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Uridine-5'-Triphosphate Stimulates Chloride Secretion via Cystic Fibrosis Transmembrane Conductance Regulator and Ca^{2+}-Activated Chloride Channels in Cultured Human Middle Ear Epithelial Cells

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사람중이점막세포에서 Uridine-5' -Triphosphate 자극에 의한 Cystic Fibrosis Transmembrane Conductance Regulator와 Ca^{2+}-Activated Chloride Channel을 통한 Chloride 분비 활성화

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Background and Objectives Nucleotide binding to purinergic P2Y receptors contributes to the regulation of fluid and ion transport in the middle ear epithelial cells. Here, we investigated the regulatory mechanism of the $P2Y_2$ receptor agonist, uridine-5'-triphosphate (UTP), on Cl^- transport in cultured normal human middle ear epithelial (NHMEE) cells.
Materials and Method Electrophysiological measurements were performed in monolayers of cultured NHMEE cells. Short circuit currents (*Isc*) were measured from the cells mounted in Ussing chambers under various conditions.
Results Apical addition of UTP in presence of amiloride evoked a transient rise and a sustained response in *Isc* due to Cl^- efflux. Application of different Cl^- channel blockers to the apical side of the cells significantly decreased UTP-induced *Isc*. Niflumic acid (NFA), a known blocker of Ca^{2+}-activated chloride channels (CACC), and $CFTR_{inh172}$, a selective inhibitor of cystic fibrosis transmembrane conductance regulator (CFTR), partially inhibited the UTP-induced Cl^- secretion, respectively.
Conclusion Cl^- transport across the airway epithelia plays a predominant role in regulating airway hydration. In this study, UTP is shown to increase both CACC and CFTR-dependent Cl^- secretion in NHMEE cells, suggesting their role in fluid and ion transport in the middle ear epithelium. Korean J Otorhinolaryngol-Head Neck Surg 2011;54:840-6

Key Words UTP · Chloride · Ion channel · Middle ear.

Introduction

Inflammation in the middle ear mucosa, caused usually by bacterial and viral pathogens, is the primary event in the middle ear predisposing the development of otitis media with effusion (OME).[1)] However, factors leading to retention of effusion still need to be elucidated. The middle ear mucosa is considered an extension of the respiratory tract epithelium and its surface is covered with a thin layer of mucus and airway surface liquid (ASL). The volume and composition of ASL in lower airway is known to be meticulously maintained by various ion transport systems such as epithelial sodium channel (ENaC) and cystic fibrosis transmembrane conductance regulator (CFTR) to allow effective mucociliary clear-

ance.[2-4] Recent studies report similar ion transport systems in the middle ear epithelium and their role in the pathogenesis of OME.[5-8] Alterations in the function of these ion channels are proposed to result in increased secretion or reduced absorption of ASL and lead to development of OME.

Chloride ion (Cl^-) transport is thought to play predominant roles in airway fluid secretion. Cl^- enters polarized airway epithelial cells through basolateral Na^+-K^+-Cl^-Cotransporter (NKCC) and is secreted by two apical channels: CFTR and Ca^{2+}-activated chloride channel (CACC). CFTR is a well-known cAMP-regulated Cl^- conductance and plays a critical role of maintaining ASL volume under basal conditions.[9] CACC, a recently identified anion channel, is stimulated by increased intracellular Ca^{2+}concentration due to various stimuli in both human and murine airways.[10,11] Contribution of CACC as an alternative Cl^- channel in fluid transport and thus ASL maintenance is considered even more important in cystic fibrosis patients who lack CFTR.[12] Previous studies have identified NKCC and CFTR in the middle ear epithelium, but exact mechanism regulating ion channel function remains unclear.[6,13]

Luminal nucleotides such as purines and pyrimidines are known to stimulate anion secretion in airway epithelia by activating P2 receptors in the apical cell membrane.[14] In the respiratory system, $P1A_{2b}$, $P2Y_2$, $P2Y_6$, some P2X receptor subtypes are expressed and control mucociliary clearance, ion transport, ciliary beat frequency and mucin release.[14,15] $P2Y_2$ and $P2Y_6$ receptors have been identified in the middle ear mucosa.[7] Also, UTP has been shown to induce mucin secretion in middle ear epithelial cells.[7] However, little is known about the role of purinergic receptors in the ion and fluid transport in the middle ear mucosa. Since abnormal fluid retention and mucin hypersecretion are the main pathologic processes, the role of purinergic receptors on Cl^- secretion may be important in understanding the pathology of OME.

The aim of this study was to examine the effect of UTP on Cl^- -secretion in cultured normal human middle ear epithelial (NHMEE) cells.

Materials and Method

Cell culture

Primary cultures of NHMEE cells were prepared as described previously.[16] All procedures were approved by the Institutional Review Board of Yonsei Medical Center. Passage-2 NHMEE cells were plated on a collagen-coated semi-permeable membrane with a pore size of 0.45 μm (Transwell-clear, Costar Co., Cambridge, MA, USA) at a density of 1.0×10^4 cells/cm^2. The cells were maintained in a 1 : 1 mixture of bronchial epithelial growth medium and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and all supplements.[16] Cultures were grown submerged for the first 9 days, at which time, the air-liquid interface was created by removing the apical medium and feeding the cultures from the basal compartment only and further cultured for 7 to 10 day for complete differentiation. Confluence of passage-2 NHMEE cells (8−9 days after seeding) was verified by measurement of transepithelial resistance (R_t; >1,000 Ω/cm^2 at room temperature) using endohm meter.

Ussing chamber study

Passage-2 NHMEE cells were grown at an air-liquid interface on Snapwell permeable supports with a surface areas of 1.13 cm^2 (Costar Co., Cambridge, MA, USA) for 4 additional days after confluence until they formed a tight epithelium. The cells were then mounted in modified Ussing chambers (World Precision Instruments, Sarasota, FL, USA). The epithelium was bathed on both sides with 5 mL of warmed (37℃) HCO_3^- -buffered NaCl solution circulated by gas lifts with 95% O_2−5% CO_2. 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 $NaHCO_3$ at pH 7.4. The osmolarity of all solutions was adjusted to 310 mosmol/kg with the major salt prior to use. Solution pH was maintained at 7.4. The epithelial culture was voltage clamped with an automatic voltage clamp and the short-circuit current (*Isc*) was measured. A 15-min equilibration was

Table 1. Function of chemicals

Chemicals	Function	Concentration	Reference
Amiloride	ENaC inhibitor	100 mM	Garty and Palmer (1997)[25]
TEA (tetraethylammonium)	Apical K+ channel blocker	5 mM	Rechkemmer and Halm (1989)[26]
UTP (uridine-5'-triphosphate)	P2 receptor agonist	100 uM	von Kugelgen and Wetter (2000)[27]
$CFTR_{inh172}$	CFTR inhibitor	100 mM	Ma et al. (2002)[28]
BAPTA-AM (2-bis 2-aminophenoxy ethane-N,N,N',N'-tetraacetic acid -acetoxy methylester)	Ca^{2+}-chelating agent	50 uM	Dormer (1984)[29]
Niflumic acid	CACC inhibitor	100 mM	Gruber et al. (1998)[30]

CFTR: cystic fibrosis transmembrane conductance regulator, CACC: Ca^{2+}-activated chloride channels

achieved to stabilize the transepithelial current. Then amiloride (100 μM) was added to the apical bath to block ENaC-dependent current. Data were acquired and analyzed with Acquire and Analysis (version 1.2) software (Physiologic Instruments, Inc., San Diego, CA, USA).

Chemicals

Table 1 enlists chemicals and their functions. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Collection and preparation of middle ear effusion

To evaluate *in vivo* presence of adenosine triphosphate (ATP) in the middle ear, middle ear effusions were collected after myringotomy in 6 patients with OME undergoing ventilation tube insertion procedures. The samples were frozen immediately after removal at surgery and stored at −20℃.

Bioluminescence detection of ATP in middle ear effusion

ATP assay was performed according to previous studies.[17,18] Briefly, Standard curves of ATP (Sigma, St. Louis, MO, USA) at known concentrations were performed with 2 mg/mL luciferase-luciferin reagent in OptiMEM-I medium by serial dilution from a 0.5 M ATP stock (made fresh at the time of performing standard curves) to approximate the concentrations of ATP released from cells. The same mixture of luciferase-luciferin reagent was mixed in a 1 : 1 volume with the collected middle ear effusion aspirate, and luminescence was assayed. Luminescence was corrected for the total volume of the middle ear effusion, and the concentration of ATP in a given middle ear effusion sample was determined through comparison to the standard curve.

Statistical analysis

The results of at least four different experiments are presented as mean±SD. Statistical analysis was performed by paired Student's t-test. A value of $p<0.05$ was considered statistically significant.

Results

Apical UTP-induced *Isc* (short-circuit current)

To examine the effect of apical UTP on ion transport in

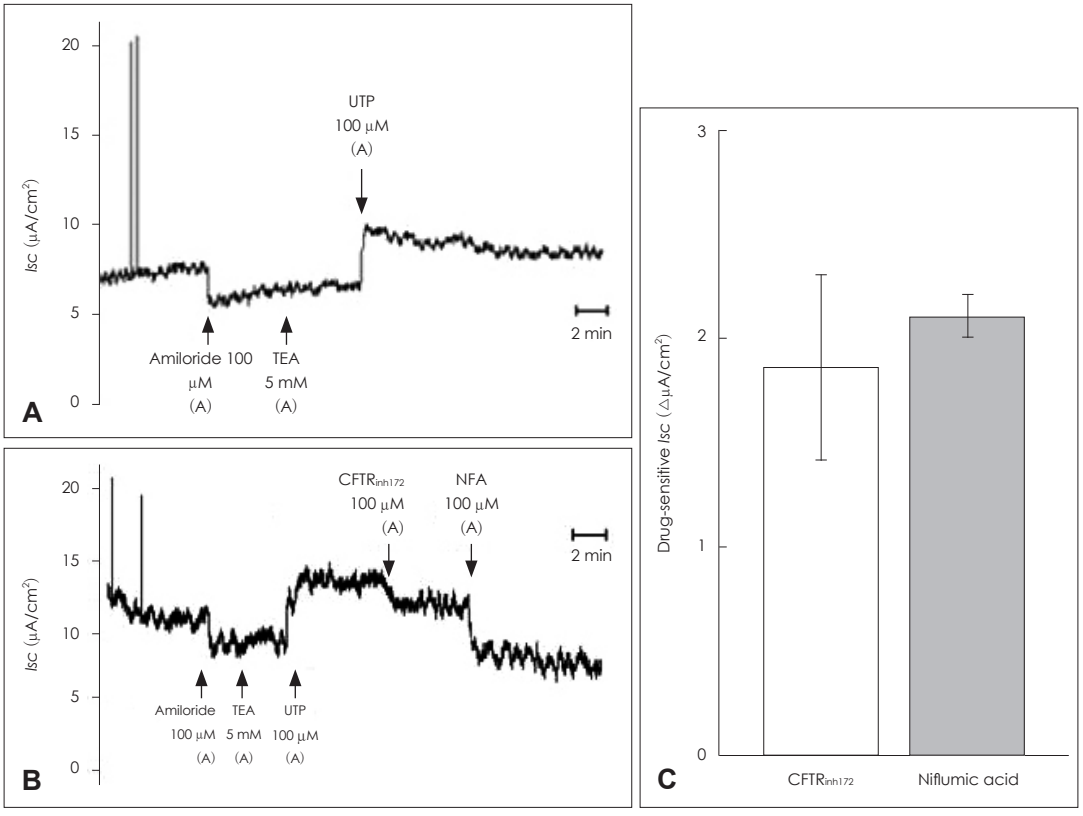

Fig. 1. Apical UTP-induced short-circuit current (*Isc*) in NHMEE cells. After pretreatment with amiloride (100 μM) and tetraethylammonium (TEA, 100 μM), apical addition of UTP (100 μM) induced a significant increase of *Isc* (A). UTP-induced *Isc* was inhibited by $CFTR_{inh172}$ (100 μM) and niflumic acid (100 μM)(B). Drug-sensitive portion of UTP-induced currents are shown (C). UTP: uridine-5'-triphosphate, NHMEE: normal human middle ear epithelial, CFTR: cystic fibrosis transmembrane conductance regulator, NFA: niflumic acid.

NHMEE cells, the short circuit current (*Isc*) was measured. The cells were pretreated: amiloride (100 μM) was applied to the apical side of the epithelia to block Na^+ channels and tetraethylammonium (TEA, 5 mM) was applied to the apical side of the epithelia to block apical K^+ channels. Apical addition of UTP (100 μM) induced a significant and sustained increase in *Isc*. UTP-induced peak response was 3.98±0.48 μA/cm^2 (Fig. 1A). The cells were exposed to different Cl^- channel inhibitors during plateau phase induced by UTP. CFTR-specific inhibitor $CFTR_{inh172}$ (100 μM) reduced *Isc* by 46.43±5.86%, and $CFTR_{inh172}$-sensitive portion of UTP was measured as 1.86±0.44 μA/cm^2. CACC inhibitor, niflumic acid (100 μM), reduced *Isc* by 53.73±5.64% and CACC-sensitive portion of UTP-induced current was measured as 2.11±0.10 μA/cm^2 (Fig. 1B and C). Cotreatment with $CFTR_{inh172}$ and niflumic acid reduced UTP-induced *Isc* to baseline. The findings suggest that the UTP increases Cl^- secretion through both CACC and CFTR in NHMEE cells.

Effects of P2Y receptor antagonists on UTP-induced *Isc*

The cells were exposed to different P2Y receptor antagonists to confirm that UTP-induced Cl^- current was due to $P2Y_2$ receptor activation. When the cells were pretreated with suramin, which acts as a strong antagonist to $P2Y_{1,2,11,12,13}$, and a much weaker antagonist to $P2Y_4$ and $P2Y_6$, UTP-induced *Isc* was significantly decreased to 0.83±0.14 μA/cm^2 (p=0.0002) (Fig. 2A and C). However, the UTP-induced *Isc* was relatively unchanged (3.73±0.21 μA/cm^2, p=0.19) after treatment with pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate, which acts as a strong inhibitor to P2X and $P2Y_1$ and only weakly on $P2Y_2$, $P2Y_{11}$ and $P2Y_1$ (Fig. 2B and C). The findings suggest that UTP activates $P2Y_2$ receptors.

Effect of Ca^{2+}-chelating agent on UTP-induced *Isc*

To examine whether the UTP-induced Cl^- current is dependent on intracellular Ca^{2+} concentration, the cells were pretreated with 2-bis 2-aminophenoxy ethane-N,N,N',N'-tetraacetic acid -acetoxy methyl ester (BAPTA-AM), a Ca^{2+}-chelating agent. UTP-induced current was decreased to 2.88±0.51 μA/cm^2. As expected $CFTR_{inh172}$-sensitive portion of UTP-induced current remained relatively unchanged (2.15±0.37 μA/cm^2, p=0.37) and niflumic acid-sensitive portion was significant-

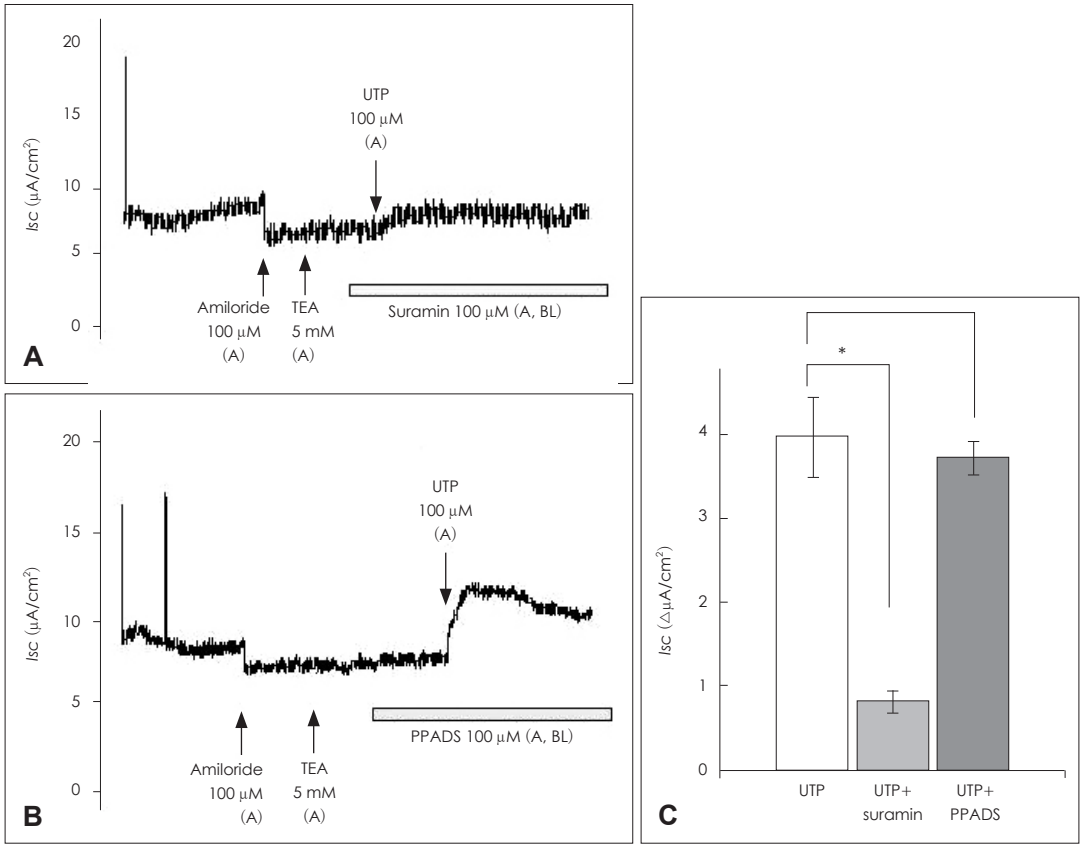

Fig. 2. Effects of P_2Y receptor antagonists on UTP-induced *Isc*. UTP-induced current was significantly decreased in presence of suramin (A), but remained unchanged in presence of PPADS (B). UTP-induced *Isc* in various conditions are shown (C). *p<0.05. UTP: uridine-5'-triphosphate, TEA: tetraethylammonium, PPADS: pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate.

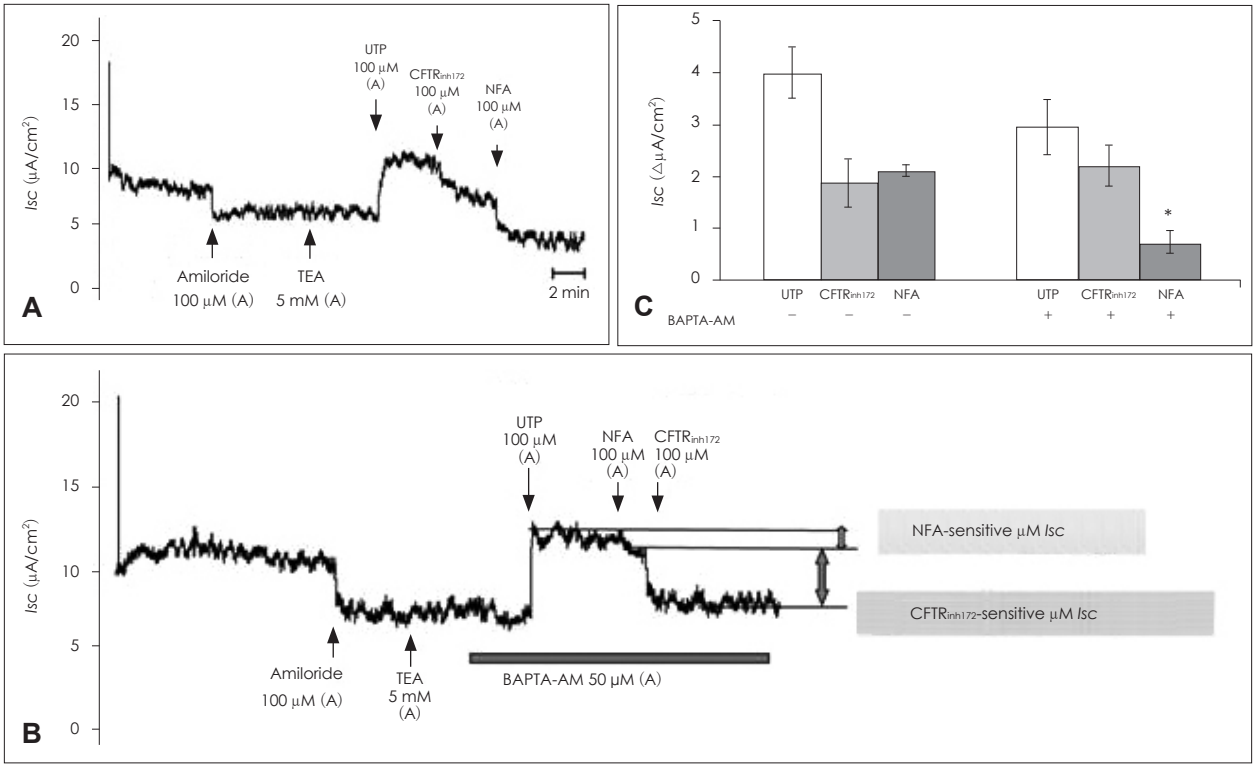

Fig. 3. Effect of Ca^{2+}-chelating agent on UTP-induced *Isc*. Representative tracings of *Isc* in conditions without and with BAPTA-AM pretreatment (A and B). UTP-induced Cl^- current was decreased when the cells were pretreated with a Ca^{2+}-chelating agent BAPTA-AM (C). UTP: uridine-5'-triphosphate, NFA: niflumic acid, CFTR: cystic fibrosis transmembrane conductance regulator, BAPTA-AM: 2-bis 2-aminophenoxy ethane-N,N,N',N'-tetraacetic acid -acetoxy methylester, TEA: tetraethylammonium.

ly decreased to 0.69±0.21 μA/cm² (p=0.0002)(Fig. 3).

Identification of ATP in middle ear effusion

One of the limitations of the Ussing chamber experiments is that UTP was added to the apical bathing solution to simulate the local effects of the purine agonist in the middle ear mucosa. To be able to extrapolate the physiological importance of our study in the clinical situation, the question whether extracellular nucleotides are present in adequate amounts in the ASL in the middle ear mucosa needs to be addressed. However, it is technically difficult to assess UTP and we investigated whether another nucleotide ATP is present the middle ear in patients with chronic OME. The middle ear mucosa is normally covered with a thin layer of ASL which is difficult to collect for ATP assay. Therefore we collected middle ear effusions from 6 patients and bioluminescence assay of ATP was performed. ATP was detected in the middle ear aspirates in all six patients (Fig. 4). The concentrations varied over a wide range (mean 853.67 nM, range 97 pM−3.75 nM). Our preceding experiments showed that an outward chloride current was induced by both UTP and ATP in cultured NHMEE cells (data not shown).

Discussion

Purinergic receptors play a major role in regulating epithelial electrolyte and fluid transport, prerequisite for mucociliary clearance and host defense.[3,19] Major functions comprise upregulation of Cl^- and mucin secretion and ciliary motility.[20,21] $P2Y_2$ receptors are the dominant purinergic receptors in the airway.[14] Extracellular purinergic agonists ATP and UTP play significant regulatory roles in various biological responses by activating $P2Y_2$ receptors. Stimulation of $P2Y_2$ receptors in airway cells activates Ca^{2+}-dependent Cl^- secretion and inhibits Na^+ absorption by the epithelial sodium channel ENaC.[4,15] As a part of respiratory tract, the middle ear mucosa also expresses $P2Y_2$ receptors.[13] UTP has been shown to upregulated mucin secretion via Ca^{2+}-dependent pathway in the middle ear mucosa.[13] The present study addresses the question whether UTP influences ion transport responses as well as intracellular Ca^{2+} signaling in the middle ear mucosa.

The experiments were designed to focus on electrogenic Cl^- ion transport in the middle ear epithelium. By blocking apical Na^+ and K^+ channels, the electrophysiological measurements reflect the isolated effect of UTP on chloride secretion

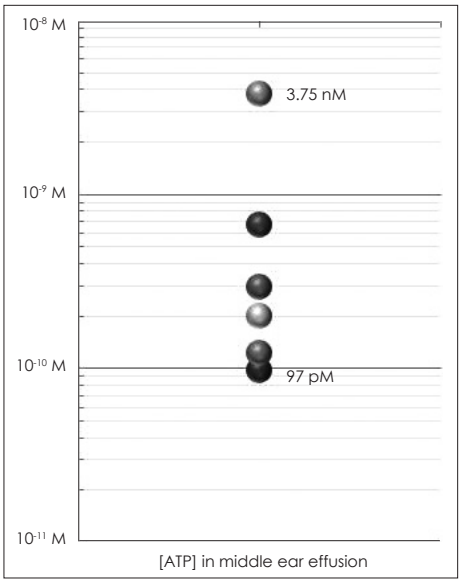

Fig. 4. A middle ear effusion luminometry scatterplot of [ATP] detected in middle ear effusions. Bioluminescence assay of ATP confirmed the presence of ATP in variable concentrations in middle ear effusions (mean 853.67 nM, range 97 pM−3.75 nM). ATP: adenosine triphosphate.

across the middle ear epithelial cells. As shown in Fig. 2, UTP induced a significant and sustained increase in *Isc* attributed to an outward current. Using specific inhibitors of different Cl^- channels, the suramin-inhibitable UTP-induced current can be explained as CFTR- and CACC-dependent. Treatment with both $CFTR_{inh172}$ and niflumic acid (a selective inhibitor of CACC) nearly abolished UTP-induced Cl^- current. As expected, the niflumic acid-sensitive portion of UTP-induced current was dependent on $[Ca^{2+}]_i$, while the $CFTR_{inh172}$-sensitive portion remained unchanged. Preliminary results suggest the CFTR-dependent portion of UTP-induced Cl^- current is partly sensitive to PKC inhibition (not shown). In various tissues, involvement of cAMP-dependent protein kinase (PKA) and PKC pathways in CFTR regulation has been studied. Phosphorylation of the CFTR channel by the PKA, regulates CFTR, and Ca^{2+}-dependent and Ca^{2+}-independent isoforms of PKC activate a recombinant CFTR Cl^- channel.[9] Synergistic modulation of CFTR activity by both PKA and PKC has been identified in a heterologous expression system.[22] P2-receptor activation has been reported to stimulate both Ca^{2+}-dependent Cl^- channels and Ca^{2+}-insensitive, CFTR-like Cl^- channels in rat submandibular gland.[22,23] Further experiments are needed to understand the mechanism by which UTP stimulation of $P2Y_2$ receptor might modulate CFTR activity in the middle ear epithelia.

The sources for extracellular nucleotides such as ATP and UTP are considered as the airway epithelial cells themselves. Respiratory epithelial cells release ATP and UTP both apically and basolaterally under basal conditions and in response to various stimuli.[19] Particularly, membrane stretch which occurs during coughing leads to a transient accumulation of nucleotides within the thin ASL, which reaches sufficiently high concentrations locally liquid.[24] A similar scenario can be postulated in the middle ear mucosa. Although the presence of extracellular nucleotides in the middle ear cavity could not be identified in healthy ears, ATP was detected in significant concentrations in the middle ear effusion aspirates collected from all six patients with OME. Since disruption of ion/fluid transport leading to abnormal retention of fluid and inflammatory exudates contribute to the pathogenesis of OME, it can be presumed that nucleotides are involved in regulating epithelial responses crucial to maintaining effective mucociliary clearance. ATP concentrations in the middle ear effusion varied over a wide range. Different characteristics of the effusion samples collected may correlate with the relative abundance or lack of ATP detected, although no definite observation could be made due to the small number of samples. Retained effusion in the middle ear cavity has variable viscosity due to variable mucin and serous composition, which may also change over time during the disease course. ATP release can be expected to vary depending on the cellular response to various inflammatory stimuli.

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