Interferon-γ activates outwardly rectifying chloride channels in the human bronchial epithelial cell line BEAS-2B

Ning Ge*, Youichi Nakamura*, Yutaka Nakaya†, and Saburo Sone*

*Third Department of Internal Medicine, and †Department of Nutrition, The University of Tokushima School of Medicine, Tokushima Japan

Abstract: The mechanism of increased chloride currents by inflammatory cytokine, interferon-gamma (IFN-γ), was investigated in cultured a human bronchial epithelial cell line (BEAS-2B) using cell-attached and inside-out patch configurations. The channel sensitive to chloride ion was activated by forskolin, an activator of adenylate cyclase, or 100 μM dibutyryl 5'-cyclic monophosphate in cell-attached configurations. The conductance of this channel was 40 ±4 pS in symmetrical 150 mM chloride solution between membrane potentials of 0 to +50 mV, and this channel was blocked by 500 μM 4,4'-diisothiocyanatostilbene-2, 2'-disulfonic acid (DIDS), suggesting that this channel was an outwardly rectifying chloride channel (ORCC). Treatment of 10-1000 U/ml IFN-γ for 3 hours, but not IFN-α, significantly increased channel activities of ORCC, and this activation was observed at least 24 hours after treatment. Erythromycin, a macrolide antibiotic, at a concentration of 100 μM inhibited the activation of ORCC induced by IFN-γ. The findings of the present study indicate that increased mucus secretion during inflammation might be partly due to activation of chloride permeability by cytokine and erythromycin might improve oversecretion of mucus from bronchial epithelium by blocking ORCC. J. Med. Invest. 48 : 97-101, 2001

Keywords : *chloride channel ; epithelial cell ; outwardly rectifying chloride channel, cystic fibrosis transmembrane conductance regulator*

INTRODUCTION

Infection and inflammation increases mucus production, but a large amount of sputum may cause air flow limitation and recurrent respiratory infection due to impaired mucociliary transport functions (1). The increased secretion of fluid is partly due to the increased permeability to chloride ions (2). Previous studies showed the presence of at least three types of chloride channels in epithelial cells, i.e. cystic fibrosis transmembrane conductance regulator (CFTR) (3, 4), outwardly rectifying chloride channels (ORCC) (5) and stretch-induced chloride channels (6). Transepithelial chloride fluxes are determined mainly by the activity of chloride channels, the ORCC and the CFTR, located in the apical membrane of airway epithelial cells (7). In cystic fibrosis, the CFTR is genetically defective, and transepithelial electrolyte transport in the airway epithelium becomes inadequate resulting in impaired mucociliary clearance and ultimately respiratory failure (8). Therefore, much attention has been paid to the CFTR. However, there have been few studies on ORCC and its characteristics have not been clarified completely. The present study aimed to clarify the ionic mechanism of increased chloride secretion by the inflammatory cytokine, interferon-gamma (IFN-γ). IFN-γ is a pleiotropic cytokine that has a large number of immunologic and non-immunologic functions, which may play an important role in respiratory pathological conditions. Cytokines are also known to modulate ionic currents (9-11). However, there have been no studies on the effect of cytokines, including IFN-γ on chloride channel activities of bronchial epithelial cells. The purpose of the present study was

Received for publication December 25, 2000 ; accepted January 19, 2001.

Address correspondence and reprint requests to Saburo Sone, MD., Third Department of Internal Medicine, The University of Tokushima School of Medicine, Kuramoto-cho, Tokushima 770-8503, Japan and Fax : +81-88-633-7113.

to evaluate the effects of IFN-γ on the ORCC, which participates in the transepithelial chloride fluxes. The results of the study might explain the mechanism of excess mucus secretion in various pathological conditions in airway tracts.

MATERIALS AND METHODS

Isolation and culture of human bronchial epithelial cells

A human bronchial epithelial cell line (BEAS-2B) transformed with SV40 virus was purchased from the American Type Culture Collection (Rockville, MD). The cells at passages 43-45 were used for experiments. The cells were plated in tissue culture plates with 16 mm diameter wells coated with type 1 collagen gel (Vitrogen 100 ; Celtrix, Santa Clara, CA), and incubated in LHC9/RPMI-1640 medium with gentamycin at 37°C, under 5% CO_2 in air. The medium was changed after 24 hr and then every other day. When the cells reached confluency, they were replated on new dishes with cover slips coated with type 1 collagen gel. Cells grown on the cover slips were used for experiments. At the same time, they were confirmed to be entirely epithelial cells by their positive staining by the avidin-biotin complex method (ABC kit ; VECTOR Lab., Burlingame, CA). No cells reacted with monoclonal murine anti-swine vimetin antibody (DAKO-Vimetin ; DAKO. Santa Barbara, CA), which was demonstrated to stain fibroblast IMR-90 cells provided by Tokyo Metropolitan Institute of Gerontology.

Electrical recording

The cover slips with cultured human bronchial epithelial cells (BEAS-2B) were transferred to an organ bath on an inverted microscope. Membrane currents were recorded in inside-out and cell-attached configurations with a patch clamp amplifier (EPC7 List Medical Electronics, Darmstat, Germany), as described by Hamill *et al*. (12). Patch electrodes were pulled from capillary glass tubes using a two-stage puller (PP-83, Narishige, Tokyo, Japan), coated with sylgard. The electrical resistance of the patch pipette was 5-7 MΩ for single channel recordings. Experiments were conducted at a temperature of 35-37°C. The patch electrodes were maneuvered to the cellular membrane surface, and, when contact was made, negative pressure was applied until a gigaohm-seal was formed. Measurements were filtered and stored on the hard disk of a personal computer for subsequent analysis.

Solutions and chemical

In cell-attached and inside-out patches, the pipette contained 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM Ca^{2+} and 10 mM 3-(N-morpholino) propane sulfonic acid (MOPS) (Sigma Chemical Co. St. Louis, USA), pH 7.2. In inside-out patches, the bath contained a similar solution except for10^{-8} M Ca^{2+}. The standard bath solution for the cell-attach patches was modified normal Tyrode's solution of the following composition (in mM) ; 137 NaCl, 2.7 KCl, 7.5 MOPS, 1.2 $CaCl_2$, 1 $MgCl_2$, 11.5-glucose, pH 7.2. To create a 50 mM concentration of chloride, equimolar chloride was replaced by Na_2SO_4, pH 7.2. Dibutyryl 5'-cyclic monophosphate, forskolin, and 4,4'-diisothiocyanatostilbene-2, 2'-disulfonic acid (DIDS) were purchased from Sigma Chemical Co. (St. Louis, USA).

Data analysis

The data acquisition and analysis of single-channel currents was conducted using pCLMP version 6.0 (Axon Instruments, Foster City, CA). The open probability (NP_0) was determined from current amplitude histograms and was calculated using the following equation :

$$NP_0 = \Sigma\ (n \times P_n)$$

where n is the number of channels in the patch and P_n is the integrated channels opening. Values are presented as means ± SE. Paired and unpaired *t* tests were used to study significance between two groups. $P<0.05$ was considered to be significant.

RESULTS

Characterization of the chloride channels

In cell-attached configurations, un-stimulated epithelial cells did not show any current activities. When 5'-cyclic monophosphate (db-cAMP), was added to the bathing solution, channel activities were observed in 8 of 10 cells (Fig. 1b). Similar findings were obtained using 100 μM forskolin (n=6). The open probability was increased from 0.01±0.02 to 0.22±0.10 (n=8, $p<0.05$). A similar current was observed in inside-out configurations without stimulation and Fig. 1c shows typical currents recorded and the I-V relation from inside-out patches. The slope conductance was 40± 4 pS between a membrane potential of -0 and + 50mV with symmetrical 150 mM chloride solution (n=6). The reversal potential was 0 mV. Reducing the chloride concentration to one third by replacement with SO_4^- caused the I-V relation to shift by −25 mV, suggesting that this channel was relatively selective to

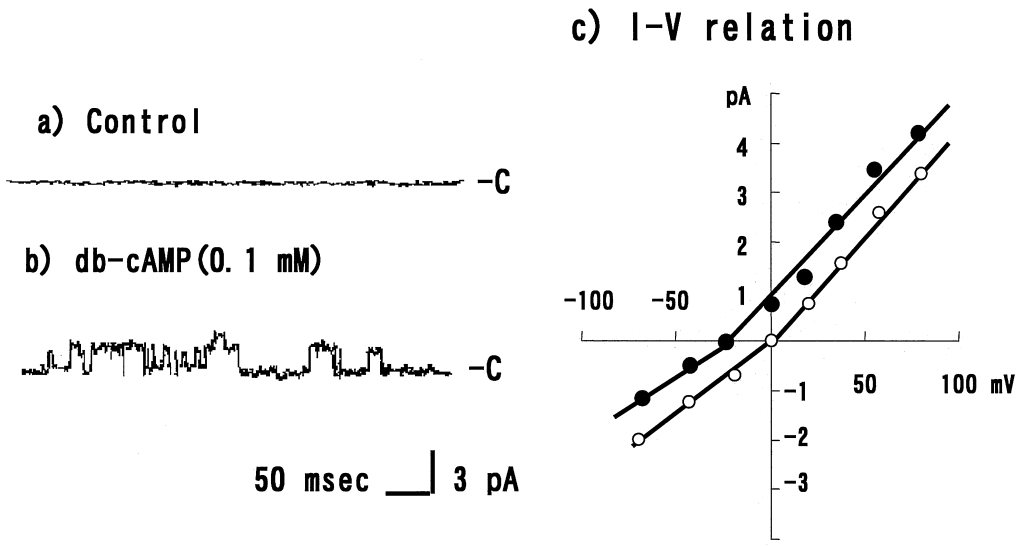

Fig.1. Single channel current of outwardly rectifying chloride channel (ORCC). Administration of db-cAMP activated ORCC (a to b) in cell-attached configurations at a pipette voltage of -50mV (membrane potential= resting potential plus 50 mV). Bath solution was normal Tyrode's solution and the pipette contains 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM Ca^{2+} and 10 mM 3-(N-morpholino) propane sulfonic acid (MOPS). Panel c shows current-voltage relationship of this channel in inside-out configurations. Open circle shows that of 150 mM symmetrical chloride solutions and closed circle shows that of 150/50 mM chloride solutions (pipette/bath). C indicates closed level in this and other figures.

chloride. Similar findings were obtained when KCl and K-aspartate solution were used (not shown). Two types of cAMP sensitive chloride channels are reported, i.e. cystic fibrosis transmembrane conductance regulator (CFTR) and outwardly rectifying chloride channels (ORCC). To identify whether this channel is ORCC or CFTR, we tested the effect of 4,4'-diisothiocyanatostilbene-2, 2'-disulfonic acid (DIDS), a chloride channel blocker sensitive to ORCC. The channel activities were inhibited by 500 μM DIDS, from 0.42±0.08 to 0.02±0.05 (n=6, $p<0.05$, n=4). The above observations show that epithelial cells express a chloride channel, which is usually infrequent in the cell-attached recording configurations and can be activated by the formation of inside-out patches or when stimulated by cAMP.

Effect of interferon-γ on epithelial chloride channels

In cell-attached configurations application of IFN-γ (100 U/ml) did not activate chloride channels, but when the cells were pretreated with 100 U/ml IFN-γ for more than 3 hours, channel activity was observed 6 in 12 cell-attached patches in the control solution (Fig. 2b) at a pipette potential of +50 mV (membrane potential = resting membrane potential minus 50 mV). The chloride channels are activated 3 hours after application of interferon-γ (from 0.01±0.02 to 0.18± 0.03, n=6 ; $p<0.01$), and this activation was observed more than 24 hours after application of IFN-γ. DIDS at a concentration of 500 μM also blocked this channel (Fig. 2c). Fig. 2d summarizes the dose-dependent activation of chloride channels by IFN-γ at 3 hours after application. We also tested the effects of other cytokines on chloride channels. IFN-α did not activate this channel (data not shown).

Effect of erythromycin

Erythromycin is reported to inhibit chloride secretion across canine tracheal epithelial cells. We studied the effect of erythromycin on single channel current of ORCC. Fig. 3 shows that 100 μM erythromycin inhibited completely channel activities induced by IFN-γ in inside-out patches (n=5).

DISCUSSION

We have shown that IFN-γ activated chloride channels in time- and dose-dependent manners in cultured human bronchial epithelial cells. The conductance of the channel was 40 pS and the channel activity was inhibited by DIDS, suggesting that this channel was ORCC. In addition, erythromycin could inhibit IFN-γ -induced channel activities. These findings indicated that ORCC might be related to the transepithelial ion flux during inflammation, and that IFN-γ may play an important role in production of mucus during inflammation.

Chloride channels are present in the cell membranes of most cells, and a large variety of chloride channels have been documented (13). Chloride channels may play a role in complex cellular regulation including

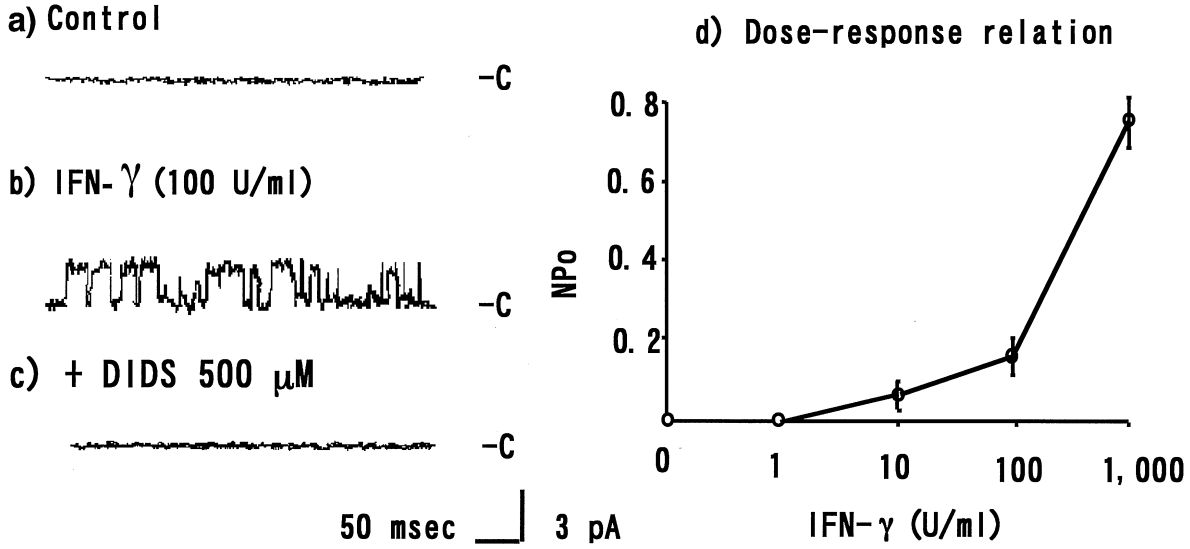

Fig.2 Interferon-gamma (IFN-γ) at concentration of 100 U/ml activated chloride channels and this channel is inhibited by 500 μM 4,4'-diisothiocyanatostilbene-2, 2'-disulfonic acid (DIDS). Panel d shows dose-response relations of IFN-γ induced chloride channel activities. Experiment was done in cell-attached patch configurations. Solution and holding pipette potentials were as in Fig. 1a and b.

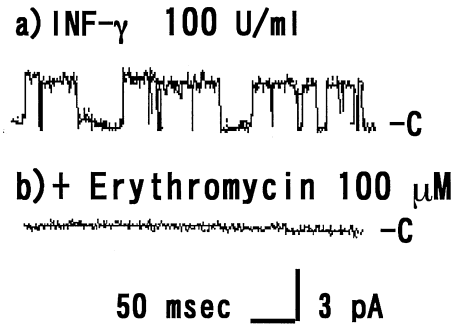

Fig.3. Effect of erythromycin on IFN-γ induced channel activities in cell-attached configurations. Erythromycin at a concentration of 100 μM inhibited chloride channel activities. Solutions and holding potentials were as in Fig. 1a and b.

ionic secretion, cell volume control, and endocrine secretions. In the present study, chloride channels were activated spontaneously when the membrane patches were excised especially at depolarized potentials (14), but this was infrequent for the case of cell-attached patches. In agreement with these findings, in the present study, no channel activities were observed in cell-attached recordings in the absence of any reagent in the bath solution, although in contrast, more than half of the inside-out patches showed activities, suggesting the participation of cellular components or mechanisms which keep the channel closed. This channel is also activated by cAMP, suggesting that this channel is CFTR or ORCC. The conductance (40 pS) and sensitivities to DIDS strongly suggest that this channel is ORCC.

Cytokines are known to modulate ionic channel currents. Colgan *et al.* (15) reported that chloride secretion, as measured by the epithelial short circuit current, was diminished as much as 70% by IL-4. Schlichter (16) found that IFN-α can increase the K^+ current near the resting potential. In addition, they also found that IFN-α induced nonselective cationic channels. However, cytokine's effects on chloride channels have not yet been studied extensively. The present study firstly demonstrated that IFN-γ activated chloride channels in epithelial cells. IFN-α did not modulate the channel activities, suggesting that this action is specific to IFN-γ.

The findings of the study indicated that ORCC was one of the regulators of various stimuli, including IFN-γ, and plays important functions in chloride secretion, although the mechanism of activating the chloride channel by IFN-γ was not examined in the present study. The findings of the present study also indicate that the inhibition of chloride channels might be essential for the treatment of chronic inflammation. The macrolide antibiotics, such as erythromycin, clarithromycin and roxythromycin, have been shown to be effective in the treatment of patients with acute bronchitis and chronic airway diseases including asthma and chronic bronchitis (17-20). The efficacy of antibiotics can be attributed not only to their antimicrobial activities but also to their immunomodulatory action on neutrophils and macrophages. In addition, many patients receiving erythromycin show a reduction in sputum volume and dryness of sputum, the mechanism of which may partly be associated with the direct inhibition of airway secretion by this drug (21). Recently, Ikeda *et al.* (22) reported using whole-cell patch

configuration that the acetylcholine-evoked ionic currents were significantly inhibited by 14-membered macrolides, erythromycin and roxythromycin, in a concentration-dependent manner. The present findings also indicated that erythromycin directly inhibited chloride currents, which might explain the reduction of mucus secretion and improvement of chronic infection by erythromycin.

REFERENCES

1. Widdicombe JH, Widdicombe JG : Regulation of human airway surface liquid. Respir Physiol 99 : 3-12, 1995
2. Frizzell RA, Halm DR, Rechkemmer G, Shoemaker RL. Chloride channel regulation in secretory epithelial. Fed Proc 45 : 2727-2731, 1986
3. Cliff WH, Schoumacher RA, Frizzell RA : cAMP-activated Cl channels in CFTR-transfected cystic fibrosis pancreatic epithelial cells. Am J Physiol 262 : C1154-C1160, 1992
4. Anderson MP, Gregory RJ, Thompson S, Souza DW, Paul S, Mulligan RC, Smith AE, Welsh MJ : Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. Science 253 : 202-205, 1991
5. Zhang ZH, Jow F, Numann R, Hinson J : The airway-epithelium : a novel site of action by guanylin. Biochem Biophys Res Commun 244 : 50-56, 1998
6. Jeulin C, Fournier J, Marano F, Dazy AC. Effects of hydroxyl radicals on outwardly rectifying chloride channels in a cultured human bronchial cell line. Eur J Physio 439 : 331-338, 2000
7. Schwiebert EM, Flotte T, Cutting GR, Guggino WB : Both CFTR and outwardly rectifying chloride channels contribute to cAMP-stimulated whole cell chloride currents. Am J Physiol 266 : C1464-C1477, 1994
8. Fuller CM, Benos DJ : CFTR Am J Physiol 263 : C267- C286, 1992
9. Martin DK, Bootcov MR, Campbell TJ, French PW, Breit SN : Human macrophages contain a stretch-sensitive potassium channel that is activated by adherence and cytokines. J Membr Biol 147 : 305-315, 1995
10. Zhu J, Mix E, Olsson T, Link H : Influence of ion channel modulation on in vitro interferon-gamma induced MHC class I and II expression on macrophages. Immunopharmacol Immunotoxicol 17 : 109-136, 1995
11. Visentin S, Agresti C, Patrizio M, Levi G : Ion channels in rat microglia and their different sensitivity to lipopolysaccharide and interferon-gamma. J Neurosci Res 42 : 439-451, 1995
12. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ : Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391 : 85-100, 1981
13. Jentsch TJ, Gunther W : Chloride channels : an emerging molecular picture. Bioessays 19 : 117-126, 1997
14. Hwang TC, Lu L, Zitlin PL, Gruenert DC, Hungnir R, Guggino WB : Chloride channels in CF : lack of activation by protein kinase C and cAMP-dependent protein kinase. Science 244 : 1351-1353, 1989
15. Colgan SP, Resnick MB, Parkos CA, Delp-Archer C, McGuirk D, Bacarra AE, Weller PF, Madara JL : IL-4 directly modulates function of a model human intestinal epithelium. J. Immunol 153 (5) : 2122-2129, 1994
16. Schlichter LC : Acute exposure to human interferon-alpha affects ion currents in human natural killer cells. Can J Physiol Pharmacol 70 (3) : 365-376, 1992
17. Ichikawa Y, Ninomiya H, Koga H, Tanaka M, Kinoshita M, Tokunaga N, Yano T, Oizumi K : Erythromycin reduces neutrophils and neutrophil-derived elastolytic-like activity in the lower respiratory tract of bronchiolitis patients. Am Rev Respir Dis 146 : 196-203, 1992
18. Shirai T, Sato A, Chida K : Effect of 14-membered ring macrolide therapy on chronic respiratory tract infections and polymorphonuclear leukocyte activity. Intern Med 34 : 469-474, 1995
19. Kudoh S : Erythromycin treatment in diffuse panbronchiolitis. Curr Opin Pulm Med 4 : 116-121, 1998
20. Yanagihara K, Tomono K, Kuroki M, Kaneko Y, Sawai T, Ohno H, Miyazaki Y, Higashiyama Y, Maesaki S, Kadota J, Kohno S : Intrapulmonary concentrations of inflammatory cytokines in a mouse model of chronic respiratory infection caused by pseudomonas aeruginosa. Clin Exp Immunol 122 : 67-71, 2000
21. Tamaoki J, Isono K, Sakai N, Kanemura T, Konno K : Erythromycin inhibits Cl secretion across canine tracheal epithelial cells. Eur Respir J 5 : 234-238, 1992
22. Ikeda K, Wu D, Takasaka T : Inhibition of acetylcholine-evoked choride currents by 14-membered macrolide antibiotics in isolated acinar cells of the guinea pig nasal gland. Am J Respir Cell Mol Biol 13 : 449-454, 1995