INTRODUCTION

Nasal polyps are a prevalent medical condition, affecting 1-4% of the population. Controversies persist regarding the management of nasal polyps because the exact etiology remains unclear. Corticosteroids are beneficial in reducing the size of polyps and preventing recurrences, but proper control of nasal polyposis is a challenging task in clinical situations. It has been reported that the recurrence rate is as high as 60% in patients with severe nasal polyposis (1). Nasal polyps are characterized by stromal edema and increased inflammatory cells. Hyperabsorption of Na+ and increased Cl– permeability may account for stromal edema formation (2).

There are two main families of purinergic receptors (adenosine and P2 receptors). The P2 receptors can in turn be divided into two families (ligand-gated P2X and the G protein-coupled P2Y receptors). To date, seven P2X receptors (P2X1-7) and eight P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11-14) have been cloned and characterized, and are accepted as valid members in mammals (3). The activation of chloride secretion via purinergic receptors has been reported in the ciliated respiratory epithelial cells cultured from nasal polyps (4). On the other hand, there are reports that the nitric oxide (NO) inhibits Na+ absorption in rat cortical collecting ducts (5) and rat distal lung epithelium (6), while NO activates the cystic fibrosis transmembrane conductance regulator (CFTR) activator (genistein), nitric oxide substrate (L-arginine), and nitric oxide donor (sodium nitroprusside) had no significant effect on the short circuit current. (7)

OBJECTIVES. To examine possible modulators of the ion transport through the apical membrane of the nasal polyps.

METHODS. The study was conducted using the freshly-excised nasal polyps from the patients with chronic sinusitis. A voltage-sensitive vibrating probe technique was introduced to monitor the short-circuit current across the apical membrane of the polyp at 37°C.

RESULTS. In the presence of amiloride, Adenosine 5′-triphosphate induced 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid-sensitive chloride current. Uridine 5′-diphosphosphate was less potent than Uridine 5′-triphosphate, and adenosine increased chloride secretion, which was blocked by the antagonist, 8-(p-sulfophenyl) theophylline on adenosine receptor. Based on the pharmacologic profiles, multiple purinergic receptors, including P2Y1, P2Y6, and P1 receptors, were functionally expressed. However, P2X receptor agonists (α, β-methyleneadenosine 5′-triphosphate and 2′ & 3′-O-[4-benzoyl-benzoyl] adenosine 5′-triphosphate), Cystic fibrosis conductance regulator (CFTR) activator (genistein), nitric oxide substrate (L-arginine), and nitric oxide donor (sodium nitroprusside) had no significant effect on the short circuit current.

CONCLUSION. Among tested drugs, P2Y receptor agonists were major modulators of ion transport in nasal polyps in situ.

KEY WORDS. Nasal polyps, CFTR, Purinergic receptors, Genistein, Nitric oxide, Vibrating Probe
MATERIALS AND METHODS

The polyps were freshly obtained from patients with chronic sinusitis during endoscopic sinus surgery and were immediately delivered to the laboratory in cold physiologic saline. No local anesthetic agents were introduced before extirpating the polyps. The submucosa of the polyp was removed under a stereomicroscope and the tissue was divided into several small pieces. The tissue was folded with the apical membrane of the surface ciliated epithelium facing outward and transferred into a recording chamber. Our Institutional Committee approved the use of these tissues with informed consent.

The vibrating probe is a technique for measuring extracellular electrical currents that are steady or slowly changing. The diameter of the vibrating probe tip is about 20 \( \mu \text{m} \) and allows the detection of voltages in the low nanovolt range; vibration between two positions within the line of current flow yields voltages that correspond to current flow through resistive physiologic saline. The technique has been described in detail elsewhere (10). Briefly, the short circuit current (Isc) was monitored by vibrating a platinum-iridium wire microelectrode that was insulated with parlene-C (Micro Electrodes, Gaithersburg, MD, USA) and which had been coated with platinum black on the exposed tip. The vibration was about 20 \( \mu \text{m} \) along both a horizontal (X) and vertical (Z) axis. The X-axis was perpendicular to the face of the epithelium. The probe was positioned 30-40 \( \mu \text{m} \) from the apical surface of the epithelium with computer-controlled, stepper-motor manipulators (Applicable Electronics, Forestdale, MA, USA) and specialized probe software (ASET, version 2.0, Science Wares, East Falmouth, MA, USA). The bath references were 26-gauge platinum-black electrodes. Calibration was performed in physiologic saline (see below) using a glass microelectrode (tip, < 1 \( \mu \text{m} \) OD) filled with 3 M KCl as a point source of the current. The frequencies of vibration were in the range of 300-400 Hz and were well-separated for the two orthogonal directions. The signals from the oscillators driving the probe were also fed to a dual channel phase-sensitive detector. The signal of the X and Z detectors were connected to a 16 bit analog to digital converter (CIO-DAS1602/16, ComputerBoards, Mansfield, MA, USA) in a Pentium IV computer. The sampling interval was 0.6s, which is the minimum in this software. The electrode was positioned such that Isc showed a maximum X value and a minimum Z value; data are expressed as the vector length of the current density and plotted with Origin software, version 6.1 (OriginLab Software, Northampton, MA, USA). The output from the vibrating probe depends not only on the specific short circuit current of the epithelium, but also on the position of the probe from the surface of the tissue and the exact geometry of each tissue sample. The current density reported here refers to the flux at the position of the probe and represents only a fraction of the current crossing the epithelium. No changes in the relative position of the probe due to swelling or shrinking of the tissue during experimental treatments were observed.

In all experiments, both sides of the polyp were perfused with physiologic saline containing (in mM) 130 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 5 glucose, and 10 HEPES (pH 7.4). Adenosine 5’-triphosphate (ATP), uridine 5’-triphosphate (UTP), 2’- and 3’-O-(4-benzoyl-benzoyl)adenosine 5’-triphosphate (BzATP), \( \alpha \beta \)-methyleneadenosine 5’-triphosphate \( \alpha \beta \text{meATP} \), 4,4-diisothiocyanatostilbene-2,2’-disulfonic acid disodium salt hydrate (DIDS), L-arginine, sodium nitroprusside (SNP), and 8-(p-sulphophenyl) theophylline (8-SPT) were directly dissolved in physiologic saline just before use. Uridine 5’-diphosphate (UDP) was pre-incubated for 1.5 h with hexokinase (1 unit/mL) and glucose (5 mM) because the commercial preparations of UDP may be supplied with a minor component of UTP (11). Amiloride, N-phenylanthranilic acid (DPC), genistein, and ouabain were dissolved in DMSO and then diluted to 0.1% DMSO in the control solution prior to application. DMSO at this concentration had no effect on the Isc.

Each tissue was mounted in a perfusion chamber on the stage of an inverted microscope and continuously perfused at 37°C at an exchange rate of 0.7 times/sec. Ouabain (10 \( \mu \text{M} \)), an inhibitor of Na\(^+\), K\(^-\)-ATPase, was tested to estimate the time for the perfusate to migrate to the basolateral membrane of the surface epithelium. Application of ouabain decreased the Isc from 8.6 \( \pm \) 2.5 \( \mu \text{A/cm}^2 \) (n=15), which indicates that the ion channel modulators used here acted in a short time on the apical membrane of surface epithelium.

For analysis, the peak Isc was chosen. Data are expressed as the mean±S.E.M. (n=number of tissues) of the Isc. An increase or decrease in Isc were considered significant at a level of P<0.05. A paired t-test was used.

RESULTS

The Isc measured in physiologic saline was -124.0 \( \pm \) 17.0 \( \mu \text{A/cm}^2 \) (n=33). Perfusion of amiloride (100 \( \mu \text{M} \)), an epithelial sodium channel (ENaC) blocker, significantly decreased the Isc by 78.7 \( \pm \) 3.1% (to -18.8 \( \pm \) 2.6 \( \mu \text{A/cm}^2 \), n=33). In the presence of amiloride, DIDS (1.5 mM) slightly decreased the Isc by 11.1 \( \pm \) 2.5% (n=17) and subsequent application of DPC (0.5 mM) significantly decreased the Isc by 88.0 \( \pm \) 2.6% (n=17, Fig. 1A). In the presence of amiloride, 100 \( \mu \text{M} \) ATP significantly increased the Isc by 460.1 \( \pm \) 171.8% (n=7), which was inhibited by DPC (92.1 \( \pm \) 1.8%), but not by DIDS (17.9 \( \pm \) 6.7%, Fig. 1B).

ATP and UTP increased the Isc in a dose-dependent manner (Fig. 2). The EC50 of ATP and UTP was 5.3 \( \pm \) 0.3 (n=5) and 7.7 \( \pm \) 0.8 \( \mu \text{M} \) (n=5), respectively. UDP, a P2Y receptor agonist, also increased the Isc to 44.5 \( \pm \) 8.9% of the UTP response (n=5, Fig. 3A). Adenosine, a purinergic receptor type 1 (P1) receptor agonist, increased the Isc by 340.1 \( \pm \) 167.9% (from -21.4 \( \pm \) 6.6
to -50.0±5.5 μA/cm², n=7, Fig. 3B). In the presence of 100 μM 8-SPT, a P1 receptor blocker, the response of adenosine was decreased by 90.0±3.7% (from -50.0±5.5 to -23.4±6.5 μA/cm², n=7, Fig. 3B).

P2X receptor agonists and other modulators for ion transport were introduced to test the effects on polyps (Fig. 4). The addition of αβmeATP and BzATP (100 μM) had no significant response in the presence of amiloride (from -13.2±4.5 to -13.9±3.9 μA/cm² and -12.7±2.5 to -12.4±2.5 μA/cm², respectively, n=5 each, Fig. 4A). The addition of genistein (20 μM), the CFTR
activator (12), had no effect on the $I_{sc}$ (from -14.1 ± 2.5 to -14.4 ± 2.9 μA/cm², n=6, Fig. 4A). Perfusion of L-arginine (1 mM, substrate for nitric oxide synthase, n=5) and sodium nitroprusside (1 mM, nitric oxide donor, n=7) showed no response in the absence (-101.7 ± 20.7 μA/cm² to -95.8 ± 22.3 μA/cm², -122.1 ± 19.6 μA/cm² to -118.8 ± 20.5 μA/cm², respectively) and presence (from -19.8 ± 4.6 to -18.9 ± 4.3 μA/cm², -18.8 ± 4.6 to -18.9 ± 4.3 μA/cm², respectively, Fig. 4B) of amiloride.

DISCUSSION

The $I_{sc}$ measured in polyps largely represents the current from the ciliated epithelial cells because the superficial epithelium of the polyp is characterized by ~80% ciliated cells (13). The blockade of Na$^+$ absorption by amiloride is consistent with a previous report involving nasal polyps in situ (14). In the presence of amiloride, DIDS had little effect on the $I_{sc}$, but DPC abolished the $I_{sc}$. Although DIDS and DPC are non-specific anion channel blockers, current pharmacology indicates that DIDS does not act on the CFTR chloride channel, even at high concentrations (15), and DPC induces a voltage-dependent blockade of CFTR when used at micromolar concentrations (16). Therefore, after the addition of amiloride the $I_{sc}$ is likely to be dominated by CFTR-mediated chloride conductance. ATP strongly stimulated DPC-sensitive chloride secretion and its potency was similar to UTP. This is consistent with the activation of the human P2Y2 receptor (17) because ATP acts as a competitive antagonist on the human P2Y4 receptor (18). The EC50 of ATP and UTP measured in this study was 5.3 and 7.3 μM, respectively, which was similar to the EC50 reported in cultured cells in terms of anion transport (ATP, 1.0 μM) (19) and ciliary beat frequency (UTP,
4.7 μM (20). UDP, the principal agonist for P2Y<sub>6</sub>, increased the chloride secretion to 44% of the UTP response, which is similar magnitude in magnitude in terms of ciliary beat frequency (20). This finding indicates that the P2Y<sub>6</sub> receptor is likely to be active. Besides, we have eliminated the possibility of UTP contamination from UDP preparation by addition of hexokinase and glucose. This point was not clearly noted in the previous reports concerning P2Y<sub>6</sub> characterization in the airway epithelium (20). Another P1 purinergic agonist, adenosine, also activated chloride secretion, which was inhibited by a non-specific adenosine receptor blocker, 8-SPT (21).

We examined further how much P2X receptors are functionally implicated in nasal polypl using αβmeATP and BzATP. The αβATP is an agonist for P2X<sub>1</sub> and P2X<sub>3</sub>, and BzATP is for P2X<sub>1</sub>, P2X<sub>3</sub>, and P2X<sub>7</sub> (22). P2X receptors could not be detected pharmacologically, which indicates that P2X receptors do not have a significant role compared to P2Y receptors. This is contrary to the report which described these agonists having large responses in lung-derived cultured cells (23). The CFTR activator, genistein (24), did not induce a CFTR current, which may be attributed to an altered localization of CFTR in the nasal polyp (25). If NO, generated by the application of NO substrate (L-arginine) or NO donor (SNP), has a regulatory effect on the CFTR or ENaC, it can serve as a medical, therapeutic strategy for nasal polyps. However, there was no beneficial effect at high concentrations of L-arginine and SNP, both in the absence and presence of amiloride in nasal polyps, which is similar to the observation in cultured human nasal epithelium (26).

In conclusion, the multiple P2Y receptors were functioning at the apical membrane of the nasal polyp in situ. However, P2X agonists, the CFTR activator, and NO-producing drugs did not have a beneficial effect on nasal polyps in situ.

REFERENCES

1. Wynn R, Har-El G. Recurrence rates after endoscopic sinus surgery for massive sinus polyposis. Laryngoscope. 2004 May;114(5):811-3.
2. Bernstein JM, Yankaskas JR. Increased ion transport in cultured nasal polyp epithelial cells. Arch Otolaryngol Head Neck Surg. 1994 Sep;120(9):993-6.
3. Boeynaems JM, Communi D, Gonzalez NS, Robaye B. Overview of the P2 receptors. Semin Thromb Hemost. 2005 Apr;31(2):139-49.
4. Lazarowski ER, Tarran R, Grubb BR, van Heusden CA, Okada S, Boucher RC. Nucleotide release provides a mechanism for airway surface liquid homeostasis. J Biol Chem. 2004 Aug 27;279(35):36855-64.
5. Stoos BA, Garcia NH, Garvin JL. Nitric oxide inhibits sodium reabsorption in the isolated perfused cortical collecting duct. J Am Soc Nephrol. 1995 Jul;6(1):89-94.
6. Compeau CG, Rotstein OD, Tohda H, Maranaka Y, Rafii B, Slutsky AS, et al. Endotoxin-stimulated alveolar macrophages impair lung epithelial Na+ transport by an L-Arg-dependent mechanism. Am J Physiol. 1994 May;266(5 Pt 1):C1330-41.
7. Dong YJ, Chao AC, Koutaya K, Hsu YP, Bocian RC, Moss RB, et al. Activation of CFTR chloride current by nitric oxide in human T lymphocytes. EMBO J. 1995 Jun 15;14(12):2709-7.
8. Ramis I, Lorente J, Roselló-Catafau J, Quersada P, Geli E, Bulbena O. Differential activity of nitric oxide synthase in human nasal mucosa and polyps. Eur Respir J. 1996 Feb;9(2):202-6.
9. Kang BH, Huang NC, Wang HW. Possible involvement of nitric oxide and peroxynitrite in nasal polyposis. Am J Rhinol. 2004 Jul-Aug;18(4):191-6.
10. Jaffe LF, Nuccitelli R. An ultrasonic vibrating probe for measuring steady extracellular currents. J Cell Biol. 1974 Nov;63(2 Pt 1):614-28.
11. Nicholas RA, Watt WC, Lazaronowski ER, Li Q, 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. 1996 Aug;50(2):224-9.
12. Al-Nakkash L, Hu S, Li M, Hwang TC. A common mechanism for cystic fibrosis transmembrane conductance regulator protein activation by genistein and benzimidazolone analogs. J Pharmacol Exp Ther. 2001 Feb;296(2):464-72.
13. Boucher RC, Stutts MJ, Knowles MR, Cantley L, Gatzy JT. Na+ transport in cystic fibrosis respiratory epithelia. Abnormal basal rate and response to adenylylcyclase activation. J Clin Invest. 1989 Nov;78(5):1245-52.
14. Knowles MR, Stutts MJ, Spock A, Fischer N, Gatzy JT, Boucher RC. Abnormal ion permeation through cystic fibrosis respiratory epithelium. Science. 1983 Sep 9;221(4615):1067-70.
15. Stutts MJ, Fitz JG, Paradiso AM, Boucher RC. Multiple modes of regulation of airway epithelial chloride secretion by extracellular ATP. Am J Physiol. 1994 Nov;267(5 Pt 1):C1442-51.
16. McCarty NA, McDonough S, Cohen BN, Rondan JR, Davidson N, Lester HA. Voltage-dependent block of the cystic fibrosis transmembrane conductance regulator Cl- channel by two closely related arylyaminobenzoates. J Gen Physiol. 1993 Jul;102(1):1-23.
17. Nicholas RA, Lazaronowski ER, Watt WC, Li Q, Boyer J, Harden TK. Pharmacological and second messenger signalling selectivities of cloned P2Y receptors. J Auton Pharmacol. 1996 Dec;16(6):319-23.
18. Kennedy C, Qi AD, Herold CL, Harden TK, Nicholas RA. ATP, an agonist at the rat P2Y(4) receptor, is an antagonist at the human P2Y(4) receptor. Mol Pharmacol. 2000 May;57(5):926-31.
19. Paradiso AM, Ribeiro CM, Boucher RC. Polarized signaling via purinoceptors in normal and cystic fibrosis airway epithelia. J Gen Physiol. 2001 Jan;117(1):53-67.
20. Morse DM, Smullen JL, Davis CW. Differential effects of UTP, ATP, and adenosine on ciliary activity of human nasal epithelial cells. Am J Physiol Cell Physiol. 2001 Jun;280(6):C1485-97.
21. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev. 1998 Sep;50(3):413-92.
22. Khakh BS, Burnstock G, Kennedy C, King BF, North RA, Séguela P, et al. International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. Pharmacol Rev. 2001 Mar;53(1):107-18.
23. Taylor AL, Schwiebert LM, Smith JJ, King C, Jones JR, Sorscher EJ, Schwiebert EM. Epithelial P2X purinergic receptor channel expression and function. J Clin Invest. 1999 Oct;104(7):875-84.
24. Al-Nakkash L, Hu S, Li M, Hwang TC. A common mechanism for cystic fibrosis transmembrane conductance regulator protein activation by genistein and benzimidazolone analogs. J Pharmacol Exp Ther. 2001 Feb;296(2):464-72.
25. Jang YJ, Lee JH et al.: Localization of cystic fibrosis transmembrane conductance regulator in epithelial cells of nasal polyps and postoperative polypoid mucosa. Acta Otolaryngol. 2001 Jan;121(1):93-7.