Effects of Fluoranthene, a Polycyclic Aromatic Hydrocarbon, on cAMP-Dependent Anion Secretion in Human Airway Epithelia

Yasushi Ito, Masami Son, Shinji Sato, Takamasa Ohashi, Masashi Kondo, Kaoru Shimokata, and Hiroaki Kume

Division of Respiratory Diseases, Department of Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan

Received August 24, 2003; accepted November 4, 2003

ABSTRACT

The human respiratory tract is constantly exposed to polycyclic aromatic hydrocarbons (PAHs) through inhalation of atmospheric pollutants. We examined the effects of three PAHs (benzo[a]pyrene, anthracene, and fluoranthene) on the airway ion transport, which is essential for lung defense and normal airway function, using human airway epithelia (Calu-3). These three PAHs had no significant effect on the basolateral membrane, accompanied by a sustained increase in the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]. In the presence of fluoranthene, however, a much larger hIK-1-dependent IC\(_{\text{sc}}\) was identified by the application of 8-bromo-cAMP without concomitant elevation of [Ca\(^{2+}\)]. These results suggest that fluoranthene switches from cAMP-dependent HCO\(_3\^-\) secretion to CI\(^-\) secretion through the hIK-1 channel, whose sensitivity to protein kinase A may be up-regulated by the sustained [Ca\(^{2+}\)]\(_i\) elevation produced by this chemical.

The human respiratory tract is constantly exposed to noxious materials, including atmospheric pollutants and microorganisms. Among the atmospheric pollutants, polycyclic aromatic hydrocarbons (PAHs) are ubiquitous (Kendall et al., 2002). Although cigarette smoke is one of the major sources of PAHs (Hoffmann and Hoffmann, 1997), human exposure to these chemicals also occurs from nontobacco sources (Waldman et al., 1991). The air in urban areas contains high percentages of PAHs. In lung biopsies, the pulmonary PAH levels increased with age (Lodovici et al., 1998). Another major source of PAHs in the air is diesel exhaust particles (DEP) emitted by motor vehicles (Fromme et al., 1998). The PAHs absorbed in the fine particles (1 \(\mu\)m) of DEP remain in the body for quite a long time (Sun et al., 1984; Lee et al., 1987). Epidemiologically, it has been shown that cigarette smoke and DEP are well associated with various health hazards, including airway infections, bronchial asthma, and chronic bronchitis (Sydbom et al., 2001). Recent investigations have revealed that DEP containing PAHs can cause asthma-like conditions, such as airway inflammation, enhanced IgE production, proliferation of goblet cells, increased mucus secretion, and bronchoconstriction (Sagai et al., 1996; Diaz-Sanchez et al., 2000). Notwithstanding, little has been documented regarding the effects of PAHs on the airway ion transport system, which is required for efficient mucociliary clearance, a primary host defense mechanism (Shimura, 2000).

This work was supported by Research Grant Funds from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Hibino Memorial Research Fund, and the Aichi Health Promotion Foundation (to Y.I.).

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. DOI: 10.1124/jpet.103.059089.

ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; BMT, bumetanide; 8Br-cAMP, 8-bromo-cAMP; CFTR, cystic fibrosis transmembrane conductance regulator; ChTx, charybdotoxin; DEP, diesel exhaust particle; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; FLT, fluoranthene; ISO, isoproterenol; NBC1, 4,4′-dinitrostilbene-2,2′-disulfonic acid-sensitive Na\(^+\)-HCO\(_3\^-\) cotransporter; NKCC1, bumetanide-sensitive Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter; PD, potential difference; PKA, protein kinase A; PSS, physiological saline solution; IC\(_{\text{sc}}\), short-circuit current; DMSO, dimethyl sulfoxide; hIK-1 channel, human intermediate conductance Ca\(^{2+}\)-activated K\(^+\) channel.
The ion transport system is implicated in mucociliary clearance by producing a thin layer of aqueous fluid called the airway surface liquid (Jayaraman et al., 2001). Since the volume of bronchial gland cells exceeds that of surface epithelial cells by a ratio of 30:1 in the human airway, the volume and composition of airway surface liquid are mainly regulated by submucosal gland cells (Shimura, 2000). Thus, in the present study, we used Calu-3 human airway cells, which are functionally and morphologically analogous to human airway serous cells, expressing abundant cystic fibrosis transmembrane conductance regulators (CFTR) (Haws et al., 1994). This cell line produces HCO_3^- and Cl^- secretion in response to various stimuli like primary submucosal gland cells do (Devor et al., 1999).

The present study focused especially on one of the PAHs, fluoranthene, whose toxicological effects have been less studied to date in spite of its environmental ubiquity and high bioactivity (Yamaguchi et al., 1996). A recent investigation found that this chemical is the most abundant PAH in human lung samples: 0.151 ng/g of wet tissue (Goldman et al., 2001).

Materials and Methods

Cell Culture. The Calu-3 cells (American Type Culture Collection, Rockville, MD) were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen), 100 μg/ml streptomycin, and 100 U/ml penicillin (Invitrogen) in culture flasks (T_25) at 37°C in a humidified atmosphere containing 5% CO_2. When 80 to 90% confluent, cells were detached with a solution of 0.05% trypsin (Invitrogen) and seeded onto porous polyester membranes [0.4-μm pore size on Snapwell or Transwell inserts (1.1 cm^2; Costar, Cambridge, MA)] at a density of 10^5 cells/well. The inserts had been collagen-coated overnight with 0.2% human placental collagen type VI (Sigma-Aldrich, St. Louis, MO). On day 1, the medium remaining on the apical side was removed to establish an air interface, which markedly improves the differentiation of human airway epithelia in a well polarized fashion. The cells were fed by replacement of the basolateral medium every 48 h. Experiments were performed after 7 to 13 days in culture.

Solutions. The physiological saline solution (PSS) used in the present study was composed of 115 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 2 mM CaCl_2, 10 mM glucose, 10 mM Heps, and 25 mM NaHCO_3. The pH of the solution was adjusted to 7.4 (at 37°C) by NaOH before addition of NaHCO_3. The pH of the solution was kept at pH 7.4 when gassed with a mixture of 5% O_2/5% CO_2. HCO_3^- free buffers consisted of 140 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 2 mM CaCl_2, 10 mM glucose, and 10 mM Heps (pH adjusted to 7.4 at 37°C). This solution was circulated with air. For Cl^- free buffers, Cl^- was replaced by chloride. In this solution, Ca_2^+ was increased to 4 mM to compensate for the Ca_2^+ chelating capacity of the gluconate. This solution was bubbled with 5% CO_2/95% O_2.

Bioelectric Studies. When cells had grown confluent, the Snapwell inserts were rinsed with PSS and mounted in modified Ussing chambers (EasyMount Chamber; Physiologic Instruments, San Diego, CA) connected to a VCC MC2 voltage-clamp apparatus (Physiologic Instruments). The monolayers were continuously open-circuited to monitor transepithelial potential differences (PD), and every 20 s, a bidirectional 2-μA pulse was imposed across the epithelium for 0.5 s to cause voltage deflections (ΔPD). This procedure enabled us to calculate transepithelial conductance (G_E) by Ohm's law (G_E = 2 μA/ΔPD). The short-circuit current (I_SC) was recorded by clamping PD to 0 mV by VCC MC2. I_SC represents the net flow of negative charges, which is mostly composed of anion current from the basolateral to the apical compartment. To pharmacologically distinguish between HCO_3^- and Cl^- dependent transport, the I_SC was measured for 10 min after basolateral application of the Na^+-2HCO_3^- cotransporter inhibitor 4,4'-dinitrostilbene-2,2'-disulfonic acid (NDNS; 3 mM) and the Na^+ - K^+ -2Cl^- cotransporter inhibitor bumetanide (50 μM), called NDNS- and bumetanide-sensitive I_SC, respectively.

Measurement of Basolateral K^+ Current. The K^+ current, which is across the basolateral membrane (I_B) was estimated after permeabilization of the apical membrane with nystatin (50 μM) for more than 30 to 40 min and establishment of an apical-to-basolateral K^+ concentration gradient (Devor et al., 1999; Ito et al., 2002a,b). Apical NaCl was replaced by equimolar K-glucolate, whereas basolateral NaCl was substituted with equimolar Na-glucolate. Cl^- was removed from these solutions. Previous investigations have demonstrated that major K^+ conductance on the basolateral membrane is produced by the human intermediate conductance (10–31 pS) Ca^2^+-activated K^+ (hIK-1) channel (Devor et al., 1999; Gerlach et al., 2000). To assess the hIK-1 channel-dependent K^+ current, we measured I_K reduction for 10 min after basolateral application of charybdotoxin (ChTX; 100 nM); this is called the ChTX-sensitive I_K.

Measurement of Intracellular Ca^2+ Concentration. For intracellular Ca^2+ imaging, Calu-3 cells were subcultured on the porous membranes of Transwell inserts (Costar) in 12-well plates. We grew airway epithelial cells as a monolayer on transparent permeable membrane supports to measure [Ca^2+], in response to extracellular PAHs. This culture strategy yields an airway epithelial morphology that closely mimics that in vivo. Before experiments, the apical and basolateral sides of the confluent monolayers were rinsed twice with PSS and incubated for 1.5 h at 37°C in the same buffer containing 5 μM 1,2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxyl-2-(2'-amino-5'-methylphenoxyl)ethane-N,N',N'-N'-tetracetic acid, pentacetoxy methyl ester (Diojindo Laboratories, Kumamoto, Japan) and 0.01% pluronic F127 (Molecular Probes, Eugene, OR). After the fluorescence loading, cell monolayers were rinsed twice with PSS to wash residual dyes outside the cells, and thereafter, 0.5 and 1 ml of PSS were added to the apical and basolateral membranes, respectively. Fluorescence signals were collected for 20 ms at 6-s intervals using a fluorometer (Fluoroscan Ascent CF, Labsystem, Helsinki, Finland) at the excitation wavelength of 485 nm and the emission wavelength of 538 nm. The maximum signal (F_max) was obtained by adding 10 μM ionomycin, and the minimum signal (F_min) was obtained by adding 10 mM EGTA to the cell monolayer. The [Ca^2+] was calculated according to the following formula:

\[ [Ca^{2+}] = K_d (F - F_{min}) / (F_{max} - F), \]

where K_d was assumed to be 390 nM. To estimate significant [Ca^2+], changes, average values before and after additions of agents during the measurements were calculated and compared.

Chemicals. Isoproterenol, forskolin, 8-bromo-cyclic AMP (8BrcAMP), bumetanide, NDNS, ellipticine, nystatin, ionomycin, and anthracene were obtained from Sigma-Aldrich. ChTX was purchased from Peptide Institute, Inc. (Osaka, Japan). Benzo[a]pyrene, fluoroanthene, and H89 were products of Wako Pure Chemicals (Tokyo, Japan). The chemical structures of benzo[a]pyrene, anthracene, and fluoroanthene are shown in Fig. 1. For permeabilization studies, nystatin was used as a 100 mM stock solution in DMSO and sonicated for 30 s just before use.

Data Analysis. Concentration-response curves in the present study were obtained using the computer program Cricket Graph version 1.5.3 for Macintosh (Computer Associates International Inc., Islandia, NY). All data are expressed as means ± S.E.M. with the number of experiments used (n). Statistical differences were determined by Student's t test or one-way analysis of variance, and p < 0.05 was considered to indicate statistical significance.
Results

Effects of PAHs on Anion Secretion Stimulated by cAMP-Related Agents. Calu-3 cells respond to β-adrenergic stimuli that contribute to anion secretion. Application of isoproterenol (10 nM) to the basolateral face led to a rapid increase in $I_{sc}$, reaching a peak in 1 to 2 min and followed by a sustained $I_{sc}$ (Fig. 2A). The increased value from the basal $I_{sc}$ to the rapid peak (peak $I_{sc}$) and to the $I_{sc}$ measured at 30 min (sustained $I_{sc}$) after isoproterenol addition were 43.2 ± 4.2 μA/cm² and 18.4 ± 3.2 μA/cm² ($n = 12$), respectively. On the other hand, exposure of cells to fluoranthene (100 μM) from the apical surface, as a model of inhalation of a PAH, resulted in no significant changes in the $I_{sc}$ for at least a 30-min observation (Fig. 2A). Nevertheless, isoproterenol-induced responses were augmented by the apical pretreatment with fluoranthene so that peak $I_{sc}$ and sustained $I_{sc}$ were 90.6 ± 8.4 μA/cm² and 60.4 ± 3.5 μA/cm² ($p < 0.0001$, $n = 9$), respectively (Fig. 2A). The effect of fluoranthene is concentration-dependent (Fig. 2B). Fluoranthene at concentrations higher than 300 μM made experimental solutions very cloudy, so we performed dose-response experiments up to 300 μM. The presence of fluoranthene at 3 μM had no significant effect on the isoproterenol-induced $I_{sc}$ ($p = 0.0085$, $n = 5$) compared with the control ($18.4 ± 3.2 μA/cm²$, $n = 12$; see Fig. 1A). Thus, considering the effects of fluoranthene at 300 μM as the maximum and the control as the minimum, the estimated EC₅₀ is between 30 and 40 μM (see Fig. 2B). This effect of fluoranthene seems unlikely to be mediated by the PAH receptor because this potentiation was unaffected by ellipticine, a PAH receptor antagonist (Fig. 2C). This notion was also supported by the results that neither benzo[a]pyrene nor anthracene, PAH agonists, applied to the apical solution had any effect (Fig. 2C). Furthermore, no significant efficacy of basolaterally applied fluoranthene (100 μM), benzo[a]pyrene (100 μM), or anthracene (100 μM) was observed in the isoproterenol-induced responses (Fig. 2D).

The effects of fluoranthene were simulated in $I_{sc}$ responses to an adenylate cyclase activator, forskolin (10 μM; Fig. 3A), and to a cell-permeable cAMP analog, 8Br-cAMP (1 mM; Fig. 3B), suggesting that fluoranthene potentiates cAMP-medi-
ated anion secretion. In addition, the fluoranthene-induced potentiation is highly sensitive to H89, a protein kinase A (PKA) inhibitor. Namely, the 8Br-cAMP-induced actions, estimated by sustained $\Delta I_{sc}$, were increased by the presence of fluoranthene from 11.3 ± 0.8 μA/cm² to 60.0 ± 1.8 μA/cm² ($n = 4$), but pretreatment with H89 (10 μM, 2 h) interrupted the augmentation by fluoranthene (sustained $\Delta I_{sc} = 12.7 \pm 1.0$ μA/cm², $n = 4$). These observations suggest that the effects of fluoranthene are subject to the activity of PKA.

**Effects of Fluoranthene on Basolateral Anion Transporters.** To help establish the ionic basis of fluoranthene effects, ion substitution experiments were performed (Fig. 4). To observe the Na⁺-K⁺-Cl⁻ cotransport function, HCO₃⁻ was removed from the physiological solution. Under this condition, the initial and sustained components of the $I_{sc}$ response to isoproterenol were potentiated by the presence of fluoranthene (Fig. 4A). In the Cl⁻-free solution used to estimate the Na⁺-2HCO₃⁻ function, however, basal and isoproterenol-induced $I_{sc}$ were remarkably decreased, and exposure of the cells to isoproterenol caused only transient responses whose $\Delta I_{sc}$ (the peak $\Delta I_{sc}$) was 13.1 ± 1.7 μA/cm² ($n = 5$; Fig. 4B). The presence of fluoranthene augmented the peak $\Delta I_{sc}$ (22.3 ± 7.5 μA/cm², $n = 5$), but this chemical had no significant effect on the $I_{sc}$ 4 to 30 min after addition of isoproterenol (Fig. 4B). Alternatively, to characterize the fluoranthene-potentiated anion secretion in the normal solution, DNDS- and bumetanide-sensitive $I_{sc}$ were evaluated in the presence and absence of fluoranthene (Fig. 5). Since sustained anion transport was produced by cAMP-related agents, including isoproterenol, this evaluation was performed 20 min after addition of isoproterenol and its vehicle (distilled water). The DNDS-sensitive component was increased by the application of isoproterenol (from 0.5 ± 0.1 μA/cm², $n = 4$ to 11.1 ± 0.7 μA/cm², $n = 4$; $p < 0.0001$), but it was abolished by the presence of fluoranthene (−0.1 ± 1.7 μA/cm², $n = 5$, $p < 0.0001$; Fig. 5A). The bumetanide-sensitive component was also up-regulated by isoproterenol (from 0.7 ± 0.2 μA/cm², $n = 5$ to 10.5 ± 0.7 μA/cm², $n = 11$; $p < 0.0001$; Fig. 5B), but, in contrast, the presence of fluoranthene further augmented the component (57.8 ± 2.4 μA/cm², $n = 5$, $p < 0.0001$; Fig. 5B). However, the effects of fluoranthene on the DNDS- and bumetanide-sensitive $I_{sc}$ were counteracted by ChTx (100 nM, basolateral) applied 10 min before additions of DNDS and bumetanide, respectively (Fig. 5, A and B).

**Effect of Fluoranthene on the hIK-1 Channel and Cytosolic Ca²⁺ Concentrations.** We measured the reduction of the sustained $I_{sc}$ generated by isoproterenol for 10 min after basolateral application of ChTx (100 nM), called the ChTx-sensitive $I_{sc}$, and that was very small (1.1 ± 0.2 μA/cm², $n = 5$; Fig. 5C). However, a larger ChTx-sensitive $I_{sc}$ was detected in the isoproterenol-stimulated monolayer when pretreated with fluoranthene for 30 min (47.3 ± 6.6 μA/cm², $n = 5$).

**Fig. 3.** Effects of FLT on the forskolin- and 8-bromo cAMP (8Br-cAMP)-induced $I_{sc}$. After apical pretreatment with fluoranthene (100 μM) or its vehicle (0.1% DMSO), forskolin (10 μM; A) or 8Br-cAMP (1 mM; B) was applied to the basolateral solution. Data are means ± S.E.M. ($n = 4$–5).

**Fig. 4.** Effects of ISO on $I_{sc}$ in nominally Cl⁻ and HCO₃⁻-free buffers in the presence and absence of FLT on the apical surface. $I_{sc}$ responses to ISO were potentiated by the presence of FLT in the HCO₃⁻-free buffers (A). In the Cl⁻-free solution, exposure of the cells to ISO caused only transient responses whose peak values are increased by the presence of FLT (B). However, FLT made no significant difference in the $I_{sc}$ 4 to 30 min after addition of ISO. FLT (100 μM) was applied to the apical compartment 30 min prior to addition of ISO. Data are means ± S.E.M. ($n = 4$–9).

**Fig. 5.** Alteration of bioelectric properties by the presence of FLT. A, DNDS (3 mM, basolateral)-sensitive $I_{sc}$ was measured 20 min after addition of isoproterenol [ISO (+)] and its vehicle [distilled water, ISO (−)]. Note that the ISO-induced increment in the DNDS-sensitive $I_{sc}$ in the absence of FLT [FLT (−)] was abolished by its presence [FLT (+)] applied 30 min before addition of ISO or its vehicle. However, pretreatment with ChTx (100 nM, basolateral) 10 min before addition of DNDS counteracted the suppression of DNDS-sensitive $I_{sc}$ [ChTx (+) + FLT (+)]. B, bumetanide (BMT; 50 μM, basolateral)-sensitive $I_{sc}$ was measured 20 min after addition of ISO and its vehicle. In contrast, the pretreatment with FLT potentiated the ISO-induced up-regulation of the BMT-sensitive $I_{sc}$. However, pretreatment with ChTx 10 min before addition of BMT counteracted the potentiation of BMT-sensitive $I_{sc}$ [ChTx (+) + FLT (+)]. C, in correlation to the BMT-sensitive $I_{sc}$, the ChTx (100 nM, basolateral)-sensitive $I_{sc}$, which is less detected under basal and ISO-unstimulated conditions [ISO (−)], was also significantly increased under the FLT plus ISO condition. Data are means ± S.E.M. ($n = 4$–11) (D). Significant differences are expressed with *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.0001$ (unpaired Student’s t test).
nystatin (50 μM), although fluoranthene itself had a modest effect on the ChTx-sensitive $I_{sc}$ (1.7 ± 0.4 μA/cm², n = 4; Fig. 5C). On the basis of these observations, we speculated that the hIK-1 channel activities on the basolateral membrane were closely related to the fluoranthene-induced changes in the bioelectric properties described above. Thus, after permeabilization of the apical membrane with nystatin (50 μM), the basolateral membrane $I_K$ was followed under the establishment of transepithelial K⁺ gradients (Fig. 6). Fluoranthene gradually increased the $I_K$, and consequently, the ChTx-sensitive $I_K$ in the absence of fluoranthene (0.4 ± 0.1 μA/cm², n = 4) was raised to 8.5 ± 0.7 μA/cm² (n = 4) 20 min after its addition ($p < 0.0001$; Fig. 6A). On the other hand, 8Br-cAMP (1 mM, basolateral) had a very small effect on the ChTx-sensitive $I_K$ (0.6 ± 0.1 μA/cm², n = 6; Fig. 6B). Nevertheless, in the apical presence of fluoranthene, 8Br-cAMP remarkably augmented the ChTx-sensitive $I_K$. Data are means ± S.E.M. ($n = 4$–7).

**Discussion**

Collection of airborne particulate samples in a city in Japan revealed that the average daily inhalations of three major PAHs were 17 ng/day/person for benzo[a]pyrene, 1 ng/day/person for anthracene, and 16 ng/day/person for fluoranthene (Matsumoto and Kashimoto, 1985). In the present study, we examined the effects of these three PAHs on the anion transport in human airway Calu-3 cells and demonstrated that only fluoranthene exposed to the apical membrane modulates the function. In polarized airway epithelia, HCO₃⁻ and Cl⁻ secretion are primarily regulated by cytosolic cAMP and Ca²⁺, respectively (Smith and Welsh, 1992; Devor et al., 1999; Ito et al., 2001). Generally, the transepithelial anion transport is maintained through two steps: anion entry across the basolateral membrane and its export across the apical membrane (Ito et al., 2001). The apical CFTR is well known as a common pathway for HCO₃⁻ and Cl⁻ export (Devor et al., 1999). Which anion will be transported across the airway is determined by the activity of basolateral anion transporters. Namely, HCO₃⁻ secretion is maintained by the DNDS-sensitive Na⁺–2HCO₃⁻ cotransporter (NBC1), and Cl⁻ secretion is done by the bumetanide-sensitive Na⁺–K⁺–2Cl⁻ cotransporter (NKCC1)(Dever et al., 1999; Inglis et al., 2002). These two anion transporters have been characterized in many mammalian tissues, including Calu-3 cells (Romero and Boron, 1999; Inglis et al., 2002; Liedtke et al., 2002). Under cAMP-stimulated conditions, both NBC1 and NKCC1 seem likely to be activated by cAMP/PKA-dependent phosphorylation (Kurihara et al., 2002; Gross et al., 2003), as suggested in Fig. 5. In the fluoranthene-pretreated epithelia, however, isoproterenol failed to increase the DNDS-sensitive $I_{sc}$ accompanied by basolateral Na⁺–K⁺–2Cl⁻ cotransporter (Figure 5C). Furthermore, the effects of fluoranthene on the DNDS- and bumetanide-sensitive $I_{sc}$ were counteracted by the presence of ChTx (Fig. 5, A and B). Thus, these results suggest that the hIK-1 channel plays a key role in the modulatory effects of fluoranthene on the cAMP-stimulated anion secretion. DeVor et al. (1999) reported that the switch between HCO₃⁻ secretion and Cl⁻ secretion is determined by...
the basolateral membrane potential regulated by the hIK-1 channel. Namely, when the basolateral membrane is hyperpolarized by hIK-1 channel activation, the driving force for HCO$_3^\text{-}$/entry via NBC1 cotransporters, which electrogenically carry negative charges into the cell, is reduced, whereas that for Cl$^\text{-}$ entry across the electroneutral NKCC1 is up-regulated. Since the hyperpolarization simultaneously provides a driving force for anion export across the apical CFTR, the activation of the hIK-1 channel would cause Cl$^\text{-}$ dominant secretion. 

In the HCO$_3^\text{-}$/free solution to estimate the NKCC1 function, the $I_{sc}\text{ }$ response to isoproterenol was potentiated by the presence of fluoroantheine, in agreement with the pharmacological data obtained using bumetanide (see Figs. 4A and 5B). In the Cl$^\text{-}$/free solution used to confirm the NBC1 function, however, basal and isoproterenol-induced $I_{sc}\text{ }$ were remarkably decreased, and exposure of the cells to isoproterenol caused only transient responses. Furthermore, the peak in the presence of fluoroantheine was rather higher that that in its absence, although there was no difference between the two groups of the $I_{sc}\text{ }$ measured 4 to 30 min after addition of isoproterenol. Possibly, the absence of the major anion Cl$^\text{-}$ produces a cytosolic charge that is negative relative to the outside, leading to a decrease in the sustained current generated by the NBC1. The fluoroantheine-induced potentiation of the transient $I_{sc}\text{ }$ under the Cl$^\text{-}$/free condition may be explained by the notion that export of remaining anions in the cytosol is up-regulated by the hIK-1-mediated driving force potentiated by fluoroantheine.

The most important factor to activate the hIK-1 channel in Calu-3 cells is elevation of [Ca$^{2\text{+}}$]$_i$. Actually, fluoroantheine caused a persistent increase in [Ca$^{2\text{+}}$]$_i$, resulting in up-regulation of the ChTx-sensitive $I_K$ (see Figs. 6A and 7A). However, the degrees of changes in [Ca$^{2\text{+}}$]$_i$ (approximately 100 nM) and hIK-1-mediated $I_{sc}\text{ }$ current had no significant effect on the $I_{sc}\text{ }$ (see Figs. 2 and 3). This suggests that the hIK-dependent up-regulation of the NKCC1 by fluoroantheine would be offset by the down-regulation of the NBC1. Furthermore, it is noteworthy that addition of 8Br-cAMP, which itself had a minor effect on the ChTx-sensitive $I_K$ (Fig. 6B), evoked a much larger augmentation in the ChTx-sensitive $I_K$ after pretreatment with fluoroantheine without significant changes in [Ca$^{2\text{+}}$]$_i$ (see Figs. 6C and 7B). Possibly, the PKA sensitization of the hIK-1 channel produced by fluoroantheine is attributable to persistent increases in [Ca$^{2\text{+}}$]$_i$ caused by the application of this chemical (see Fig. 7). The Ca$^{2\text{+}}$/dependent sensitization of the hIK-1 channel to PKA was first reported by Gerlach et al. (2000). They showed that the hIK-1 channels, which were unresponsive to cAMP-elevating agents, were markedly activated by the cAMP-elevating agents after [Ca$^{2\text{+}}$]$_i$, was elevated by ionomycin in Xenopus oocytes. Although it might be possible that fluoroantheine directly modifies the sensitivity of the hIK-1 channel to PKA, mechanisms other than the synergism between Ca$^{2\text{+}}$/ and PKA have never been reported. Thus, the Ca$^{2\text{+}}$/dependent mechanisms may be a more reasonable explanation of our results.

There is some evidence that PAHs such as anthracene, benzo[a]pyrene, and their metabolites possess the ability to cause sustained elevation of [Cu$^{2\text{+}}$]$_i$ in HPB-ALL human T-lymphocytes (Krieger et al., 1994). However, [Ca$^{2\text{+}}$]$_i$, in mammary epithelial cells was not elevated by anthracene (Tannheimer et al., 1997), and [Ca$^{2\text{+}}$]$_i$ in human small airway epithelial cells was raised by a benzo[a]pyrene metabolite but not by benzo[a]pyrene itself (Jyonouchi et al., 2001). In Calu-3 cells also, neither benzo[a]pyrene nor anthracene caused sustained [Ca$^{2\text{+}}$]$_i$ increases (unpublished data). Sustained [Ca$^{2\text{+}}$]$_i$ elevation induced by PAHs may be due to inhibition of sarcoplasmic/endoplasmic reticulum Ca$^{2\text{+}}$/ATPase, as demonstrated by Krieger et al. (1995). These previous reports, however, provided no information regarding the reasons for the species difference in the effects and the relationship between Ca$^{2\text{+}}$/-mobilizing effects and the chemical structures of PAHs. Furthermore, there has been no report of the effects of fluoroantheine and its metabolites on sarcoplasmic/endoplasmic reticulum Ca$^{2\text{+}}$/ATPase or [Ca$^{2\text{+}}$]$_i$. Thus, it is difficult to clearly define why fluoroantheine is the only PAH examined that mobilizes [Ca$^{2\text{+}}$]$_i$, and modulates anion secretion. Further investigations are necessary to clarify these points.

Yamaguchi et al. (1996) reported that fluoroantheine induced apoptosis in murine T cell hybridomas via Ca$^{2\text{+}}$/-dependent and PAH receptor-independent mechanisms. The present study demonstrated that representative PAH receptor agonists benzo[a]pyrene and anthracene had no effect on the $I_{sc}\text{ }$ responses and that an PAH receptor antagonist, ellipticine, failed to counteract the effects of fluoroantheine (Gasiewicz et al., 1996). Thus, the PAH receptor seems unlikely to be involved in Ca$^{2\text{+}}$/- and cAMP-mediated ion transport in this cell line.

As seen in Figs. 2 and 3, there is a difference in $I_{sc}\text{ }$ characteristics for isoproterenol and the other cAMP-related agents, especially in the presence of fluoroantheine. Namely, under isoproterenol-stimulated conditions, a component sustained at a lower level than the initial peak was generated, whereas immediate responses to forskolin and 8Br-CAMP were followed by a second rise (see Fig. 3). Since PKA appears to play a key role in the fluoroantheine-induced modulation of $I_{sc}\text{ }$, the different $I_{sc}$ behaviors may indicate that the responses due to forskolin and 8Br-CAMP are more dependent on cAMP/PKA than those due to isoproterenol, especially in the second sustained phase. Indeed, it was reported that isoproterenol activates CFTR by the cooperative actions of two second messengers, heterometric G protein and cAMP/PKA, whereas forskolin and 8Br-CAMP do so only through cAMP/PKA-dependent mechanisms (Reddy and Quinton, 2001).

HCO$_3^\text{-}$/ transport is important to regulate the pH on the airway surface (Devor et al., 1999; Inglis et al., 2003). Analysis of exhaled breath condensate has suggested airway acidification in patients with cystic fibrosis, chronic obstructive lung disease, bronchiectasis, and acute asthma (Hunt et al., 2000; Kostikas et al., 2002; Tate et al., 2002). Lowering of the airway pH may be responsible for the development of airway inflammation (Kostikas et al., 2002). Thus, our results suggest that the effects of fluoroantheine may affect pH homeostasis in the respiratory tract. Furthermore, cAMP-mediated Cl$^\text{-}$/ secretion potentiated by fluoroantheine may produce large amounts of sputum, limiting airflow (Tamaoki et al., 1992).

In conclusion, the present study shows that a major environmental chemical, fluoroantheine, which permeates the apical membrane, impaired cAMP-mediated HCO$_3^\text{-}$/ secretion and potentiated Cl$^\text{-}$/ secretion, distinct from the other PAHs benzopyrene and anthracene. The modulation of anion trans-
port appeared to be mediated by the hIK-1 channel, whose sensitivity to PKA may be up-regulated by the sustained \([\text{Ca}^{2+}]_i\), elevation produced by fluoranthene. Thus, constant exposure to fluoranthene from the airway surface might be involved in airway surface acidification, resulting in exacerbation of various airway inflammatory diseases.

References


Address correspondence to: Dr. Yasushi Ito, Division of Respiratory Diseases, Department of Medicine, Nagoya Graduate School of Medicine, Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. E-mail: itoyasu@med.nagoya-u.ac.jp