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Membrane-specific expression of functional purinergic receptors in normal human nasal epithelial cells

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Kim, Chang-Hoon, Sung-Shik Kim, Jae Young Choi, Ji-Hyun Shin, Jin Young Kim, Wan Namkung, Jeung-Gweon Lee, Min Goo Lee, and Joo-Heon Yoon. Membrane-specific expression of functional purinergic receptors in normal human nasal epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 287: L835–L842, 2004. First published June 18, 2004; 10.1152/ajplung.00285.2003.—Extracellular purines and pyrimidines regulate various physiological responses via the cell surface receptors known as purinoreceptors and may exert autocrine or paracrine effects on ion transport, fluid transport, ciliary beat frequency, and mucin secretion. Therefore, this study aims to investigate the expression patterns of the purinoreceptors in normal human nasal epithelial (NHNE) cells. In RT-PCR, the mRNAs for several P2X ($P2X_3$, $P2X_4$, $P2X_7$) and P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$) receptors were identified in NHNE cells. Functional localizations of P2 receptors were investigated by measuring intracellular calcium concentration ($[Ca^{2+}]_i$) increases in membrane-specific manner using a double-perfusion chamber. Absence of the responses of αβ-methylene ATP and 2-methylthio-ATP excluded functionally active $P2X_3$, $P2X_4$, and $P2Y_1$ receptors as far as $[Ca^{2+}]_i$ increase is concerned. Applications with ATP and UTP revealed that luminal membranes of NHNE cells express $P2Y_2$ and $P2Y_6$ receptors and basolateral membranes express $P2Y_2$ receptor. Expressions of $P2Y_2$ and $P2Y_6$ receptors in NHNE cells were further verified by immunoblotting using specific antibodies. In addition, the results with 2,3-O-(4-benzoyl)-benzoyl-ATP indicate that the $P2Y_{11}$ receptor may be present on the luminal side. In conclusion, the NHNE cells express functionally active $P2Y_2$, $P2Y_6$, and $P2Y_{11}$ receptors in a membrane-specific pattern, which may play an important role in the control of mucin and fluid secretion in NHNE cells.

purinoreceptor; calcium; secretion

THE NASAL EPITHELIUM actively secretes most of the mucus and determines the electrolyte composition of nasal secretions. Nasal hypersecretion is a common feature in various nasal and paranasal sinus diseases such as rhinitis and sinusitis. In general, nasal secretion results from stimulation of secretagogues and inflammatory mediators (29). Secretagogues stimulate the release of the serous and mucous components inside cells as an early event of secretion before the activation of the secretory genes. Representatives of the secretagogues are the extracellular triphosphate nucleotides. The extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP, UTP) regulate the various physiological responses via the cell surface receptors termed purinoreceptors and exert autocrine or paracrine effects on ion transport (1, 2, 15), fluid transport (1, 2), ciliary beat frequency (15, 18), and mucin secretion (10, 12). Thus the purinoreceptors in nasal epithelial cells may play an important role in the early stages of secretion under inflammatory conditions.

Up to now, nine G protein-coupled (P1, P2Y) and eight ligand-gated (P2X) purinoreceptors have been identified in mammalian cells (8, 21). ATP and UTP regulate the cellular processes via interactions with the cell-surface ion-gated (P2X) and G protein-coupled (P2Y) receptors. ATP is an effective agonist of the $P2Y_2$ and $P2Y_{11}$ receptors, whereas UTP activates the $P2Y_2$ and $P2Y_4$ receptors (18). Certain P2Y receptors are activated principally by nucleoside diphosphate. For example, ADP activates the $P2Y_1$ receptor, and UDP activates the $P2Y_6$ receptor (18).

Until recently many investigations have been carried out to characterize the P2 receptors that are expressed in various kinds of cells and tissues including submandibular acinar cells (14) and pancreatic duct cells (16). However, there are only a few reports on the expression of the purinoreceptors in nasal epithelial cells (13). Because most of those studies were based only on a pharmacological approach for determining the potency of their agonists and/or antagonists, the characterization of the P2 receptors in the airway epithelial cells has not been fully clarified. Therefore, the aims of this study were, first, to investigate which mRNAs and proteins of the P2X and P2Y receptors are expressed in normal human nasal epithelial (NHNE) cells using RT-PCR and Western blot analysis and, second, to examine which of the P2X and P2Y receptor subtypes are functionally active on the intracellular calcium levels using various agonists and antagonists.

MATERIALS AND METHODS

Chemicals and solutions. Fura-2-AM was purchased from Molecular Probes (Eugene, OR), and pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) was obtained from Tocris Cooksom (Bristol, UK). All other chemicals, including ATP, UTP, UDP, 2-methylthioadenosine 5′-triphosphate (2MeS-ATP), 2′,3′-O-(4-benzoyl)-benzoyl-ATP (BzATP), αβ-methylene ATP (αβ-Me ATP), and caffeine, were purchased from Sigma. The standard perfusion solution (luminal and basolateral) contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, and 10 mM HEPES (pH 7.4 with NaOH).

Cell culture and specimen preparation. Passage 2 NHNE cells were prepared as previously described (28). The Ethics Committee of Yonsei University College of Medicine approved all the procedures used in this study. The *passage 2* NHNE cells (5.0×10^4 cells) were plated on a Transwell clear (Costar, Cambridge, MA) culture insert

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with a 0.45-μm pore size. The cells were cultured in a 1:1 mixture of bronchial epithelium growth medium/DMEM containing various supplements until confluence. After confluence was achieved, the functionally polarized monolayers of the NHNE cells were loaded with fura-2 (5 μM at 37°C for 25 min) before being mounted in a miniature Ussing chamber attached to the stage of the objective microscope.

RT-PCR for receptor subtypes of P2X and P2Y. RT-PCR was used to detect the expression of the mRNAs for human P2X ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_6$, $P2X_7$, $P2X_M$) and P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$). The oligonucleotide primers were designed for the mRNA of each gene product using Gene Runner software version 3.00 and the previously published sequences. The oligonucleotide amplimers for β_2-microglobulin (β_2M) for control PCR reaction, purchased from Clontech Laboratories (Palo Alto, CA), generated a 335-bp PCR fragment. The RT-PCRs were performed using a Perkin Elmer Cetus DNA Thermal Cycler according to the manufacturer's protocol. The total RNA (1 μg per 20-μl reaction volume) was reverse-transcribed into complementary DNA (cDNA) with the random hexanucleotide primers and Moloney murine leukemia virus reverse transcriptase. Negative controls were obtained by omitting the reverse transcriptase from the RT reaction to verify that the amplified products were from the mRNA and not from the genomic DNA contamination. No PCR products were observed in the absence of reverse transcriptase. Specific amplifications of the P2 receptor mRNAs were confirmed by sequencing (dsDNA Cycle Sequencing System; GIBCO-BRL, Gaithersburg, MD) of the PCR fragments. Forty percent for each P2 receptor or 4% for β_2M of the resulting cDNA was amplified with 0.2 nmol/l of each primer. The optimized concentration of $MgCl_2$ in the PCR was 1.5 mmol/l for P2X and P2Y receptors. Human embryonic kidney-293 cells were used as positive controls for the $P2X_3$ and $P2X_4$ receptors. Human skeletal muscle cells were used as positive controls for $P2X_M$. Human platelet cells were used as positive controls for $P2Y_{12}$. Normal human brain cells were used as positive controls for the remaining receptors tested. The oligonucleotide primers designed for the purinoreceptors used in this experiment and their reaction conditions are listed in Tables 1 and 2.

Measurement of [Ca^{2+}]. The measurements of the [Ca^{2+}] in the monolayers were performed based on the previously reported protocols with a slight modification (11, 19). After reaching confluence, the cells were loaded with fura-2 by incubating (30 min, 37°C) them in medium containing 3 μM fura-2-AM. The membranes bearing the fura-2-loaded NHNE cells were mounted in a miniature Ussing chamber (AKI Institute, University of Copenhagen, Copenhagen, Denmark) attached to the stage of an inverted microscope. The chamber is consisted of top (luminal) and bottom (basolateral) half-chambers (volume = 250 μl each) made from light-absorbing polyacetal. The membrane containing the polarized epithelial monolayer was located in between the two half-chambers, separating the chamber into a luminal (upper) and a basolateral (lower) compartment. Effective sealing was achieved using rubber O-rings embedded in the grooves of the two half-chambers, which were screwed tightly together. A transparent coverslip was placed at the bottom of the perfusion chamber, which allowed fluorescence measurements from the dye-loaded monolayers using objective lenses having a long working distance (>2 mm). The luminal chamber was open to the atmosphere. Both half-chambers had inlet and outlet ports to allow the solution to flow. The luminal and basolateral perfusates were heated to 37°C and delivered to the chamber by gravity flow (rate = 3–5 ml/min). The fura-2 fluorescence ratio was recorded (PTI Delta Ram, Photon Technology International) from an area in the center of the epithelium. The fura-2 fluorescence was recorded at excitation wavelengths of 350 and 380 nm, and the results are expressed as the 350/380 fluorescence ratio.

Immunoblot analyses for $P2Y_2$ and $P2Y_6$ receptors. NHNE cells were lysed with 2× sample buffer [250 mM Tris·Cl (pH 6.5), 2% SDS, 4% 2-mercaptoethanol, 0.02% bromphenol blue, and 10% glycerol]. Equal amounts of whole cell lysates were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 5% skim milk in Tris-buffered saline [TBS, 50 mM Tris·Cl (pH 7.5), 150 mM NaCl] for 2 h at room temperature. This blot was then incubated overnight with primary antibody ($P2Y_2$, $P2Y_6$, 1:500; Alomone Labs, Jerusalem, Israel) in TTBS (0.5% Tween 20 in TBS). Negative control experiment with the control peptide (preincubation with the same amount of antibody for 1 h at room temperature) used for immunization was done to check specificity of the primary antibody. After washing the blot with TTBS, we further incubated the blot for 45 min at room temperature with anti-goat antibody (1:2,000; Vector Laboratories, Burlingame, CA) in TTBS and then visualized it by using the ECL system (Amersham-Pharmacia, Piscataway, NJ). The same experiments were repeated three times with nearly identical results.

Table 1. *RT-PCR primers and the reaction conditions for the P2X receptors*

Primers	GenBank No.	Reaction Conditions, °C/30 s	Expected Size, bp
$P2X_1$		94	
Sense: CTGGCTGAGAAGGGTGGAGTGGTTGG	AF020498	65	392
Anti: TGGCCCCATGTCCTCAGCGTATTTG		72	
$P2X_2$		94	
Sense: GCTGCTCATCCTGCTCTACTTCGTGTGG	NM016318	65	399
Anti: GGGGTAGTGGATGCTGTTCTTGATGAGG		72	
$P2X_3$		94	
Sense: ATCAACCGAGTAGTTCAGC	Y07683	50	695
Anti: GATGCACTGGTCCCAGG		72	
$P2X_4$		94	
Sense: GAGATTCCAGATGCGACC	U83993	50	296
Anti: GACTTGAGGTAAGTAGTGG		72	
$P2X_6$		94	
Sense: AGTTCAACTTCTCTAAGTCCAATGC	AF065385	50	470
Anti: CTCTATCCACATACAGCAGTAGC		72	
$P2X_7$		94	
Sense: CCCCGGCCACAACTACACCACGAGAAAC	NM002562	67	440
Anti: CCGAAGTAGGAGAGGGTTGAGCCGATG		72	
$P2X_M$		94	
Sense: GCTTGGCTGGGCGTGGTCACCTTTTTCTG	AB002058	67	332
Anti: CCCGCCCTTCCCAGCCCCCAACTCTC		72	

Table 2. *RT-PCR primers and the reaction conditions for the P2Y receptors*

Primers	GenBank No.	Reaction Conditions, °C/30 s	Expected Size, bp
$P2Y_1$		94	
Sense: CCCTGGGCCGGCCTCAAAAAGAAGAATG	NM002563	65	389
Anti: CAAGCCGGGCCCTCAAGTTCATCGTTTTC		72	
$P2Y_2$		94	
Sense: GCTACAGGTGCCGCTTCAACGAGGACTTC	NM002564	65	428
Anti: GGCAGGCCAGCACCAACACCCACAC		72	
$P2Y_3$		94	
Sense: CCACCTGGCATTGTCAGACACC	X91852	63	424
Anti: GAGTGACCAGGCAGGGCACGC		72	
$P2Y_6$		94	
Sense: CCCTGCTGGCCTGCTACTGTCTCCTG	U52464	62	455
Anti: CTAATTCTCCGCATGGTTTGGGGTTGG		72	
$P2Y_{11}$		94	
Sense: CCCCCGCTGGCCGCCTACCTCTATCC	AF030335	67	396
Anti: CGCAGCCCAACCCCGCCAGCACCAG		72	
$P2Y_{12}$		94	
Sense: CTAAGATTCTCTCTGTTGTCATCTG	AF313449	50	432
Anti: ACAGAGTGCTCTCTTTCACATAG		72	

RESULTS

P2 receptor mRNA expressions by RT-PCR. The expression pattern of the mRNA transcripts for the P2X and P2Y receptors in NHNE cells was investigated, as has been previously reported for certain subtypes of the P2 receptors in different human epithelial cells (18). As shown in Fig. 1*A*, only the $P2X_3$, $P2X_4$, and $P2X_7$ receptor mRNAs were expressed among the seven subtypes of the P2X receptors tested (Fig. 1*A*). In addition, the mRNAs for the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{12}$ receptors were identified in NHNE cells (Fig. 1*B*). However, it is still unclear whether or not all these mRNAs for the P2 receptors expressed in the NHNE cells are functionally active in terms of the intracellular calcium response.

Presence of P2X and P2Y receptors in luminal and basolateral membranes of nasal epithelial cells. Three different concentrations of ATP, a P2 receptor agonist, were used to determine whether P2 receptors are located in the luminal or basolateral membrane of the NHNE cells. The intracellular calcium level in response to luminal or basolateral ATP administration was increased to a similar level (Fig. 2, *A* and *B*). However, the intracellular calcium level in response to luminal ATP administration was slightly higher than the level in response to basolateral ATP administration at lower ATP concentrations (1 and 10 μM).

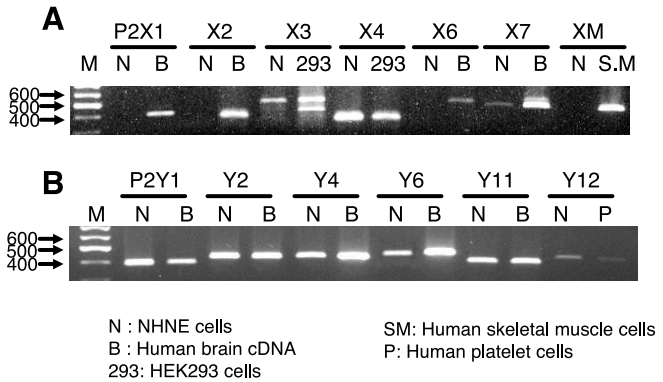

Fig. 1. P2 receptor mRNA expression with RT-PCR. The mRNAs for $P2X_3$, $P2X_4$, $P2X_7$ (*A*), $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{12}$ receptors (*B*) were expressed in normal human nasal epithelial (NHNE) cells.

We next investigated whether the P2X and P2Y receptors are located in the luminal or basolateral side by use of their functional antagonists. When the cells were pretreated with 40 mM caffeine, which blocks the intracellular calcium concentration ($[Ca^{2+}]_i$) response of metabotropic P2Y receptors by inhibiting inositol 1,4,5-trisphosphate receptors, the intracellular calcium levels in response to luminal or basolateral ATP administration abruptly decreased (Fig. 2, *C* and *D*). When the cells were pretreated with 10 μM PPADS, a nonspecific antagonist of ionotropic P2X receptors, the intracellular calcium levels in response to luminal or basolateral ATP administration were not altered (Fig. 2, *E* and *F*). These results suggest that NHNE cells have functionally active P2Y receptors, but not P2X receptors, in luminal and basolateral membranes.

Agonists that do not evoke $[Ca^{2+}]_i$ response. The roles of the functional P2X and $P2Y_1$ receptors were examined using more selective agonists for P2 receptors in the luminal and basolateral sides of the NHNE cells. Interestingly, applications of αβ-Me ATP (Fig. 3, *A* and *B*) or 2MeS-ATP (Fig. 3, *C* and *D*) did not evoke any $[Ca^{2+}]_i$ response in NHNE cells. αβ-Me ATP and 2MeS-ATP can activate $P2X_3$, $P2X_4$, and $P2Y_1$ receptors (7) among the P2 receptors identified by RT-PCR analysis in NHNE cells.

Effects of P2Y receptor agonists. Because the activation of most P2Y receptors increases the intracellular calcium level, the presence and cellular localization of the P2Y receptors can be partially determined by measuring the effects of the P2Y receptor agonists on the intracellular calcium level. This study analyzed the response in the luminal and basolateral sides of the NHNE cells to UTP ($P2Y_2$, $P2Y_4$, and $P2Y_6$ agonist) and UDP ($P2Y_6$ agonist) (20). When the luminal or basolateral side of the cells was treated with UTP, the $[Ca^{2+}]_i$ was increased to a similar level as with ATP (Fig. 4, *A* and *B*). Interestingly, basolateral application of UDP (10 μM) did not evoke $[Ca^{2+}]_i$ signals (Fig. 4*B*), whereas luminal application of the same

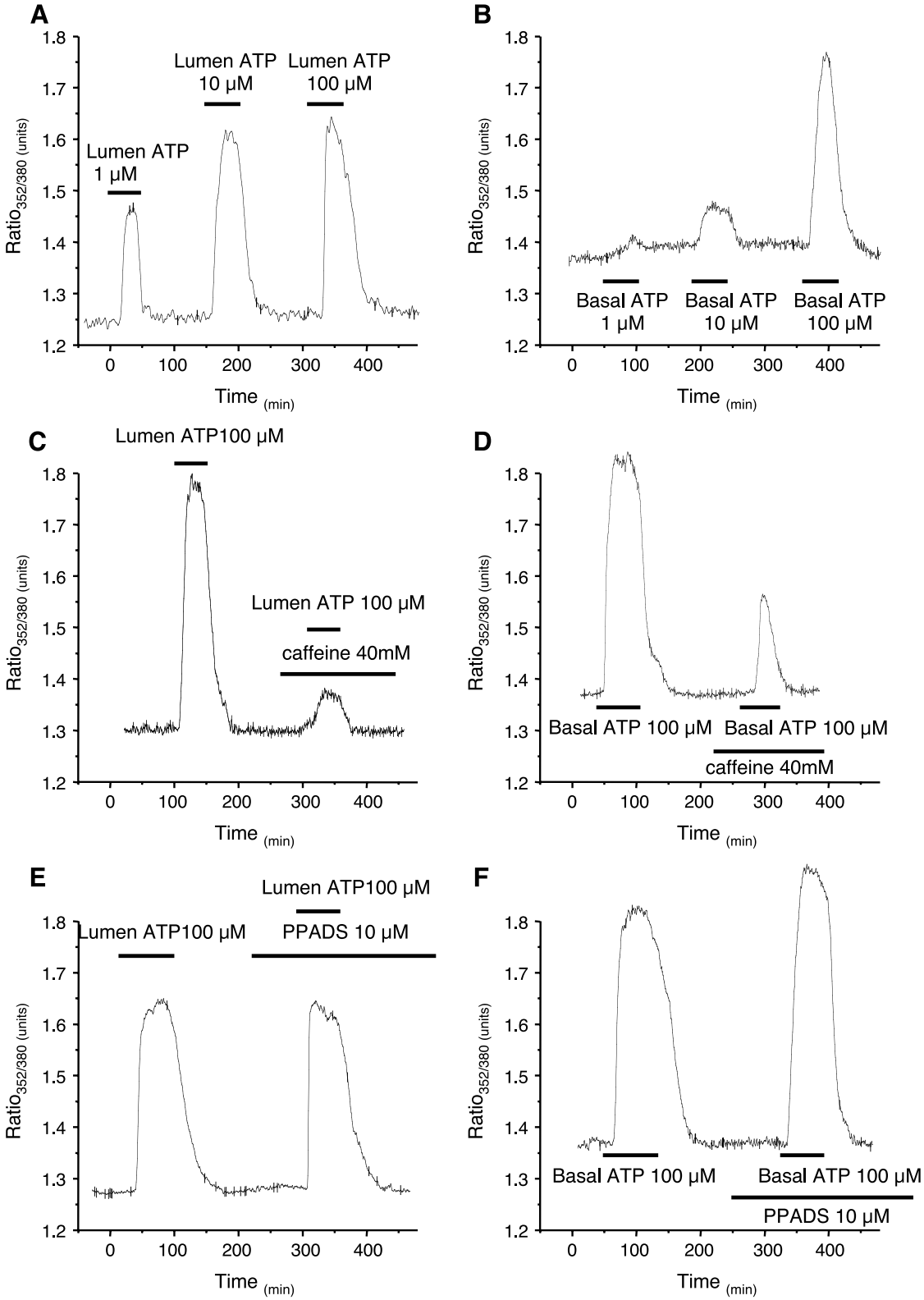

Fig. 2. Presence of functionally active P2Y receptors in NHNE cells. Three different ATP concentration (1, 10, 100 μM) successfully increased the intracellular calcium levels on both the luminal and basolateral sides (*A*, *B*), indicating the presence of the P2 receptors on both sides of the NHNE cells. The higher intracellular calcium induced by 100 μM ATP was successfully blocked by 40 mM caffeine on both sides of the NHNE cells (*C*, *D*), indicating the presence of the P2Y receptors. In contrast, 10 μM of pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) could not block the increased intracellular calcium level induced by 100 μM ATP on both sides of the NHNE cells (*E*, *F*), which indicates the absence of functionally active P2X receptors.

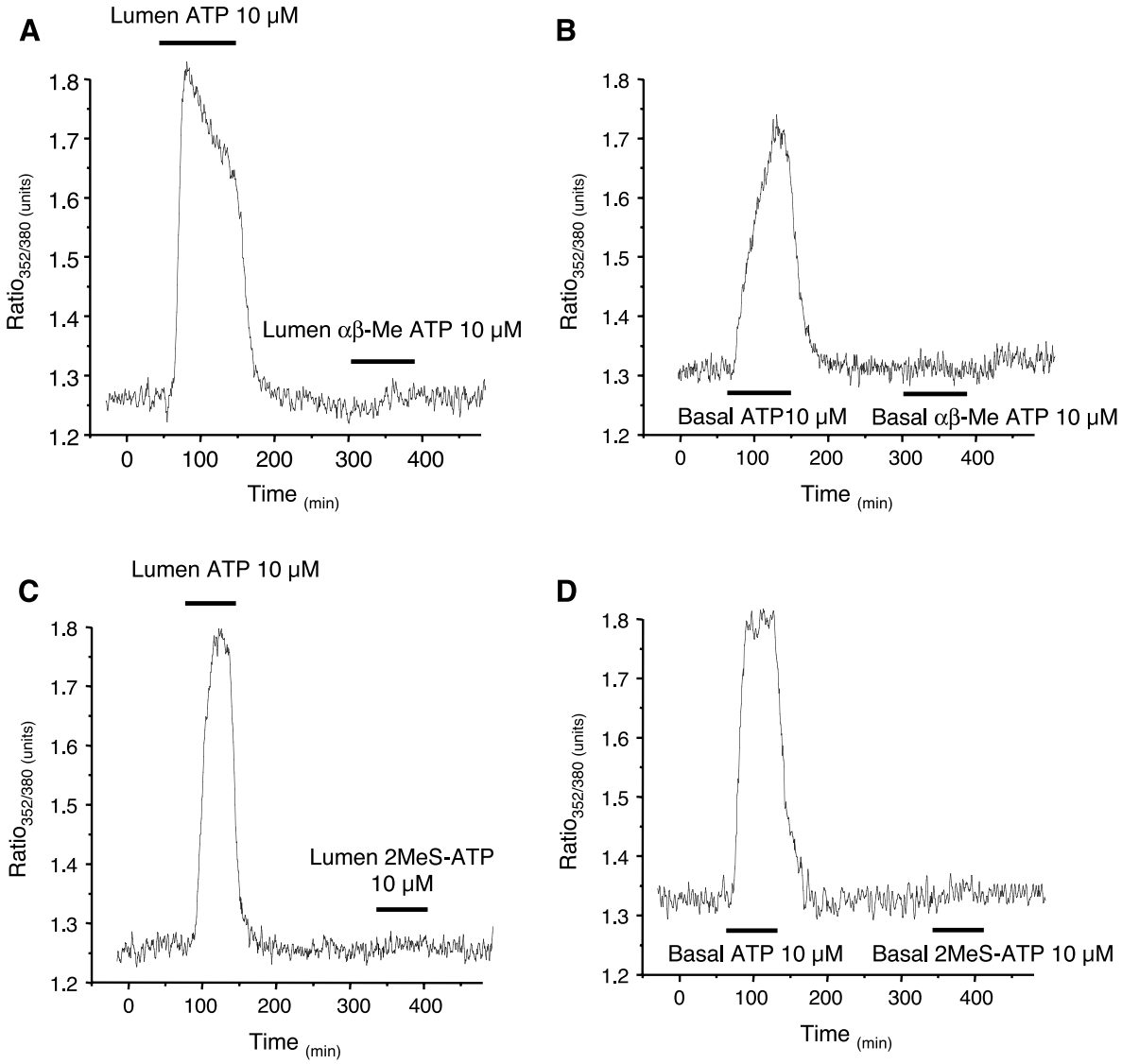

Fig. 3. Agonists that do not evoke calcium signals. Stimulation of both sides of the NHNE cells with 10 μM of αβ-methylene ATP (αβ-Me ATP) showed no changes in the intracellular calcium level (*A*, *B*). Similar responses were observed when the 2-methylthioadenosine 5′-triphosphate (2MeS-ATP) was added on both sides of the NHNE cells (*C*, *D*).

concentration of UDP significantly increased $[Ca^{2+}]_i$ in NHNE cells (Fig. 4*A*).

Next, the response to 100 μM of BzATP, which can activate $P2X_7$ and $P2Y_{11}$ receptors (27), was investigated in the luminal or basolateral sides of NHNE cells. The intracellular calcium level in response to the administration of BzATP was increased only on the luminal side, not on the basolateral side (Fig. 4*C*). Furthermore, pretreatment with caffeine abolished >80% of the BzATP-induced $[Ca^{2+}]_i$ response (Fig. 4*D*), which suggests that the $P2Y_{11}$ rather than the $P2X_7$ receptor is responsible for the BzATP-induced effects.

Immunoblot analyses for P2 receptors in NHNE cells. The presence of proteins for $P2Y_2$ and $P2Y_6$ receptors, which revealed functionally active purinoreceptors, was investigated by Western blotting (Fig. 5). For the $P2Y_2$ receptor, a band of ~35 kDa, compatible with the deduced protein size, was observed. In addition, extra bands ~55 and 120 kDa were found in the immunoblot of $P2Y_2$, which were also reported in previous studies (4, 23, 26). In a recent report, it was suggested that the multiple bands of $P2Y_2$ receptors could be the results of posttranscriptional modification of protein, oligomerization of receptor, or formation of heteromers with other proteins (26). In the immunoblot of $P2Y_6$, a clear band of ~42 kDa, compatible with the deduced size of $P2Y_6$, and a faint band ~68 kDa were observed similar to a previous report (26). Importantly, the specificities of $P2Y_2$ and $P2Y_6$ immunoblots were verified with relevant peptide antigens (Fig. 5, *right*).

DISCUSSION

Mucus secretion is closely related to the intracellular calcium level (3, 9, 25). The secretagogues induce the exocytosis of the preformed secretory granules usually within 5–10 min after stimulation (3). On the other hand, it takes at least 8–12

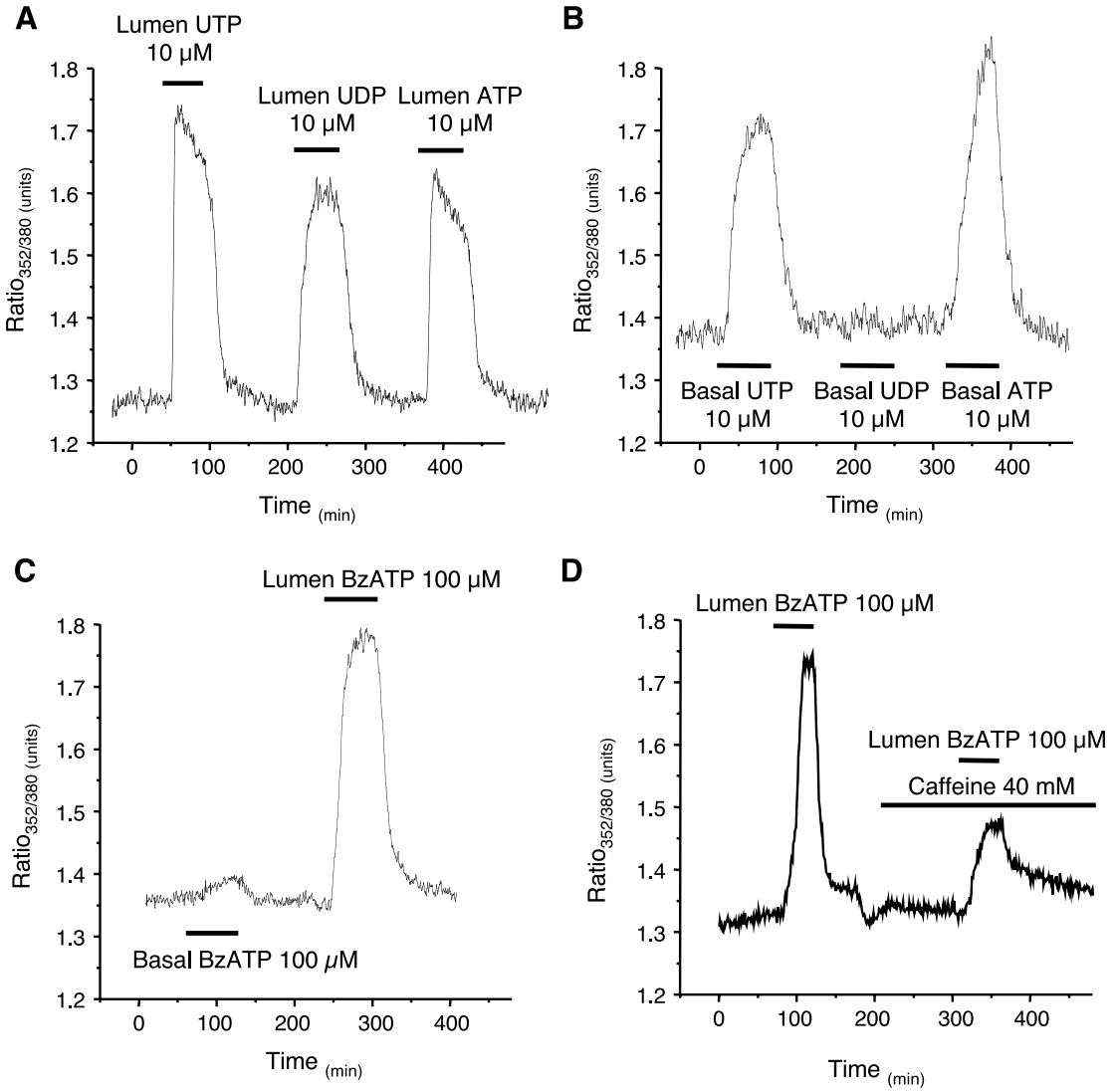

Fig. 4. Effects of the P2Y receptor agonists. Both sides of the NHNE cells were stimulated with 10 μM of UTP (agonist for $P2Y_2$ and $P2Y_4$ receptors) and UDP (agonist for $P2Y_6$ receptors) along with 10 μM of ATP for the control. The intracellular calcium levels were elevated to a similar level for both UTP and UDP in the luminal side (*A*), but UDP did not elevate the intracellular calcium level in the basolateral side of the NHNE cells (*B*). These results suggest that the $P2Y_2$ receptor is present in both the luminal and basolateral side of the NHNE cells, but the $P2Y_6$ receptor is present only in the luminal side of the NHNE cells. Stimulating the NHNE cells with 100 μM 2′,3′-O-(4-benzoyl)-benzoyl-ATP (BzATP) resulted in an increased intracellular calcium level only on the luminal side of the NHNE cells (*C*), which was successfully blocked by pretreatment with 40 mM caffeine (*D*). This indicates that a certain type of P2Y receptor is present only on the luminal side of the NHNE cell, most likely the $P2Y_{11}$ receptor.

h to induce exocytosis of the newly formed secretory granules made by the inflammatory mediators (25). Accordingly, it is possible that there are two secretory patterns. Secretagogues induce the early stages of secretion, and the inflammatory mediators induce the late stages. In the present study we examined the effects of the purinoreceptor agonists, which are the potent secretagogues, on the intracellular calcium level, since calcium signals are involved in the control of epithelial fluid and ion secretion as well as mucus secretion (9). For example, recently we have shown that purinergic stimulation contributes to the Cl^--driven fluid secretion by activating $Na^+,K^+,2Cl^-$ cotransporter in the basolateral membrane of NHNE cells (24).

In the present study, the expression patterns of the P2 receptor subtypes were analyzed in NHNE cells first by RT-PCR. Previous studies have shown that epithelial cells express multiple P2 receptor subtypes such as in pancreatic duct cells (16), human keratinocytes (7), and rat lens epithelial cells (17). In addition, cell lines originated from airway epithelia, such as 1HAEo-, 16HBE14o-, and A549 cells, have been reported to express the mRNAs for the $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors, but not for the $P2Y_1$ or $P2Y_{11}$ receptors (5). These results imply that the expression patterns can differ among the epithelial cells of various organs, and even between epithelial cells from the upper and lower airways.

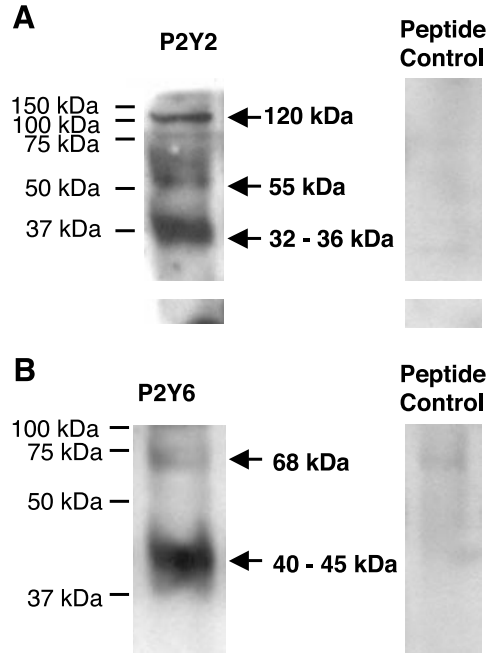

Fig. 5. Immunoblotting of $P2Y_2$ and $P2Y_6$ receptor subtype in NHNE cells. In the immunoblot of $P2Y_2$, a band ~35 kDa, compatible with the deduced protein size, was observed. In addition, extra bands ~55 and 120 kDa were found in the immunoblot of $P2Y_2$. However, when the anti-$P2Y_2$ antibody was preincubated with control peptide antigen, no band was found (*A*). In the immunoblot of $P2Y_6$, a clear band ~42 kDa, compatible with the deduced size of $P2Y_6$, and a faint band ~68 kDa were observed. However, when the anti-$P2Y_6$ antibody was preincubated with control peptide antigen, no band was found (*B*).

Although we initially investigated the P2R expression patterns using RT-PCR, the technique has significant limitations. RT-PCR does not generate quantitative data and can detect a very small amount of messages that do not perform a significant role in the cells. For that reason, expression patterns of functionally active P2Rs and their membrane-specific localizations were further examined using $[Ca^{2+}]_i$ measurements. The results suggest that the P2 receptors are present on both the luminal and basolateral sides of the NHNE cells. Moreover, it was shown that functionally active P2Y receptors were present on both the luminal and basolateral sides of the NHNE cells by blocking the effect of ATP with caffeine. However, in this study, PPADS did not inhibit the ATP-induced increase in intracellular calcium level, which suggests that there are no functionally detectable P2X receptors in the luminal or basolateral side of the NHNE cells, although the $P2X_3$, $P2X_4$, and $P2X_7$ receptor mRNAs were identified in the RT-PCR. This indicates that either the functional P2X receptor is not present in the NHNE cells or the function of the P2X receptor may be extremely weak as far as $[Ca^{2+}]_i$ increase is concerned.

This study also examined the effects of various purinergic agonists in the luminal and basolateral membranes of NHNE cells (Fig. 3). Difficulties in determining with certainty which of the P2Y receptors are functional in human nasal epithelial cells begin with the cross-reactivity of most P2 receptor agonists with various P2Y receptors. Nevertheless, the $P2Y_1$ receptor is quite sensitive to stimulation by 2MeS-ATP, and the potency of this agonist to increase intracellular calcium level is high (5). Even after the cells were treated with 10 μM 2MeS-ATP, there was also no change in the intracellular calcium level in response either to luminal or to basolateral 2MeS-ATP administration (Fig. 3*B*). This suggests that the $P2Y_1$ receptor is not active in NHNE cells.

The intracellular calcium level increased significantly in response to luminal ATP, UTP, and UDP administration (Fig. 4*A*). However, when the same agonists were applied to the basolateral side, the intracellular calcium level was increased only in response to ATP and UTP, but not to UDP (Fig. 4*B*). ATP is an effective agonist of the $P2Y_2$ and $P2Y_{11}$ receptors, UTP is an agonist for the $P2Y_2$ and $P2Y_4$ receptors, and UDP is an agonist for the $P2Y_6$ receptors (18, 22). Generally, $P2Y_4$ receptor responds to UTP much more sensitively than to ATP (22). In an experiment measuring the potencies for $[Ca^{2+}]_i$ increase, ED_{50} of UTP (luminal 2.8 ± 1.2 μM, basolateral 19 ± 4 μM) did not show significant difference from that of ATP (luminal 2.3 ± 0.9 μM, basolateral 23 ± 5 μM). The equal potency of ATP and UTP in the luminal and basolateral membranes implies that the $P2Y_4$ receptor is not the predominant P2Y receptor in NHNE cells. Because UDP evoked calcium signals only on the luminal side, it was concluded that the luminal membrane of NHNE cells expresses the $P2Y_6$ receptor. Therefore, the above results suggest that both $P2Y_2$ and $P2Y_6$ receptors are present on the luminal side, but only the $P2Y_2$ receptor is present on the basolateral side of NHNE cells. In addition, immunoblotting using specific antibodies and the relevant blocking peptides demonstrated the expression of $P2Y_2$ and $P2Y_6$ receptor proteins in NHNE cells (Fig. 5).

Lastly, to identify the presence of $P2Y_{11}$ receptors, the response to 100 μM BzATP in the luminal or basolateral membranes of human nasal epithelial cells was analyzed, since BzATP at 100 μM concentration acts as a $P2Y_{11}$-selective agonist (6). The intracellular calcium level increased in response to BzATP administration only to the luminal membrane, but not to the basolateral (Fig. 4*C*). The effect of luminal BzATP administration was successfully blocked when the cells were pretreated with caffeine, which excluded the possibility of $P2X_7$ activation (Fig. 4*D*). This suggests that the $P2Y_{11}$ receptor may be present and active only in the luminal membrane of the NHNE cells.

In summary, we found functionally active $P2Y_2$, $P2Y_6$, and $P2Y_{11}$ receptors in the luminal membrane and the $P2Y_2$ receptor in the basolateral membrane of the NHNE cells, which may play an important role in controlling the mucus and fluid secretion. In addition, NHNE cells do not appear to have functional P2X receptors to evoke significant $[Ca^{2+}]_i$ signals. Future studies using P2 receptor subtype-specific agonists and/or antagonists will elucidate the precise role of each P2 receptor on the secretory mechanisms in NHNE cells.

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REFERENCES

1. **Boucher RC.** Human airway ion transport. *Am J Respir Crit Care Med* 150: 271–281, 1994.
2. **Boucher RC.** Human airway ion transport. *Am J Respir Crit Care Med* 150: 581–593, 1994.
3. **Choi JY, Cho KN, and Yoon JH.** Effect of uridine 5′-triphosphate on mucin and lysozyme expression in human middle ear epithelial cells. *Acta Otolaryngol (Stockh)* 123: 362–366, 2003.
4. **Christofi FL, Wunderlich J, Yu JG, Wang YZ, Xue J, Guzman J, Javed N, and Cooke H.** Mechanically evoked reflex electrogenic chloride secretion in rat distal colon is triggered by endogenous nucleotides acting at P2Y1, P2Y2, and P2Y4 receptors. *J Comp Neurol* 469: 16–36, 2004.
5. **Communi D, Paindavoine P, Place GA, Parmentier M, and Boeynaems JM.** Expression of P2Y receptors in cell lines derived from the human lung. *Br J Pharmacol* 127: 562–568, 1999.
6. **Communi D, Robaye B, and Boeynaems JM.** Pharmacological characterization of the human P2Y11 receptor. *Br J Pharmacol* 128: 1199–1206, 1999.
7. **Dixon CJ, Bowler WB, Littlewood-Evans A, Dillon JP, Bilbe G, Sharpe GR, and Gallagher JA.** Regulation of epidermal homeostasis through P2Y2 receptors. *Br J Pharmacol* 127: 1680–1686, 1999.
8. **Harden TK, Boyer JL, and Nicholas RA.** P2 purinergic receptors: subtype-associated signaling responses and structure. *Annu Rev Phamacol Toxicol* 35: 541–579, 1995.
9. **Ishihara H, Shimura S, Sato M, Masuda T, Ishide N, Miura M, Sasaki T, Sasaki H, and Takishima T.** Intracellular calcium concentration of acinar cells in feline tracheal submucosal glands. *Am J Physiol Lung Cell Mol Physiol* 259: L345–L350, 1990.
10. **Kim SS, Kim KS, Lee JG, Park IY, Koo JS, and Yoon JH.** Levels of intracellular protein and mRNA of mucin and lysozyme in normal human nasal and polyp epithelium. *Laryngoscope* 110: 276–280, 2000.
11. **Ko WH, Law VW, Wong HY, and Wilson SM.** The simultaneous measurement of epithelial ion transport and intracellular free Ca^{2+} in cultured equine sweat gland secretory epithelium. *J Membr Biol* 170: 205–211, 1999.
12. **Koo JS, Jetten AM, Belloni P, Yoon JH, Kim YD, and Nettesheim P.** Role of retinoid receptors in the regulation of mucin gene expression by retinoic acid in human tracheobronchial epithelial cells. *Biochem J* 338: 351–357, 1999.
13. **Lazarowski ER, Paradiso AM, Watt WC, Harden TK, and Boucher RC.** UDP activates a mucosal-restricted receptor on human nasal epithelial cells that is distinct from the P2Y2 receptors. *Proc Natl Acad Sci USA* 94: 2599–2603, 1997.
14. **Lee MG, Zeng W, and Muallem S.** Characterization and localization of P2 receptors in rat submandibular gland acinar and duct cells. *J Biol Chem* 272: 32951–32955, 1997.
15. **Leuba D, De Ribaupierre Y, and Kucera P.** Ion transport, ciliary activity, and mechanosensitivity of sinusal mucosa: an in vitro study. *Am J Physiol Lung Cell Mol Physiol* 271: L349–L358, 1996.
16. **Luo X, Zheng W, Yan M, Lee MG, and Muallem S.** Multiple functional P2X and P2Y receptors in the luminal and basolateral membranes of pancreatic duct cells. *Am J Physiol Cell Physiol* 277: C205–C215, 1999.
17. **Merriman-Smith R, Tunstall M, Kistler J, Donaldson P, Housley G, and Eckert R.** Expression profiles of P2-receptor isoforms P2Y1 and P2Y2 in the rat lens. *Invest Ophthalmol Vis Sci* 39: 2791–2796, 1998.
18. **Morse DM, Smullen JL, and Davis CW.** Differential effects of UTP, ATP, and adenosine on ciliary activity of human nasal epithelial cells. *Am J Physiol Cell Physiol* 280: C1485–C1497, 2001.
19. **Namkung W, Lee JA, Ahn W, Han W, Kwon SW, Ahn DS, Kim KH, and Lee MG.** Ca^{2+} activates cystic fibrosis transmembrane conductance regulator- and Cl^--dependent HCO_3^- transport in pancreatic duct cells. *J Biol Chem* 278: 200–207, 2003.
20. **Nicholas RA, Watt WC, Lazarowski ER, Li Q, and Harden K.** Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol Pharmacol* 50: 224–229, 1996.
21. **Ralevic V and Burnstock G.** Receptors for purines and pyrimidines. *Pharmacol Rev* 50: 413–492, 1998.
22. **Robaye B, Ghanem E, Wilkin F, Fokan D, Van Driessche W, Schurmans S, Boeynaems JM, and Beauwens R.** Loss of nucleotide regulation of epithelial chloride transport in the jejunum of P2Y4-null mice. *Mol Pharmacol* 63: 777–783, 2003.
23. **Sage CL and Marcus DC.** Immunolocalization of P2Y4 and P2Y2 purinergic receptors in strial marginal cells and vestibular dark cells. *J Membr Biol* 185: 103–115, 2002.
24. **Shin JH, Namkung W, Choi JY, Yoon JH, and Lee MG.** Purinergic stimulation induces Ca^{2+}-dependent activation of Na^+-K^+-$2Cl^-$ cotransporter in human nasal epithelia. *J Biol Chem* 279: 18567–18574, 2004.
25. **Song KS, Lee WJ, Chung KC, Koo JS, Yang EJ, Choi JY, and Yoon JH.** IL-1 beta and TNF-alpha induce MUC5AC overexpression through a mechanism involving ERK/p38 MAP kinases-MSK1-CREB activation in human airway epithelial cells. *J Biol Chem* 278: 23243–23250, 2003.
26. **Wang L, Karlsson L, Moses S, Hultgardh-Nilsson A, Andersson M, Borna C, Gudbjartsson T, Jern S, and Erlinge D.** P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. *J Cardiovasc Pharmacol* 40: 841–853, 2002.
27. **Wilkin F, Duhant X, Bruyns C, Suarez-Huerta N, Boeynaems JM, and Robaye B.** The P2Y11 receptor mediates the ATP-induced maturation of human monocyte-derived dendritic cells. *J Immunol* 166: 7172–7177, 2001.
28. **Yoon JH, Kim KS, Kim SS, Lee JG, and Park IY.** Secretory differentiation of serially passaged normal human nasal epithelial cells by retinoic acid: expression of mucin and lysozyme. *Ann Otol Rhinol Laryngol* 109: 594–601, 2000.
29. **Yoon JH, Kim KS, Linton JA, Kim HU, and Lee JG.** Effects of TNF-α and IL-β on mucin, lysozyme, IL-6 and IL-8 in passage-2 normal human nasal epithelial cells. *Acta Otolaryngol (Stockh)* 119: 905–910, 1999.