AE activity was 0.080±0.018 ΔpH unit/min, which was progressively decreased to 0.066±0.019 ΔpH unit/min and 0.049±0.014 ΔpH unit/min in 100 μM and 1 mM DPC (Fig. 7C, 7D).

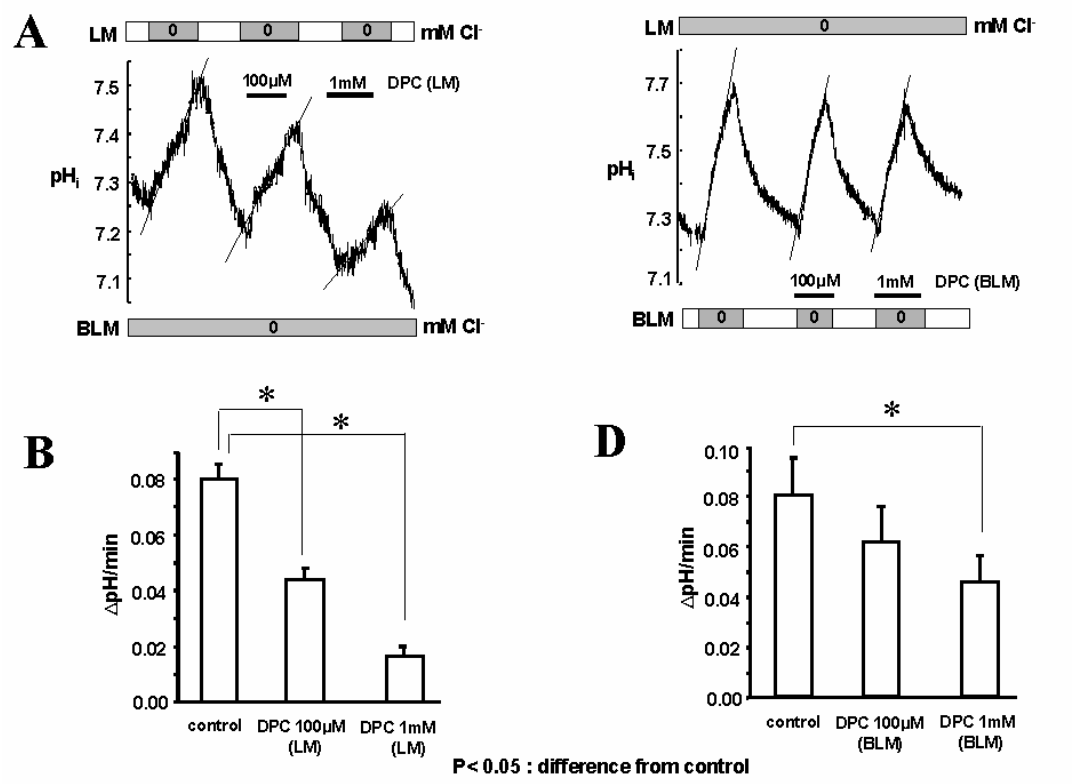

Fig. 7. Effect of DPC on AE functional activity. We used DPC, a Cl^- channel inhibitor, on the LM (A, B) and the BLM (C, D) to examine the effect of an anion antagonist on AE activity,. The figure shows the mean ± S.D. of six separate experiments. Asterisk indicates statistical significance ($p < 0.05$).

The luminal AE activity was 0.080±0.006 ΔpH unit/min, which was nearly abolished to 0.005±0.001 ΔpH unit/min in 500 μM DIDS (Fig. 8A, 8B). The basolateral AE activity was 0.078±0.010 ΔpH unit/min, which was also decreased to 0.032±0.009 ΔpH unit/min following the addition

of 500 μM DIDS (Fig. 8C, 8D).

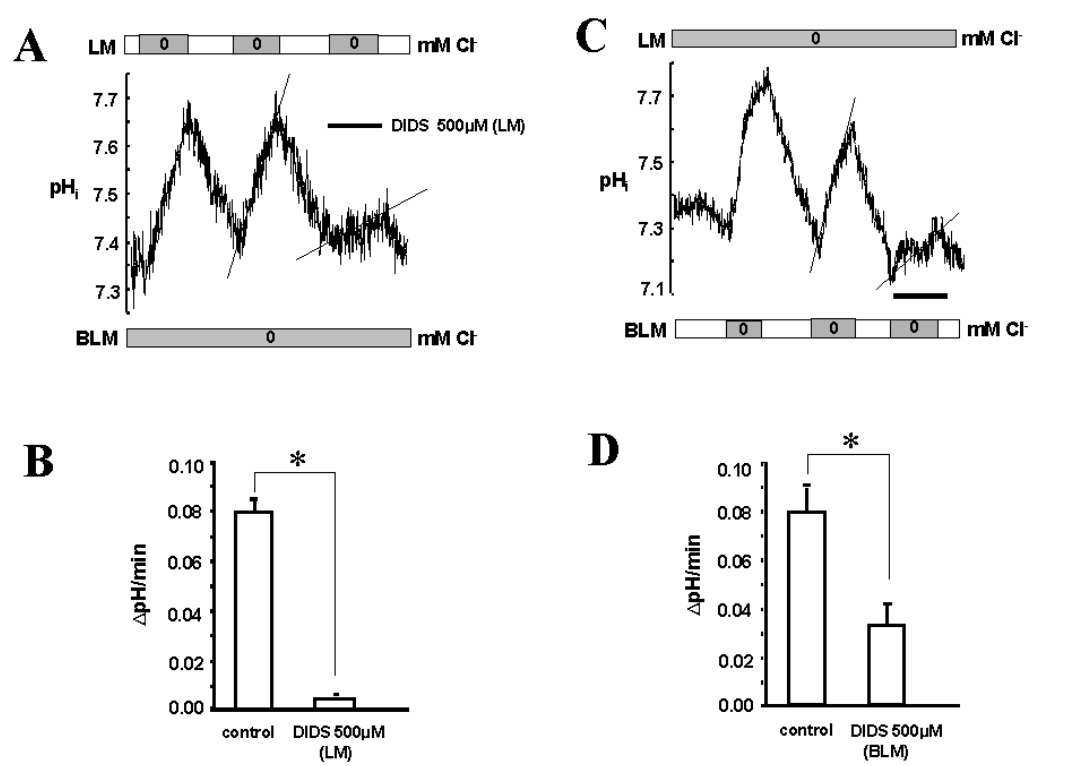

Fig. 8. Effect of DIDS on AE functional activity. To examine the effect of an anion antagonist on anion exchange activity, we used DIDS, a AE inhibitor, on the LM (A, B) and the BLM (C, D) to examine the effect of an anion antagonist on anion exchange activity,. The figure shows the mean ± S.D. of six separate experiments. Asterisk indicates statistical significance ($p < 0.05$).

These results indicate that NHNE cells exhibit DPC and DIDS-sensitive AE functional activity in both LM and BLM.

Ⅳ. DISCUSSION

Intracellular pH regulation is important for the maintenance of respiratory tract homeostasis. Various ion transporters, such as AE, NBC, and CFTR, are located in the airway epithelia[3, 5, 16]. Among them, the AE is a ubiquitous plasma membrane ion transporter that contributes to the Cl^-/HCO_3^- exchange in the LM and BLM[6, 7]. Furthermore, it is responsible for pH*i* and ASL pH regulation with other various ion transporters[1, 3, 17, 18]. Accordingly, AE may play an important role in the regulation of fluid and pH*i* in NHNE cells.

We induced mucociliary differentiation of NHNE cells in the presence of retinoic acid[14]. To confirm that NHNE cells differentiated into mucociliary epithelium, we observed the histology of the cultured epithelium at indicated time points. On the 28th day after confluence, many ciliated cells and PAS-positive mucus-containing cells were noted. In addition, expression of cornifin-α, a squamous differentiation marker, and MUC5AC, a mucous differentiation marker, correlated to a great degree with the transfiguration of the NHNE cells.

These results showed that NHNE cells differentiated into mucociliary epithelium.

We next investigated the expression patterns of the AE isoforms as a function of mucociliary differentiation. In 10 members of the SLC4 family, which have AE, NBC and NDCBE function, only AE1, AE2, AE3 (bAE3 and cAE3) and AE4 were reported to be a Cl^-/HCO_3^- exchanger[8]. In previous studies[9,15], AE1 and cAE3 were not expressed in the human airway, and thus were excluded from this study. In addition, the SLC26 family, which is a recently cloned member of the AE superfamily, consists of 11 members and they transport not only Cl^- and HCO_3^-, but also SO_4^{2-}, I^- and OH^-. However, among them, SLC26A1, SLC26A2, SLC26A5, and SLC26A10 genes were also excluded from this study because they are known to have no Cl^- - HCO_3^- exchange function[7].

Our results showed that AE2, bAE3, AE4, SLC26A4, SLC26A6, SLC26A7, SLC26A8 and SLC26A11 mRNAs were expressed in NHNE cells. AE2 and bAE3 mRNA expression was consistent with Al-Bazzaz's reports in human bronchial and tracheal epithelium[9, 16]. However, interestingly, SLC26A3 and SLC26A9 mRNAs were not expressed although the expression of SLC26A3 was expressed in mouse trachea epithelial cells and SLC26A9 in human alveolar and the bronchial

epithelial cells [10, 20]. This discrepancy may be due to the difference in species or the difference in upper and lower airways. Furthermore, the expression levels of AE2 and SLC26A4 mRNAs decreased as a function of mucociliary differentiation, whereas AE4, SLC26A7 and SLC26A8 expression increased. These results suggest that mucociliary differentiation of the epithelial cells influences the expression of some AEs. Most electrophysiological studies researching the function of the several AEs that are influenced by differentiation use monolayered cells in the confluenced state. However, considering that the human airway epithelia *in vivo* are fully differentiated mucociliary epithelia, the results from these studies using monolayered undifferentiated cells may not be able to reflect the *in vivo* function of those AEs.

Localization and functional interaction of AE with the other anion transporters are poorly understood in human nasal epithelium. Therefore, we examined the AE functional activity in both LM and BLM using various agonists and antagonists in the monolayered state.

AE activity is affected by potassium concentration, because high K+ induces membrane depolarization[17]. To examine the effect of potassium concentration on anion exchange activity, we measured anion exchange

activity in both high (100 mM K^+) and normal (5 mM K^+) extracellular potassium conditions. In this study, anion exchange activity in high K^+ buffer solutions (100 mM K^+) was nearly two-fold higher than that in physiologic (5 mM K^+) buffer solutions in both LM and BLM. This finding was in accordance with Lee's study[17] that showed, in mouse submandibular and pancreatic duct cells, high external K^+ (100 mM) increased the rate of $HCO3^-$ influx, which was approximately two-fold higher than that with normal K^+ (5 mM) medium. This result suggests that, in physiologic conditions, electrogenic anion pathways, such as CFTR and K^+- Cl^- cotransporter(KCC), are open in the LM and BLM in NHNE cells. However, in high extracellular K^+ conditions, the electrogenic pathway is blocked by high positive charge and their activity may be underestimated. Thus, 100 mM K^+ conditions were used in all the subsequent experiments.

AE was regulated by CFTR and CFTR-dependent HCO_3^- conductance in human airway epithelial cell lines[21]. To test CFTR-mediated AE functional activity of NHNE cells, we used forskolin, a cAMP-elevating agent, because CFTR is generally known as a cAMP-activated anion channel. In agreement with other reports[22,23], anion exchange activity was increased by 5 μM forskolin in both sides of membrane, but luminal

anion exchange activity was higher than basolateral anion exchange activity when exposed to forskolin. These results suggest that AE activity is affected by cAMP-mediated anion channels such as CFTR, and CFTR-dependent HCO_3^- conductance. As the luminal AE activity was higher than the basolateral activity after exposure to forskolin, it can be conjectured that AE is mainly expressed in the LM.

CaCC increased AE activity in the human airway[23,24,]. Calcium agonist increased the $[Ca^{2+}]i$ through purinergic receptor, which was known to increase HCO_3^- secretion either directly or by way of cAMP-mediated mechanisms[18, 25]. Moreover, recently we have shown that various purinergic receptors were expressed in NHNE cells [26]. For that reason, to examine the effect of Ca^{2+}- mediated channel on anion exchange activity, we measured pH*i* in the presence of 100 μM ATP. In our results, the basal AE activity of the LM was increased about 3.5-fold by ATP, whereas basolateral anion exchange activity was not affected by basolateral ATP stimuli. According to other reports, luminal addition of ATP activates both CaCC and CFTR, whereas basolateral addition activates only CFTR in normal human airway epithelia[23]. And the majority of anion secretion is mediated by apical G-protein-coupled(P2Y)2 receptor, which leads to CaCC activation. Furthermore, we previously reported that the NHNE cells express functionally active P2Y2, P2Y6, and P2Y11 receptors in a membrane specific pattern. Therefore, the membrane specific

functional expression of P2R, CaCC, and CFTR may influence basolateral anion exchange activity by ATP stimuli in the NHNE cells.

Anion exchange activity was affected by both Cl^- and HCO_3^- movement, because the function of each anion was connected with the other anion movement. To examine the effect of each anion antagonist on anion exchange activity, we used DPC, a CFTR- mediated Cl^- channel inhibitor, and DIDS, a AE inhibitor, on both membranes. Similar to other reports[16,27], in this study, anion exchange activity was progressively decreased by 100 μM and 1 mM DPC in both LM and BLM. In addition, AE activity was nearly abolished by 500 μM DIDS in the LM and BLM. AE activity was also decreased to half of the basal activity. Moreover, inhibition rate of DIDS was higher than DPC in both sides of membrane. These results show that AE functional activity is sensitive to DPC and DIDS

V. Conclusion

In summary, we showed that epithelial cells differentiated into muco-ciliary epithelium through histological and molecular characterization and that while various AE isoforms were expressed, only some of them were affected by mucociliary differentiation. In addition, we found that anion exchange activity was affected by intracellular cAMP and calcium signaling, and that DPC & DIDS-sensitive anion exchange activity existed in both LM and BLM of NHNE cells. Moreover, the regulation of HCO_3^- transport by intracellular cAMP and calcium signaling demonstrated here may be of particular significance for the understanding of the physiologic roles of cAMP and calcium-mediated bicarbonate secretion in human nasal epithelia.

Our findings through molecular and functional studies using NHNE cells suggest that AE may play an important role in the regulation of pH_i and ASL pH.

VI. References

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<Abstract (in Korean)>

사람 정상 코점막 상피세포에서 Anion Exchangers 의 분자적, 기능적 발현에 관한 연구

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본 연구의 목적은 사람 정상 코점막 상피세포의 관내강막과 기저외측막에 있어서 점액섬모 분화에 따른 음이온 교환기의 mRNA 발현 정도의 조절을 연구하는 것이었다. 우리는 먼저 사람 정상 코점막 상피세포의 점액섬모 분화를 유도하고 이를 세포학적 및 분자적 차원에서 확인한 후, 사람 정상 코점막 상피세포에서 발현하는 음이온 교환기의 isoform들을 확인하고 이들이 점액섬모 분화에 의해 영향을 받는지에 대해 알아보았다. 마지막으로, 극성의 단층 사람 정상 코점막 상피세포의 관내강막과 기저외측막에서 이러한 음이온 교환기의 기능적 활성도에 대해서 알아보았다. 이 연구에서 우리는 상피세포가 점액섬모 상피세포로 분화되는 것을 세포학적 및 분자적 차원에서 확인하였고 다양한 음이온 교환기의 isoform들이 발현되는데 이중 일부만이 점액섬모 분화에 의해 영향을 받는다는 것을 알 수 있었다. 또한 사람 정상 코점막 상피세포의 관내강막과 기저외측막에 있어서 음이온 교환기의 활성도는 세포내 cAMP나 칼슘 신호전달체계 및 DPC와 DIDS에 의해 영향을 받는다는 것을 알 수 있었다. 본 연구에서는 사람 정상 코점막 상피세포를 이용한 분자적 및 기능적 연구를 통해 음이온 교환기가 세포내 pH 및 ASL pH조절에 중요한 역할을 한다는 것을 알 수 있었다.

핵심되는 말 : 음이온 교환기, pH, 점액섬모 분화, 코점막 상피세포