Expression and function of Na^+-K^+-$2Cl^-$ Cotransporter in Normal Human Nasal Epithelial cell

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Expression and function of Na^+-K^+-$2Cl^-$ Cotransporter in Normal Human Nasal Epithelial cell

Directed by Professor Joo-Heon Yoon

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Ji Hyun Shin

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This certifies that the Master's Thesis of Ji Hyun Shin is approved.

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모든 면에서 부족한 저를 때로는 채근하고, 때로는 격려하시며 지도해주신 윤주헌 교수님께 진심으로 감사와 존경의 말씀을 드립니다. 또한 윤주헌 교수님과 더불어 지난 2년 동안 저를 지도해주시고 아껴주셨던 이민구 교수님께도 진심으로 감사의 말씀을 드립니다. 아울러 제 논문을 심사해주신 의과대학의 이혜연 교수님께도 감사드립니다. 그리고 언제나 노력하는 모습으로 많은걸 배우게 해줬던 규남오빠, 항상 곁에서 제 마음을 이해해주던 은숙언니, 모든 면을 본받고 싶은 은진언니, 항상 저를 격려해주던 승은언니, 가장가까이에서 저를 도와주셨던 남궁완 선생님과 약리학교실의 모든 선생님들, 저에게 학문적 열의를 느끼게 해주신 김창훈 선생님과 최재영 선생님, 기한오빠, 영은, 든든한 친구인 아름, 연경, 아영, 성환, 상현, 용승 과 지은언니, 순희언니, 정식오빠, 후배 혜인이 에게도 감사의 말을 전합니다. 그리고 저의 영원한 후원자이자 세상에서 가장 소중한 우리가족, 부모님과 지예, 지희언니, 믿음직한 형부, 소중한 민석이와 겨울에 만나게 될 둘째 조카에게도 감사의 마음을 전하며 이 논문을 바칩니다.

2004년 6월

신 지 현

Table of Contents

List of Figures and Tables

LIST OF FIGURES

ABSTRACT

Expression and function of Na^+-K^+-$2Cl^-$ Cotransporter in Normal Human Nasal Epithelial cell

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Increasing evidence suggests that P2 receptors (P2Rs) in airway epithelial cells perform critical functions in auto- or paracrine regulation of fluid and mucus secretion. In the present study, we characterized the effects of P2R stimulation on Na^+-K^+-$2Cl^-$ cotransporter (NKCC) in normal human nasal epithelial (NHNE) cells. $[Ca^{2+}]_i$ and pH_i were measured in primary cultures of NHNE cells using a double perfusion chamber, which enabled us to analyze membrane-specific transporter activities. NKCC activities were estimated by the pH_i reduction due to Na^+-dependent and bumetanide-sensitive intracellular uptake of NH_4^+. NKCC activities were observed in the

basolateral membrane, but not in the luminal membrane of NHNE cells. Interestingly, P2Rs were expressed in both membranes, and the stimulation of either luminal or basolateral P2R increased NKCC activity. Blockades of luminal Cl^- channels, basolateral K^+ channels, or protein kinase C did not affect the activation of NKCC by basolateral P2R stimulation. The effects of luminal P2R stimulation were partially reduced by Cl^- channel blockers. However, chelation of intracellular Ca^{2+} by 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) treatment completely blocked the stimulatory effects of luminal and basolateral P2Rs on NKCC. In addition, increasing $[Ca^{2+}]_i$ by treatment with ionomycin- stimulated NKCC activity. These results provide evidence that stimulation of P2Rs directly activates basolateral NKCC by Ca^{2+}-dependent pathways in NHNE cells, which is an important aspect of the purinergic regulation of ion and fluid secretions in human airway epithelia under physiologic and pathologic conditions.

Key Words: normal human nasal epithelial cell, Na^+-K^+-$2Cl^-$ cotransporter, Ca^{2+} -activated Cl^- channel, purinergic receptor, 2′,7′-bis(2-carboxyethyl)-5(6)- carboxyfluorescein

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I. INTRODUCTION

The mucosal surfaces of airway tract are covered with airway surface fluid (ASF), which performs critical roles in proper functioning of airway tracts and in protecting the lining epithelia[1-3]. The hypersecretion or the contraction of ASF is associated with a wide spectrum of respiratory tract diseases from allergic rhinitis to cystic fibrosis (CF). Chronic volume contraction, either by defects in secretion or by dysregulated hyper-absorption, induces the formation of hyper-viscous mucus, which is thought to be the underlying pathogenic mechanism of CF[1,2]. On the other hand, in stimulated

conditions such as in allergic inflammation, airway epithelia actively secrete ion and fluids by locally induced neurotransmitters and inflammatory mediators[4].

It is generally accepted that transepithelial Cl^- transport is the major driving force for fluid secretions in airway epithelia[5,6]. During past decades, extensive investigations have been performed on the molecular nature and the regulatory mechanisms accounting for this important biologic process. However, most of the studies focused on the luminal Cl^- exit pathways, especially on the transporter named cystic fibrosis transmembrane conductance regulator (CFTR), in which gene mutation causes CF and other related disorders[7,8]. Equally important process is the basolateral Cl^- uptake pathways. In fact, recent evidence suggested that the activities of basolateral Na^+-K^+-$2Cl^-$ cotransporter (NKCC) are the rate- limiting step of ion and fluid secretions in Cl^- secreting epithelia[6,9]. In addition to NKCC, several other mechanisms can mediate intracellular Cl^- uptake: 1) the parallel activation of Na^+/H^+ and Cl^-/HCO_3^- exchangers; 2) other members of cation-chloride cotransporters such as K^+-Cl^- cotransporter (KCC) and Na^+-Cl^- cotransporter (NCC); and 3) a Cl^--ATPase. However, these transporters are expressed in specific tissues or are active in specific conditions, whereas NKCC is known to be ubiquitously expressed and responsible for most of the intracellular Cl^-

uptake in the secretory epithelia[5]. To date, two isoforms of NKCC were identified, NKCC1 and NKCC2. Both belong to the cation-chloride cotransporter family, which has 12 membrane-spanning domains[5,10]. Among them, NKCC1 is in general expressed in the basolateral membrane of Cl^- secreting epithelia[5,6].

As in other CFTR- and NKCC1-expressing epithelial tissues, cells in the airway tract are under control of several agonists, which include agonists in the cholinergic and adrenergic systems. Recently, the purinergic system, a different type of regulating systems, has attracted increasing attention in the regulation of transepithelial transport due to its unique properties[3]. For example, purinergic receptors (P2Rs) are expressed in the luminal membrane as well as in the basolateral membrane of many epithelial cells[11,12]. In addition, accumulating evidence suggests that activations of P2Rs is not only involved in the regulation of physiologic function but also associated with pathologic conditions[3]. Therefore, a dissection of the effects of P2R on the basolateral Cl^- uptake pathways would improve our understanding of physiological and pathological regulations of airway secretions.

We employed primary cultures of normal human nasal epithelial (NHNE) cells as a model of human airway epithelia. Alterations in nasal secretion are frequently associated with common medical problems such as

allergic rhinitis or the common cold[4]. In addition, cells in nasal mucosa share some common features with cells in other airway epithelia in both morphological and molecular aspects. For example, nasal epithelia have cilia on the luminal surface and co-express CFTR and epithelial Na^+ channel (ENaC) on the luminal membrane, similar to cells in the middle or lower airway tract[13]. Therefore, an investigation of the nasal secretory mechanism itself has significant clinical meanings, and further, the results may be extrapolated to the secretory mechanisms of cells in the middle and lower airway tracts.

In the present study, we analyzed the effects of P2R activation on NKCC in human nasal epithelia. NKCC activities were observed only in the basolateral membrane of NHNE cells and immunoblotting using specific antibodies revealed the expression of NKCC1. Interestingly, P2Rs were expressed in both luminal and basolateral membranes, and the stimulation of either luminal or basolateral P2R increased the basolateral NKCC activity. Finally, the stimulatory effects of P2R on NKCC were completely dependent on intracellular Ca^{2+} signals. These findings will provide the basis for understanding the purinergic control of secretions in human nasal and airway epithelia under physiologic and pathologic conditions.

II. MATERIALS AND METHODS

1. Chemicals and Solutions

The fluorescent probes, Fura-2-AM and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM), and the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyl ester (BAPTA-AM) were purchased from Molecular Probes (Molecular Probes, Eugene, OR, USA). The Cl^- channel blocker 5-nitro-2-(3'phenylpropyl-amino)benzoic acid (NPPB) was obtained from Alexis (Alexis, Carlsbad, CA, USA), and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt (DIDS) was from Calbiochem (Calbiochem, San Diego, CA). Ammonium gluconate was purchased from Pfaltz & Bauer (Pfaltz & Bauer, Waterbury, CT, USA). All other chemicals including ATP, ouabain, bumetanide, $BaCl_2$, Go 6976, and rottlerin were purchased from Sigma (Sigma, St. Louis, MO, USA).

The standard HEPES-buffered perfusate was termed solution A and 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 Na^+-free solution B was prepared by replacing Na^+ with *N*-methyl-D-glucamine$^+$ (NMDG) from solution A (pH 7.4 with Tris). The Cl^--free solution C contained (in mM) 140 Na^+-gluconate, 5

K^+-gluconate, 1 $MgSO_4$, 9.3 hemicalcium cyclamate, 10 D-glucose and 5 HEPES (pH 7.4 with NaOH). In the measurements of NH_4^+ uptake, 20 mM of Na^+ or *N*-methyl-D-gluconate$^+$ was replaced with equimolar NH_4^+ using NH_4Cl or NH_4^+-gluconate in each solution. The osmolarity of all solutions was adjusted to 310 mOSM with the major salt prior to use.

2. Culture of NHNE cells

Primary cultures of NHNE cells were prepared as described previously[14,15]. All procedures were approved by the Institutional Review Board of Yonsei Medical Center. Passage-2 NHNE cells were plated on a collagen-coated semipermeable membrane with a 0.45-μm pore size (Transwell-clear; Costar Co., Cambridge, MA, USA) at a density of 1.0×10^4 cells/cm^2 for intracellular pH (pH_i) and $[Ca^{2+}]_i$ measurements. The cells were maintained in 1:1 mixture of bronchial epithelial growth medium and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and all supplements[14]. Membranes bearing cultured cells (culture area, 0.3 cm^2) were floated on the culture medium in Petri dishes and incubated for 4-5 days until the cells formed a functionally polarized monolayer.

3. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ levels in the monolayers were determined using protocols reported previously with slight modification[16]. Briefly, after achieving confluency, the cells were loaded with Fura-2 by incubating in a medium containing 3 μM Fura-2-AM and 1.6 μM pluronic F127 (for 30 min, at 37°C). A membrane bearing Fura-2-loaded cells was mounted in a miniature Ussing chamber (AKI Institute, University of Copenhagen, Copenhagen, Denmark) attached to the stage of an inverted microscope. The membrane was located between the two-half chambers, which separated the chamber into a luminal (upper) and a basolateral (lower) compartment. 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 (more than 2 mm). Separate luminal and basolateral perfusates were delivered to the chamber after warming (37 °C). Fura-2 fluorescence was recorded (Delta Ram; PTI Inc., South Brunswick, NJ, USA) at excitation wavelengths of 350 and 380 nm, and the 350/380 fluorescence ratio was calibrated by exposing the cells to solutions containing high and low concentrations of Ca^{2+} and 10 μM ionomycin.

4. Measurements of Intracellular pH (pH_i) and NKCC activity

pH_i was measured in the monolayers using the pH-sensitive fluorescent

probe BCECF. Cells were loaded with BCECF for 10 min at room temperature in solution A containing 2.5 μM BCECF-AM and mounted in the miniature Ussing chamber described for $[Ca^{2+}]_i$ measurements. BCECF fluorescence was recorded and calibrated using a previously described protocol[16]. Briefly, the fluorescence at excitation wavelengths of 490 and 440 nm was recorded using a recording setup (Delta Ram; PTI Inc.), and the 490/440 ratios were calibrated intracellularly by perfusing the cells with solutions containing 145 mM KCl, 10 mM HEPES, and 5 μM nigericin with the pH adjusted to 6.2–7.6.

Na^+-K^+-$2Cl^-$ cotransporter activity was estimated from the pH_i decrease cause by the intracellular uptake of NH_4^+ using the methods of Evans and Turner[17] with modifications. As shown in Fig. 1, when challenged with 20 mM NH_4Cl solutions, cells were rapidly alkalinized (0-2 s) due to the intracellular diffusion of NH_3. Then, if the cells have NH_4^+ - transporting machineries, the pH_i of the cells would be decreased due to the intracellular uptake of NH_4^+. As detailed under "Results," most of the intracellular NH_4^+ uptake measured in ouabain (100 μM) and Na^+ (140 mM)-containing perfusates was a function of NKCC in NHNE cells. Typically, the first 10-40 s of the initial linear portion of pH_i decreases due to Na^+-dependent and ouabain-insensitive intracellular NH_4^+ uptake (shorter times were used for

more rapid decreases) was fitted to a linear equation using the Felix software (version 1.4; PTI Inc.).

Buffer capacity of NHNE cells was calculated by measuring pH_i in response to 5-40 mM NH_4Cl pulses in Na^+-free solutions[18], which applied to both luminal and basolateral sides simultaneously. In each experiment, the intrinsic buffer capacity (β_i) showed a negative linear relationship with pH_i between 6.4 and 7.6. The β_i of NHNE cells at resting state (23.1 ± 1.8 mM/pH unit at pH_i 7.2) was not significantly different from that of ATP- stimulated cells (25.8 ± 2.7 mM/pH unit). Therefore, the results of NKCC activity were expressed as ΔpH unit/min, and this value was directly analyzed without compensating for β_i.

5. Immunobolotting

NHNE cells were grown to confluence in 6-well plates. The cells were lysed with 2x lysis buffer (250 mM Tris-Cl (pH 6.5), 2% SDS, 0.1 mol/liter dithiothreitol, 0.02% bromophenol blue, 10% glycerol). Equal amounts of whole cell lysates were resolved by 6% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline (50 mM Tris-Cl, pH 7.5, 150 mM NaCl) for 2 h at room temperature. This membrane

was then incubated overnight with T4 monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) in TTBS (0.5% Tween 20 in Tris-buffered saline). After washing with TTBS, the blot was further incubated for 45 min at room temperature with appropriate secondary antibody (Cell Signaling, Beverly, MA, USA) in TTBS and then visualized using the ECL system (Amersham Biosciences, Buckinghamshire, England).

6. Statistical analysis

The results of multiple experiments are presented as means ± S.E. Statistical analysis was carried out by analysis of variance or Student's *t*-test as appropriate. $P<0.05$ was considered statistically significant.

III. RESULTS

1. NH_4^+ transport in NHNE cells

Na^+-K^+-$2Cl^-$ cotransporter activities were measured using NH_4^+ as a K^+ surrogate. As an initial step, NH_4^+ transport in the luminal and basolateral membrane of polarized NHNE cells was measured. The basal pH_i of NHNE cells was 7.15 ± 0.04 in Hepes-buffered solution A, and the application of 20 mM NH_4Cl to the luminal side evoked an abrupt increase in pH_i followed by a sustained pH_i increases (Fig. 1A). These results indicate that only NH_3 can pass through the luminal membrane of NHNE cells (see Fig. 1C). At pH 7.4 and 37 °C, 20 mM of NH_4Cl solution contains about 0.6 mM NH_3 (pKa=8.9). The charged NH_4^+ ion cannot pass through the lipid bilayer of plasma membrane, whereas non-polar NH_3 can freely diffuse into the cells. When it enters the cells, NH_3 rapidly converts into NH_4^+ by picking up H^+ until equilibrium is reached. Therefore, pH_i increases due to a reduced H^+ concentration. However, if the cell membrane expresses an NH_4^+-transporting protein, NH_4^+ enters the cells and pH_i decreases since NH_4^+ donates H^+ to achieve equilibrium with NH_3 (Fig. 1C). Interestingly, applications of 20 mM NH_4Cl to the basolateral side induced a small pH_i increase followed by rapid pH_i decreases, which suggests that the basolateral membrane of NHNE cells

have strong NH_4^+-transporting activities (Fig. 1B). The average value of initial pH_i reduction caused by basolateral challenge with 20mM NH_4Cl was 0.331 ± 0.021 ΔpH unit/min.

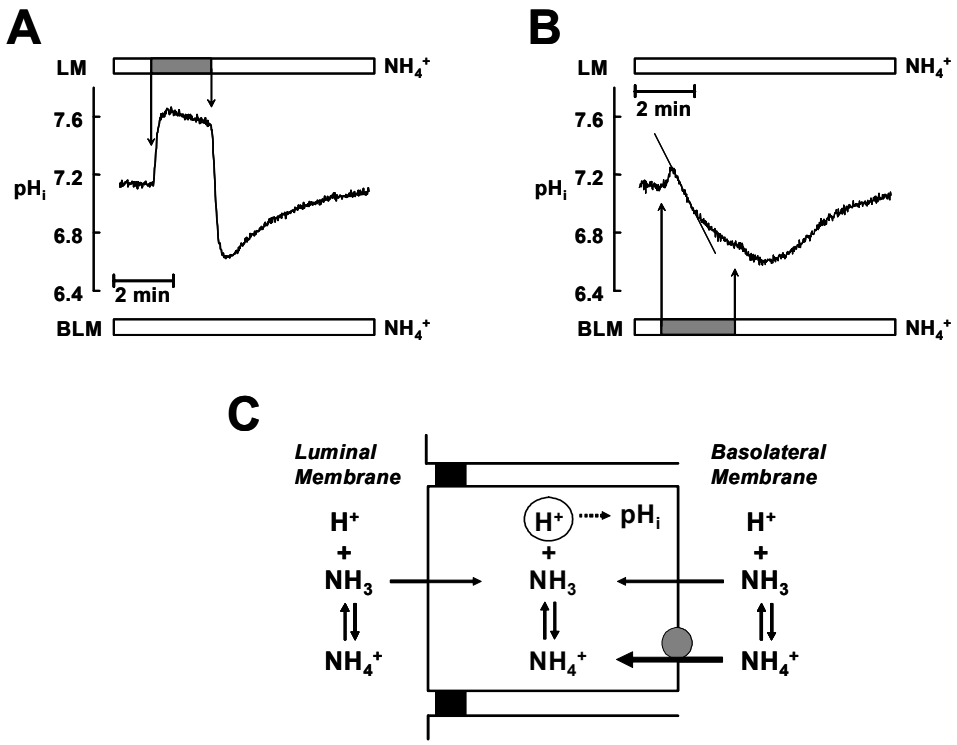

Fig.1. NH_4^+ transport in the luminal and the basolateral membranes of NHNE cells.

pH_i of NHNE cells were measured using BCECF as described in "Materials and Methods." The membrane-specific NH_4^+ -transporting activities of

polarized NHNE cells were analyzed using a double perfusion chamber with separate application of NH_4Cl (20 mM). *A*, the application of NH_4Cl to the luminal side evoked an abrupt increase in pH_i followed by a sustained pH_i increases. *LM*, luminal membrane; *BLM*, basolateral membrane. *B*, the application of NH_4Cl to the basolateral side induced a small pH_i increase followed by a rapid pH_i decreases. *C*, these results indicate that only non-polar NH_3 can pass through the luminal membrane of NHNE cells. However, the basolateral membrane of NHNE cells has strong NH_4^+-transporting activities (see "Results" for detail).

2. Characterization of basolateral NH_4^+ transporting mechanisms

To date, several transporters are known to transport NH_4^+, in particular via K^+ binding sites[19]. Therefore, we characterized the basolateral NH_4^+-transporting activities of NHNE cells. As shown in Fig. 2, we first analyzed the Na^+ and Cl^- dependence of the NH_4^+-transporting mechanisms. Incubations of the NHNE cells in the Na^+-free solutions for 5 min reduced the basal pH_i to 6.78 ± 0.07. Of interest, the basolareral application of NH_4Cl in Na^+-free solutions induced a rapid and sustained pH_i increases similar to that observed in the luminal application of NH_4Cl in Na^+-containing solutions (Fig. 1A and 2A). Therefore, it was concluded that most of the basolateral NH_4^+-

transporting activities are dependent on Na^+, which suggests that Na^+-independent transporters, such as KCC, do not contribute to the NH_4^+-transporting activity. In addition, 83% of the basolateral NH_4^+ transport was also found to be dependent on Cl^- (Fig. 2,B and C).

Basolateral NH_4^+ transport was further analyzed using inhibitors of Na^+- and K^+-coupled transporters. As shown in Fig. 3,A and B, the Na^+,K^+ ATPase inhibitor ouabain (100 μM) inhibited the NH_4^+ transport by 28%, and the addition of the NKCC inhibitor bumetanide (100 μM) inhibited by 94%. These results and the data concerning Na^+ and Cl^- dependence indicate that NKCC mediates most of the basolateral NH_4^+ transport in NHNE cells, particularly when treated with ouabain. Subsequently, the expression of NKCC1 in NHNE cells was verified by immunoblotting. Protein extracts of NHNE cells originating from two different individuals showed a band around 170 kDa when blotted with T4 anti-NKCC1 monoclonal antibody, which corresponds to the size of typical glycosylated human NKCC1 (Fig. 3C). In addition, reverse transcriptase-PCR experiments using specific primers of human NKCC1 and NKCC2 revealed that NHNE cells contain only NKCC1 mRNA (not shown).

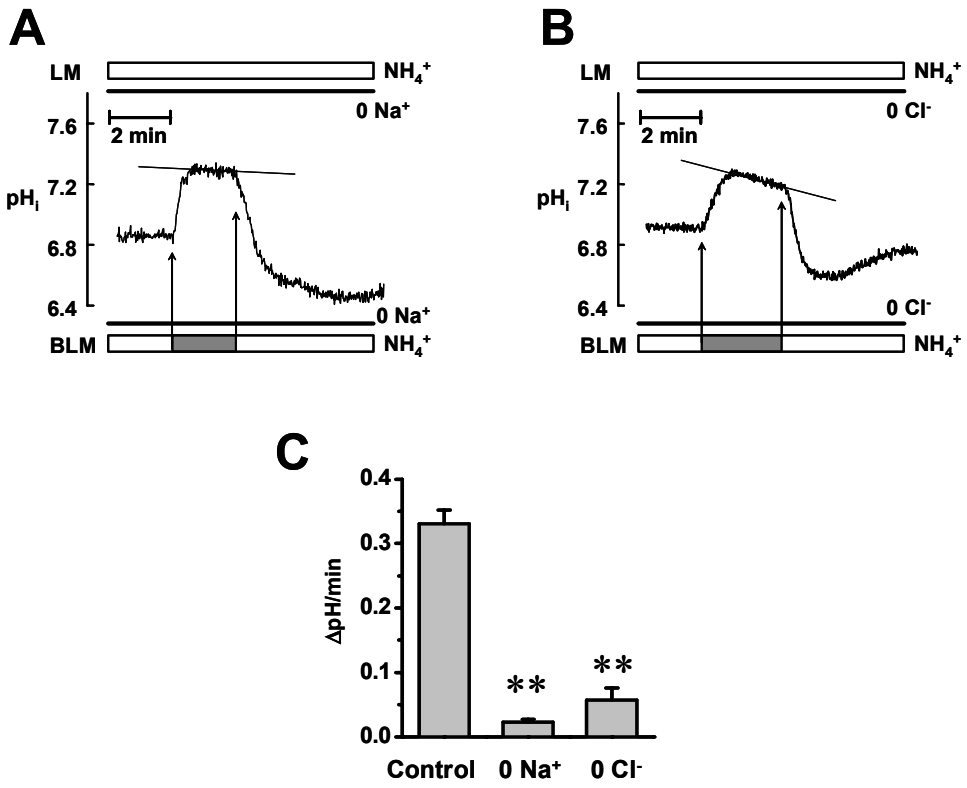

Fig.2. Na^+- and Cl^--dependence of the basolateral NH_4^+-transporting mechanisms.

The effects of Na^+ or Cl^- removal on the basolateral NH_4^+ transport were analyzed in NHNE cells. Representative trances of Na^+ removal or Cl^- removal are presented in *A* and *B*, respectively, and a summary (each n=5) is shown *C*. Incubations in the Na^+-free or in Cl^--free solutions abolished the basolateral NH_4^+ transport by 96% and 83%, respectively. **P<0.01: difference from control.

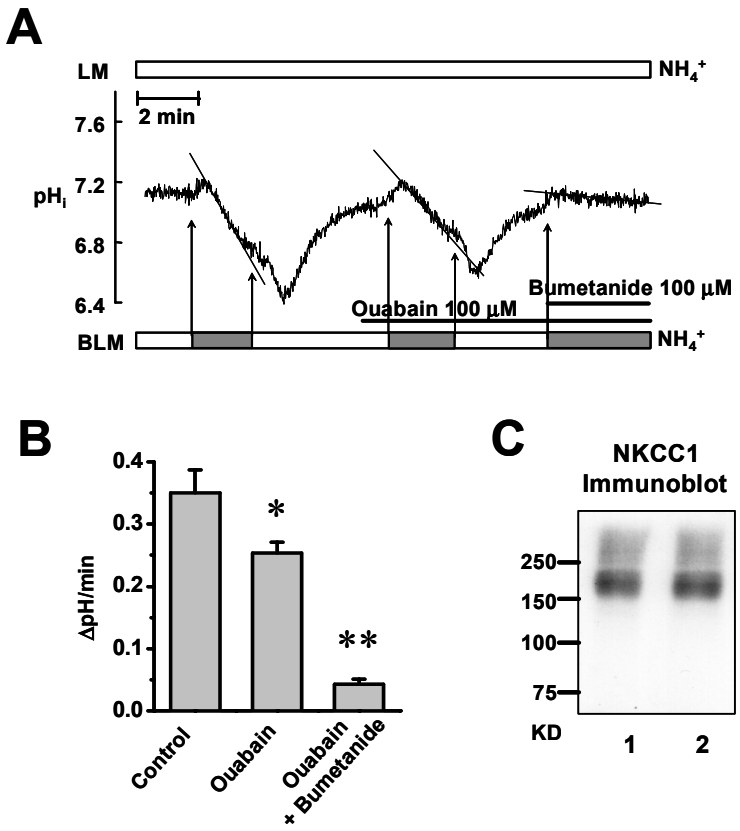

Fig. 3. Functional and molecular expression of NKCC1 in NHNE cells.

Basolateral NH_4^+ transport was measured with solutions containing the Na^+,K^+ ATPase inhibitor ouabain and the NKCC inhibitor bumetanide. *A*, a representative trace showing the effects of ouabain (100 μM) and bumetanide (100 μM). *LM*, luminal membrane; *BLM*, basolateral membrane. *B*, Summarized results of six experiments. C, immunoblotting of protein extracts of NHNE cells originating from two separate individuals using T4 anti-

NKCC1 monoclonal antibody. *P<0.05, **P<0.01, difference from control.

3. Activation of NKCC1 by purinergic stimulation

Accumulating evidence suggests that P2 receptors participate in the regulation of transepithelial ion transport in various epithelia[3]. To investigate the effects of P2R on NKCC, we first measured $[Ca^{2+}]_i$ in response to the membrane-specific applications of P2R agonists. As shown in Fig. 4A, both luminal and basolateral applications of ATP evoked $[Ca^{2+}]_i$ signals in NHNE cells. Interestingly, P2Rs in the luminal membrane (ED_{50} for ATP, 4.3 μM) showed ~ 5 times higher agonist sensitivity than those of the basolateral membrane (ED_{50} for ATP, 23.7 μM). We next evaluated the effects of P2R stimulation on basolateral NKCC activity using the protocols described above. A summary of the results is presented in Fig. 4B, and representative traces of basolateral ATP and luminal ATP stimulations are shown in Fig. 4, C and D, respectively. Importantly, the stimulation of P2Rs either in the basolateral membrane or in the luminal membrane increased the NKCC activity in NHNE cells. Control basolateral NH_4^+-transporting activity under ouabain treatment was 0.223 ± 0.017 ΔpH unit/min, and this value was increased to 0.496 ± 0.075 and 0.569 ± 0.102 by basolateral and luminal ATP applications, respectively.

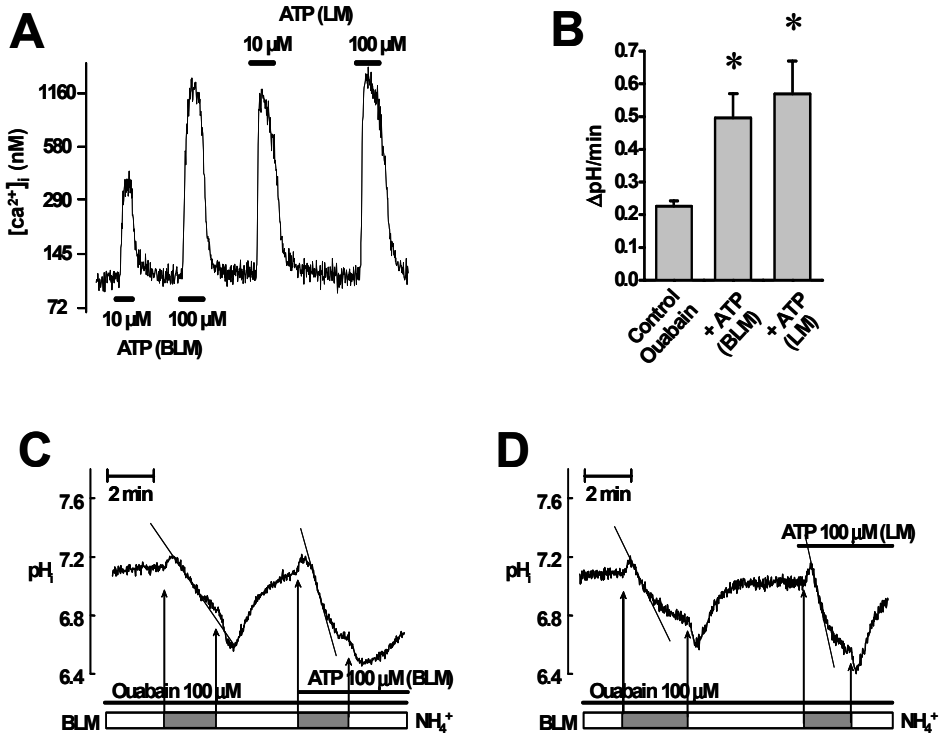

Fig. 4. Activation of NKCC by P2R stimulation.

A, $[Ca^{2+}]_i$ was measured in NHNE cells with basolateral or luminal applications of ATP. *LM*, luminal membrane; *BLM*, basolateral membrane. *B*, summarized results of NKCC measurements with basolateral(n=5) or luminal ATP stimulation(n=5). *C,* effects of basolateral P2R stimulation on NKCC was measured by comparing the pH decreases (ΔpH unit/min) due to intracellular NH_4^+ uptake in control and ATP-stimulated (BLM) conditions. To prevent Na^+,K^+ ATPase-mediated NH_4^+ uptake, ouabain (100 μM) was added

to the basolateral solutions. *D*, effects of luminal P2R stimulation on NKCC were measured using same protocols described in panel *C*, except that ATP was applied to the luminal side. *P<0.05: difference from control.

4. Effects of ion channel blockers and PKC inhibitors

Although we observed that stimulation of either luminal or basolateral P2R activated NKCC, we first analyzed the stimulatory mechanisms of basolateral P2R, which resides in the same membrane as NKCC. Previous studies have reported that various agonists can secondarily regulate NKCC activity by primarily modulating intracellular ionic compositions[5]. In particular, lowering $[Cl^-]_i$ by the activation of luminal Cl^- channels and the counter activation of basolateral K^+ channels has been suggested to be an important regulatory mechanism of NKCC[5,20,21]. Therefore, we tested whether the stimulatory effects of basolateral P2R can be inhibited by blockers of Cl^- or K^+ channels. To block luminal Cl^- channels, a mixture of the Ca^{2+}-activated Cl^- channel (CACC) inhibitor DIDS (100 μM) and of the CFTR inhibitor NPPB (30 μM) was applied to the luminal perfusates (Fig. 5C). As for the basolateral K^+ channels, a non-specific K^+ channel inhibitor Ba^{2+} (5 mM) was included in the basolateral perfusates (Fig. 5D). As shown in Fig. 5B, neither blockade of the Cl^- channel nor of the K^+ channel affected the stimulatory

effects of ATP on NKCC. In addition, the blockade of ENaC, which may affect NKCC activity by modulating the membrane potential or $[Na^+]_i$, by adding 100 μM amiloride to the luminal perfusates did not alter NKCC activity (not shown). Therefore, it was concluded that the increased NKCC activity in response to stimulation of basolateral P2R was due to the intrinsic activation of NKCC.

Recently, it was suggested that the α-adrenergic agonist methoxamine stimulates NKCC by activating protein kinase C (PKC), in particular, by activating a novel type PKC-δ isoform in Calu-3 cells originating from human lung adenocarcinoma[22]. The stimulation of P2R, especially the stimulation of G-protein- coupled P2Y receptors, can activate PKC. Therefore, we tested whether the stimulatory effects of P2R on NKCC are dependent on PKC activity in NHNE cells using two types of PKC inhibitors. Cells were pretreated for 30 min with either Go 6976 (500 nM), an inhibitor of classic PKC isoforms, or rottlerin (10 μM), an inhibitor of novel PKC isoforms. NKCC activity was then measured using the luminal and basolateral perfusates containing the same inhibitor concentrations. Unlike the findings observed in Calu-3 cells after methoxamine stimulation[22], the effects of P2R stimulation in NHNE cells were not inhibited either by Go 6976 or by rottlerin. Basolateral ATP-stimulated NKCC activity after Go 6976 treatment

was 0.462 ± 0.059 ΔpH unit/min (n=5), and after rottlerin treatment, it was 0.443 ± 0.055 (n=5). These values were not significantly different from the value of basolateral ATP stimulation presented in Fig. 4B (0.496 ± 0.075, see above).

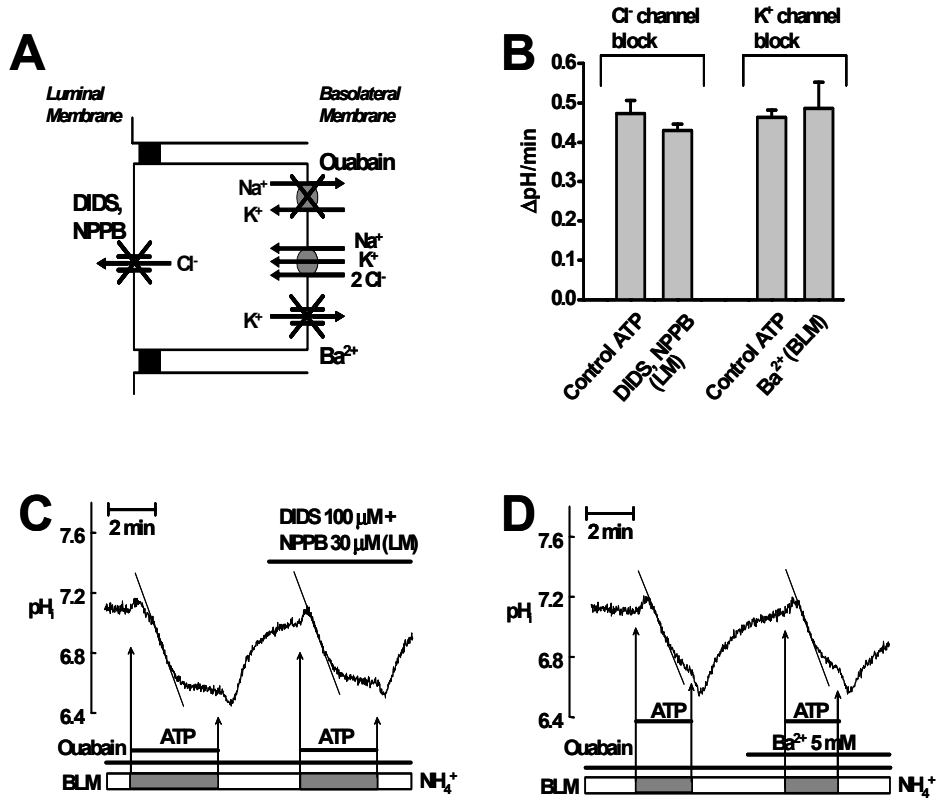

Fig. 5. Effects of Cl^- and K^+ channel blockers on NKCC activation by basolateral ATP.

A, a diagram of possible secondary activation of NKCC by activations of

luminal Cl^- channels and the counter activation of basolateral K^+ channels (see "Results" for details). *B*, summarized results of NKCC measurement with the blockers of Cl^- channels (n=5) and those of K^+ channels (n=4). *LM*, luminal membrane; *BLM*, basolateral membrane. *C,* the effects of Cl^- channels blockers were analyzed in basolaterally ATP-stimulated cells. To block luminal Cl^- channels, the mixture of CACC inhibitor DIDS (100 μM) and the CFTR inhibitor NPPB (30 μM) was applied to the luminal perfusates. *D,* effects of K^+ channel blockers. To block the basolateral K^+ channels, a non-specific K^+ channel inhibitor Ba^{2+} (5 mM) was added to basolateral perfusates.

5. The effects of basolateral P2R are dependent on $[Ca^{2+}]_i$

As shown in Fig. 4A, either luminal or basolateral ATP evoked a rapid increase in $[Ca^{2+}]_i$. Therefore, we investigated the role of calcium signals in NKCC activation. First, we measured the effects of $[Ca^{2+}]_i$ increases on NKCC using the Ca^{2+} ionophore ionomycin. As shown in Figs. 6A and C, ionomycin treatment (5 μM) acutely increased the basolateral NKCC activity by 98%. Next, we investigated whether the calcium signals are required for the P2R-induced activation of NKCC. Surprisingly, the chelation of intracellular Ca^{2+} by BAPTA-AM treatment (50 μM, for 30 min) almost completely blocked the activation of NKCC by basolateral P2R stimulation

(Figs. 6, B and C).

As depicted in Fig. 7A, stimulation of P2R caused a short-lived $[Ca^{2+}]_i$ peak followed by a small sustained $[Ca^{2+}]_i$ increase in NHNE cells. Of interest, treatment with basolateral ATP 3 min before the application of NH_4Cl solution did not increase the NKCC activity (Figs. 7, B and C). These results suggest that the P2R-induced stimulation on NKCC activity is highly correlated with $[Ca^{2+}]_i$.

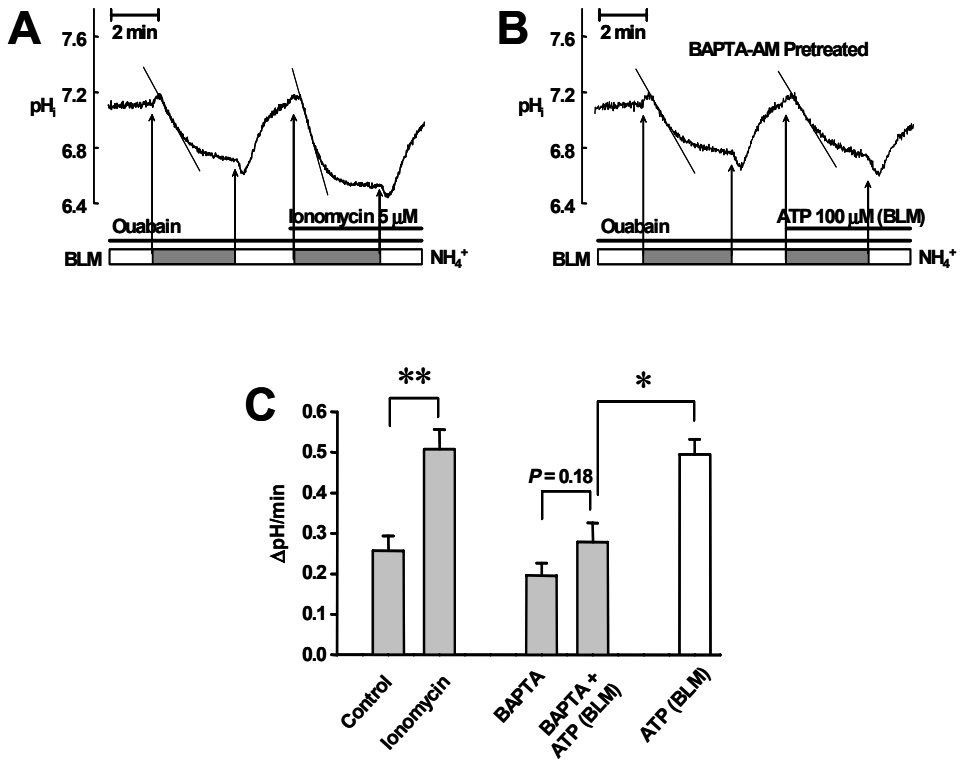

Fig. 6. Role of $[Ca^{2+}]_i$ in NKCC activation by basolateral ATP.

A, NKCC-mediated NH_4^+ transport was measured with ionomycin treatment (5μM). *BLM*, basolateral membrane. *B,* NKCC activities were measured in BAPTA-AM pretreated (50 μM, 30 min) cells. *C,* summarized results of ionomycin (n=5) and BAPTA (n=7) treatments. Basolateral ATP- stimulated NKCC activity without BAPTA treatment was adopted from Fig.4B. *P<0.05, **P<0.01, difference from control.

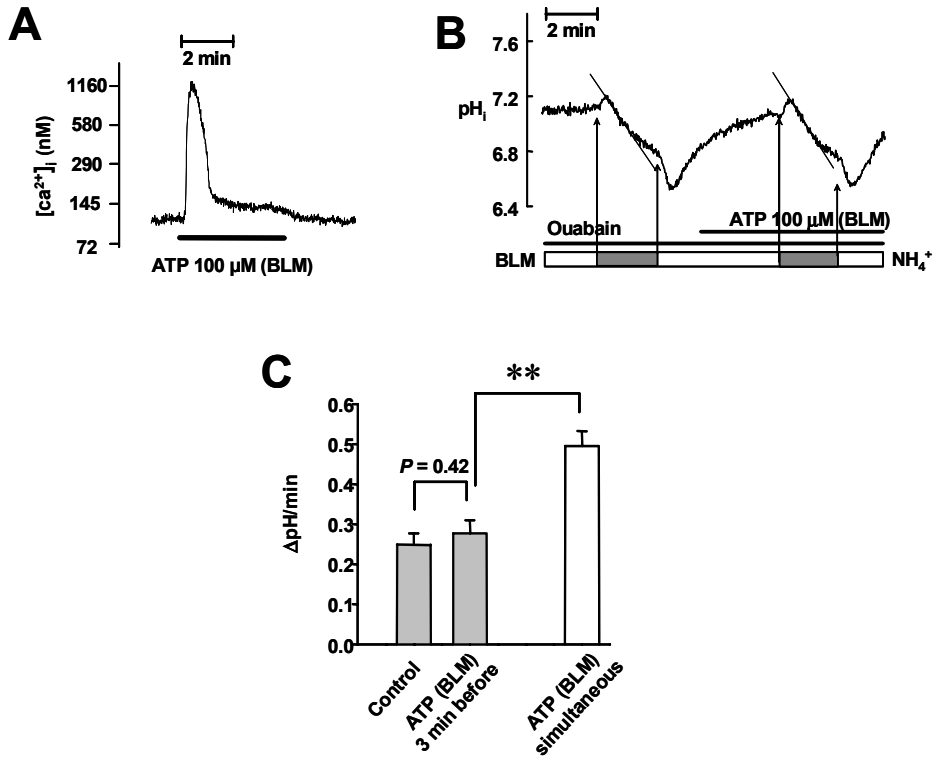

Fig. 7. Effects of long-term ATP stimulation on the NKCC activity.

A, $[Ca^{2+}]_i$ measurements with basolateral ATP for 4 min in NHNE cells. *BLM*,

basolateral membrane. *B*, basolateral ATP was applied 3 min before the NKCC measurement. *C*, a summary of the results (n=5). NKCC activity with simultaneous basolateral ATP treatment was adopted from Fig. 4B. **P<0.01: difference from control.

6. Characterization of the effects of luminal P2R on NKCC

The stimulatory effects of luminal P2R on NKCC were analyzed using protocols similar to those used for the investigation of basolateral P2R. As was observed with basolateral ATP, inhibition of ENaC and PKC did not significantly alter the effects of luminal ATP on NKCC (not shown). However, unlike that observed with basolateral ATP, the blockade of luminal Cl^- channels partially reduced the stimulatory effects of luminal ATP on NKCC (Figs. 8, A and B). The luminal ATP - stimulated NKCC activity was 0.532 ± 0.040 ΔpH unit/min, and this value was decreased to 0.401 ± 0.029 by luminal DIDS and NPPB. Finally, the effects of luminal ATP were measured in the BAPTA-pretreated cells. As illustrated in Figs. 8, C and D, the stimulatory effects of luminal ATP on NKCC were completely blocked by the chelation of intracellular calcium, as was observed in experiments with basolateral ATP.

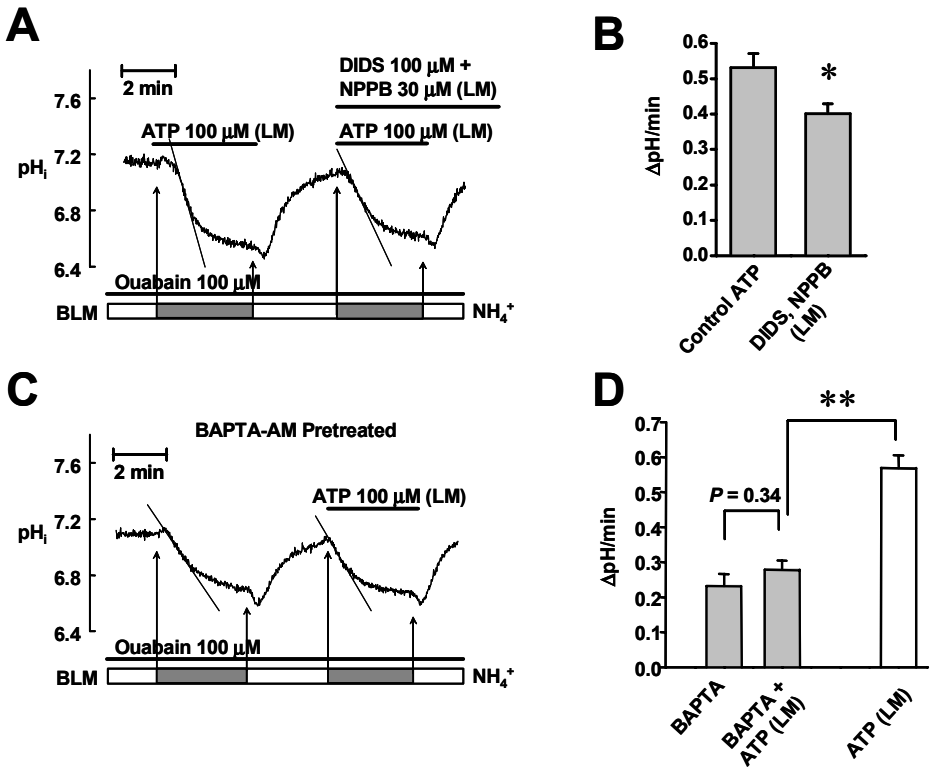

Fig. 8. Inhibition of luminal ATP-induced NKCC activation by Cl^- channel block and by $[Ca^{2+}]_i$ chelation.

A, effects of Cl^- channel blockers were analyzed in NHNE cells using the protocols described in the legend for Fig. 5 (panel B), except that the cells were stimulated with luminal ATP. *LM*, luminal membrane; *BLM*, basolateral membrane. *B,* a summary of Cl^- channel block on luminal ATP (n=5). *C,* the effects of $[Ca^{2+}]_i$ chelator BAPTA were analyzed in NHNE cells using the protocols described in the legend for Fig. 6 (panel B), except that the cells were stimulated with luminal ATP. *D,* a summary $[Ca^{2+}]i$ chelation on luminal

ATP (n=5). Luminal ATP -stimulation NKCC activity without BAPTA treatment was adopted from Fig. 4B. *P<0.05, **P<0.01, difference from control.

IV. DISCUSSION

Secretion of appropriate fluids is critical in the mucociliary clearance system, which protects the respiratory system from noxious substances. An interesting feature is that airway surface epithelium is capable of releasing ATP and UTP to the luminal space in response to a myriad of physiologic and pathologic stimuli. Moreover, neuroendocrine cells and mast cells along the airway also release ATP to the interstitial space, hence to the basolateral side of surface epithelium, with other agonists from their granules[3,23]. Therefore, purinergic signaling is thought to serve as an important regulatory mechanism of epithelial homeostasis, and to be involved in a number of pathologic conditions.

The present study provides sufficient and necessary evidence that purinergic agonists directly activate basolateral NKCC via calcium signaling in human nasal epithelia. Increased $[Ca^{2+}]_i$ by ionomycin treatment sufficiently activated the basolateral NKCC in NHNE cells (Fig. 6A). In addition, the finding that $[Ca^{2+}]_i$ chelation by BAPTA treatment completely

inhibited the P2R-induced effects (Fig. 6B and 8C) demonstrated that calcium signals are required for the P2R-induced activation of NKCC. Previously, it has been shown that an increase in $[Ca^{2+}]_i$ can modulate NKCC activity in several tissues including airway epithelia[5]. However, most of the studies have suggested that calcium signals do not directly modulate NKCC, but rather affect NKCC activity by secondarily modulating K^+ or Cl^- conductances[5,20,21]. On the other hand, the present findings that inhibitors of Cl^- or K^+ channels did not affect the stimulatory effects of basolateral ATP on NKCC (Fig. 5) and only partially inhibited those of luminal ATP (Fig. 8) strongly suggest that purinergic stimulation induced an intrinsic increase in NKCC activity.

One of the basic differences between the present study and previous studies is the method used for determining NKCC activity. We employed a technique that has a high temporal resolution by measuring the real-time changes in pH_i due to NKCC-mediated intracellular NH_4^+ uptake. Previous studies that have reported only the secondary effects of $[Ca^{2+}]_i$ used either [^{3}H] bumetanide binding or ^{86}Rb fluxes to determine NKCC activity and measured averaged NKCC activities after long term incubations with agonists[20,21]. For example, Haas *et al.*[20] reported that treatment with luminal ATP for 40 min increased [^{3}H] bumetanide binding by about 80% in canine tracheal epithelia and this increase was almost completely blocked by the Cl^-

channel blocker IAA-94. As shown in the present study, P2R stimulation evoked a short-lasting Ca^{2+} peak even in the continuous presence of ATP (Fig. 7). In native tissues, released ATP and UTP are rapidly washed away by the bulk of luminal or interstitial fluids and are instantaneously degraded by ecto-enzymes[3,23]. Therefore, repetitive short-lasting P2R stimuli rather than a continuous stimulation would be closer to actual physiological situations. In this regard, our present finding that a short-lasting Ca^{2+} peak induced by P2R acutely increases the intrinsic activity of NKCC may have important physiological implications.

A previous study on cholinergic regulation of NKCC in parotid acinar cells reported results that concur with ours (Evans and Turner[17]). These authors also determined NKCC activity by measuring pH_i decreases due to NKCC-mediated NH_4^+ uptake and found the acute up-regulation of intrinsic NKCC activity by the cholinergic agonist carbachol, which was completely inhibited by BAPTA but not by intracellular KCl loss or cell shrinkage. Recently, Brindikova *et al.*[24] reported that long term (30 min) purinergic stimulation inhibits NKCC-mediated ^{86}Rb fluxes in C11-MDCK cells but not in C7-MDCK cells. However, short term (2 min) purinergic stimulation acutely increased the NKCC activities in both MDCK cell lines, and this was completely inhibited by BAPTA treatment[24]. Together with the present

findings, the above results suggest that the acute activation of NKCC by transient increases in $[Ca^{2+}]_i$ do not seem to be a phenomenon specific to NHNE cells and that it may be a ubiquitous feature in epithelial tissues.

It is widely accepted that phosphorylation of NKCC1 is related to the increased activity of NKCC1[25,26]. Activities of various kinases such as PKA, PKC and of an unidentified kinase responding to $[Cl^-]_i$ were suggested to be associated with NKCC1 phosphorylation and activation[5,26]. In fact, it was once reported that the activity of a novel PKC-δ isoform was required for the methoxamine-induced activation of NKCC1 in Calu-3 cells[22]. However, the present results obtained using PKC inhibitors suggested that the classical and novel isoforms of PKC are not involved in the P2R-induced acute activation of NKCC in NHNE cells. The molecular mechanisms underlying P2R- and Ca^{2+}-induced activation of NKCC1 require further study.

Previously, we showed that P2R signals evoke the membrane-specific activation of ion transporters in polarized epithelial tissues[27,28]. In the present study, the stimulatory effects of luminal ATP on NKCC were partially inhibited by the blockade of luminal Cl^- channels (Fig. 8), whereas the stimulatory effects of basolateral ATP were not (Fig. 5). Since the effects of luminal ATP were completely inhibited by chelation of intracellular Ca^{2+}, Cl^- channels responding to $[Ca^{2+}]_i$ such as CACC are responsible, at least in part,

for the luminal ATP-induced activation of NKCC. The finding that luminal Cl^- channels are activated only by luminal ATP but not by basolateral ATP supports the idea of compartmentalized calcium signals in epithelial cells, as suggested by ourselves and by other researchers[11, 27-29].

V. CONCLUSION

In conclusion, we observed that the stimulation of P2R acutely activated NKCC in NHNE cells. The apical surfaces of airway tract are moistened by airway surface fluid that drives from the transcellular transport of anions, especially Cl^- ions. Since the activity of basolateral NKCC, which keeps $[Cl^-]_i$ at the levels above the predicted equilibrium potential, is actually the rate-limiting step in epithelial fluid secretion, the results described here add significantly to our understanding of airway secretions in physiologic and pathologic conditions. For example, NKCC activation due to ATP released by activated mast cells may contribute to increased nasal secretions in allergic rhinitis.

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Abstract (in Korean)

정상 사람 코점막 상피세포에서 Na^+-K^+-2Cl^- Cotransporter 의 발현과 기능

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여러 연구에서 정상 사람 코점막 상피세포에서 퓨린성 수용체(P2Rs)가 점액분비의 autocrine과 paracrine 조절에 있어서 결정적인 역할을 한다는 증거가 발견되었다. 본 연구에서 우리는 P2R 의 자극이 정상 사람 코점막 상피세포의 Na^+-K^+-2Cl^- Cotransporter의 활성에 미치는 영향을 밝혀보았다. 일차 배양한 정상 사람 코점막 상피세포의 세포 내 칼슘이온의 양과 pH는 세포막 특이적 이온 수송체의 활성을 분석할 수 있는 이중관류 chamber 를 이용하여 측정하였다. NKCC 의 기능은 Na^+의존적이며 bumetanide 에 민감한 세포 내 NH_4^+ 의 유입에 의한 세포 내 pH 의 감소를 통해 예측할 수 있다. NKCC의 기능은 정상 사람 코점막 상피세포의

기저외측막에서 관찰되었으나 관내강막 에서는 관찰되지 않았다. 흥미롭게도 퓨린성 수용체는 기저외측막과 관내강막 모두에서 발현하며 어떤 세포막의 퓨린성 수용체를 자극하더라도 NKCC 를 활성화시킨다. 관내강막 Cl^- 채널과 기저외측막 K^+ 채널, potein kinase C 의 억제는 기저외측막 퓨린성 수용체의 자극에 의한 NKCC 활성에 영향을 주지 않았으나 관내강막의 퓨린성수용체 자극에 의한 효과는 Cl^- 채널 억제제에 의해 부분적으로 감소하였다. 반면 BAPTA의 처리로 유도된 세포 내 칼슘이온의 킬레이트화는 기저외측막과 관내강막 모두에서 퓨린성 수송체의 효과에 의한 NKCC 자극을 완전히 차단한다. 또한 ionomycin 처리에 의한 세포 내 칼슘이온양의 증가는 NKCC의 기능을 활성화시킨다. 이상의 결과는 정상 사람 코점막 상피에서 퓨린성 수용체의 자극이 칼슘의존적 기저외측막 NKCC를 직접적으로 활성화시킨다는 것을 뒷받침하며, 이는 정상 사람 코점막 상피의 생리적, 병리적 상황에서 퓨린성 수용체에 의한 이온 및 점액분비의 조절 이라는 측면에서 중요성을 가진다.

핵심되는 말: 정상 사람 코점막 상피세포, 퓨린성 수용체, Na^+-K^+-$2Cl^-$ cotransporter, 기저외측막, 관내강막