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

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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 basal short-circuit current (I_{sc}). However, fluoranthene (1–100 μM) applied in the apical compartment potentiated I_{sc} in response to cAMP-related agents (isoproterenol, forskolin, and 8-bromo-cAMP). The effects of fluoranthene were unaffected by ellipticine, a PAH receptor antagonist. Estimation of the anionic composition of I_{sc} revealed that isoproterenol increased both HCO_3^- and Cl^- transport in the control, whereas it potentiated only Cl^- transport in the presence of fluoranthene. The fluoranthene-induced modulations of these anion transporters were counteracted by charybdotoxin (ChTx, a hIK-1 channel blocker). Fluoranthene gradually augmented the ChTx-sensitive K^+ current (I_K) across the basolateral membrane, accompanied by a sustained increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). In the presence of fluoranthene, however, a much larger hIK-1-dependent I_K was identified by the application of 8-bromo-cAMP without concomitant elevation of $[Ca^{2+}]_i$. These results suggest that fluoranthene switches from cAMP-dependent HCO_3^- secretion to Cl^- 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 of urban residents were higher than those of the rural residents, and the 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 μm) 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).

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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^+-$2HCO_3^-$ cotransporter; NKCC1, bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter; PD, potential difference; PKA, protein kinase A; PSS, physiological saline solution; I_{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 has 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_{75}) at 37°C in a humidified atmosphere containing 5% CO_2. When 80 to 90% confluent, cells were detached with a solution of phosphate-buffered saline, 0.04% EDTA, and 0.25% trypsin. The collected cells were passaged with a 1:4 dilution of the same solution 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 10^6 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 Hepes, 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 95% 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 Hepes (pH adjusted to 7.4 at 37°C). This solution was circulated with air. For Cl^--free buffers, Cl^- in the PSS was replaced by gluconate. 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 (Δ PD). This procedure enabled us to calculate transepithelial conductance (G_t) by Ohm's law ($G_t = 2\ \mu A/\Delta$ PD). 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} reduction was measured for 10 min after basolateral application of the Na^+-$2HCO_3^-$ cotransport inhibitor 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS; 3 mM) and the Na^+- K^+-$2Cl^-$ cotransport inhibitor bumetanide (50 μM), called DNDS- and bumetanide-sensitive I_{sc}, respectively.

Measurement of Basolateral K^+ Current. The K^+ current across the basolateral membrane (I_K) 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-gluconate, whereas basolateral NaCl was substituted with equimolar Na-gluconate. 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 polarized monolayer on transparent permeable membrane supports to measure $[Ca^{2+}]_i$ 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 aspects of the confluent monolayer 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)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentaacetoxymethyl ester (Dojindo 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 off 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 (Fluoroskan 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 $[Ca^{2+}]_i$ was calculated according to the following formula:

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

in which K_d was assumed to be 390 nM. To estimate significant $[Ca^{2+}]_i$ changes, average values before and after additions of reagents during the measurements were calculated and compared.

Chemicals. Isoproterenol, forskolin, 8-bromo-cyclic AMP (8Br-cAMP), bumetanide, DNDS, ellipticine, nystatin, ionomycin, and anthracene were obtained from Sigma-Aldrich. ChTx was purchased from Peptide Institute, Inc. (Osaka, Japan). Benzo[a]pyrene, fluoranthene, and H89 were products of Wako Pure Chemicals (Tokyo, Japan). The chemical structures of benzo[a]pyrene, anthracene, and fluoranthene 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.

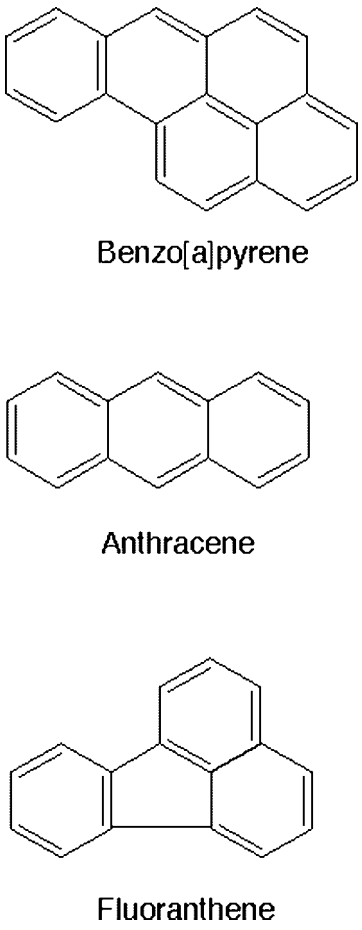

Fig. 1. Chemical structure of benzo[a]pyrene, anthracene, and fluoranthene.

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 ΔI_{sc}) and to the I_{sc} measured at 30 min (sustained ΔI_{sc}) after isoproterenol addition were 43.2 $\pm$ 4.2 $\mu A/cm^2$ and 18.4 $\pm$ 3.2 $\mu A/cm^2$ ($n = 12$), 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 ΔI_{sc} and sustained ΔI_{sc} were 90.6 $\pm$ 8.4 $\mu A/cm^2$ and 60.4 $\pm$ 3.5 $\mu A/cm^2$ ($p < 0.0001$, $n = 9$), 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} (sustained ΔI_{sc} = 18.6 $\pm$ 2.3 $\mu A/cm^2$, $n = 5$) compared with the control (18.4 $\pm$ 3.2 $\mu A/cm^2$, $n = 12$; see Fig. 1A). Thus, considering the effects of fluoranthene at 300 μM as the maximum and the control as the minimum, the estimated EC_{50} is between

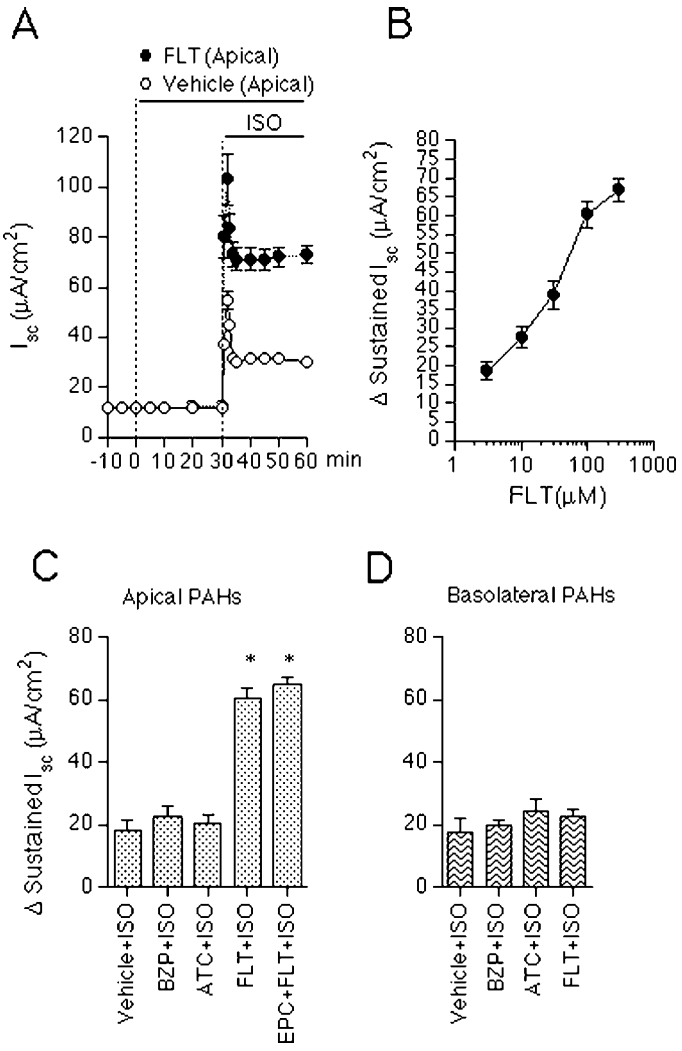

Fig. 2. Effects of fluoranthene (FLT) and other PAHs on I_{sc} in response to the β-adrenergic agonist isoproterenol in Calu-3 cells. A, isoproterenol (ISO; 10 nM) applied to the basolateral solution 30 min after adding fluoranthene (100 μM) or its vehicle (0.1% DMSO). Note that the pretreatment with FLT markedly potentiated ISO-induced I_{sc}, although the chemical itself caused no significant changes in the basal I_{sc}. B, the concentration-dependent effects of FLT on Δ sustained I_{sc}. C and D, effects of PAH agonists (benzo[a]pyrene, anthracene, and FLT) and a PAH antagonist (ellipticine) on the ISO-induced responses estimated by Δ sustained I_{sc}. Benzopyrene (100 μM), anthracene (100 μM), and FLT (100 μM) were applied to apical (C) and basolateral (D) compartments 30 min prior to addition of ISO. Ellipticine (100 μM) was apically applied 10 min before addition of FLT (namely, 40 min prior to ISO addition). Data are means $\pm$ S.E.M. (n = 4–12). *, $p < 0.0001$.

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-

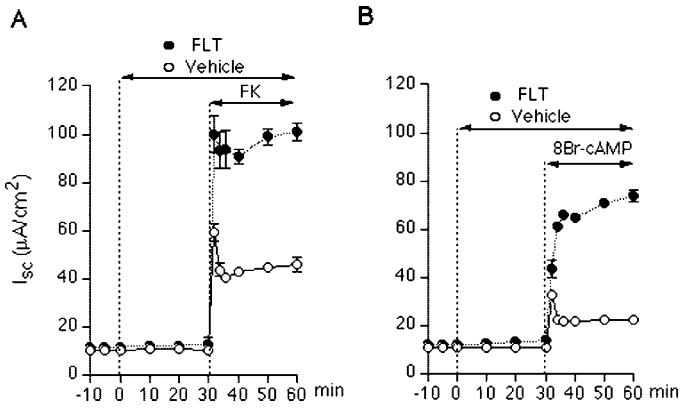

Fig. 3. Effects of FLT on the forskolin- and 8-bromo cAMP (8Br-cAMP)-induced I_{sc}. After apical pretreatment with fluoranthene (100 μM) or its vehicle (0.1% DMSO), forskolin (10 μM; A) or 8Br-cAMP (1 mM; B) was applied to the basolateral solution. Data are means ± S.E.M. (n = 4–5).

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 ΔI_{sc}, were increased by the presence of fluoranthene from 11.3 ± 0.8 μA/cm^2 to 60.0 ± 1.8 μA/cm^2 (n = 4), but pretreatment with H89 (10 μM, 2 h) interrupted the augmentation by fluoranthene (sustained ΔI_{sc} = 12.7 ± 1.0 μA/cm^2, 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, HCO_3^- 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^+-2HCO_3^- function, however, basal and isoproterenol-induced I_{SC} were remarkably decreased, and exposure of the cells to isoproterenol caused only transient responses whose ΔI_{sc} (the peak ΔI_{sc}) was 13.1 ± 1.7 μA/cm^2 (n = 5; Fig. 4B). The presence of fluoranthene augmented the peak ΔI_{sc} (22.3 ± 7.5 μA/cm^2, n = 5, $p < 0.05$), 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/cm^2, n = 4 to 11.1 ± 0.7 μA/cm^2, n = 7; $p < 0.0001$), but it was abolished by the presence of fluoranthene (−0.1 ± 1.7 μA/cm^2, n = 5, $p < 0.0001$; Fig. 5A). The bumetanide-sensitive component was also up-regulated by isoproterenol (from 0.7 ± 0.2 μA/cm^2, n = 5 to 10.5 ± 0.7 μA/cm^2, n = 11; $p <$ 0.0001; Fig. 5B), but, in contrast, the presence of fluoranthene further augmented the component (57.8 ± 2.4 μA/cm^2, 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 Ca^{2+} 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/cm^2, 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

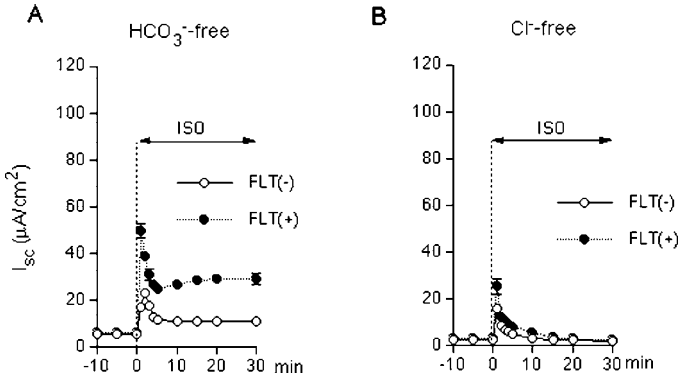

Fig. 4. Effects of ISO on I_{sc} in nominally Cl^-- and HCO_3^--free buffers in the presence and absence of FLT on the apical surface. I_{sc} responses to ISO were potentiated by the presence of FLT in the HCO_3^--free buffers (A). In the Cl^- free solution, exposure of the cells to ISO caused only transient 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).

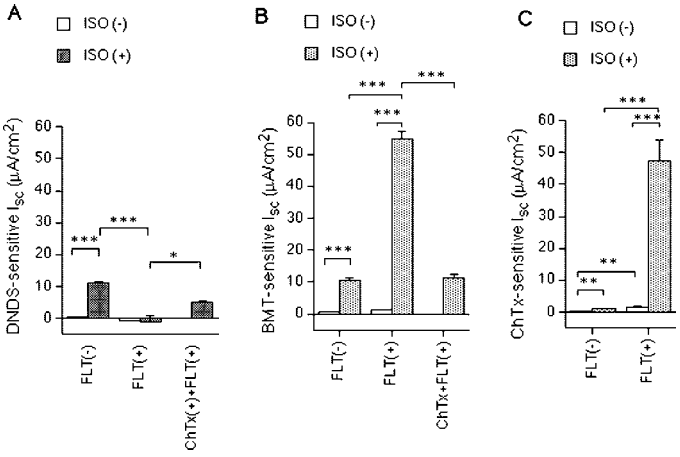

Fig. 5. Alteration of bioelectric properties by the presence of FLT. A, DNDS (3 mM, basolateral)-sensitive I_{sc} was measured 20 min after addition of isoproterenol [ISO (+)] and its vehicle [distilled water, ISO (−)]. Note that the ISO-induced increment in the DNDS-sensitive I_{sc} in the absence of 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).

$\mu A/cm^2$, $n = 8$; Fig. 5C), although fluoranthene itself had a modest effect on the ChTx-sensitive I_{sc} (1.7 ± 0.4 $\mu A/cm^2$, $n = 4$; 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 $\mu A/cm^2$, $n = 4$) was raised to 8.5 ± 0.7 $\mu A/cm^2$ ($n = 4$) 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 $\mu A/cm^2$, $n = 6$; Fig. 6B). Nevertheless, in the apical presence of fluoranthene, 8Br-cAMP remarkably augmented the ChTx-sensitive I_K (31.6 ± 1.3 $\mu A/cm^2$, $n = 6$, $p < 0.0001$; Fig. 6C). Since it is well known that hIK-1 channel activity correlates with $[Ca^{2+}]_i$, the effects of fluoranthene on the $[Ca^{2+}]_i$ were examined. Figure 7A shows a representative recording of $[Ca^{2+}]_i$ before and after addition of fluoranthene. A sustained oscillatory $[Ca^{2+}]_i$ elevation was observed immediately after addition of fluoranthene, followed by the sustained component (217.9 ± 4.6 nM to 307.8 ± 9.7 nM, $n = 9$, $p < 0.0001$). The Ca^{2+} oscillation was still sustained around 30 min after addition of fluoranthene. Subsequently, application of 8Br-cAMP (1 mM, basolateral) 30 min after application of fluoranthene (Fig. 7B) resulted in no significant change in the $[Ca^{2+}]_i$, whose levels were 346.4 ± 12.7 nM versus 395.2 ± 41.7 nM.

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, HCO_3^- and Cl^- secretion are primarily regulated by cytosolic cAMP and Ca^{2+}, 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 HCO_3^- 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, HCO_3^- secretion is maintained by the DNDS-sensitive Na^+-$2HCO_3^-$ cotransporter (NBC1), and Cl^- secretion is done by the bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter (NKCC1)(Devor 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 larger potentiation of the bumetanide-sensitive I_{sc} (Fig. 5, A and B), so that the total anion current, estimated by I_{sc}, was consequently augmented (Fig. 2). Similar results were also obtained under the 8Br-cAMP-stimulated condition, suggesting that fluoranthene impaired cAMP-dependent up-regulation of HCO_3^- uptake via NBC1, accompanied by augmentation of Cl^- uptake via NKCC1. In correlation to the up-regulated Cl^- secretion, the ChTx-sensitive component, which reflects the basolateral hIK-1 channel activity, was increased by the presence of fluoranthene (Fig. 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 HCO_3^- secretion and Cl^- secretion is determined by

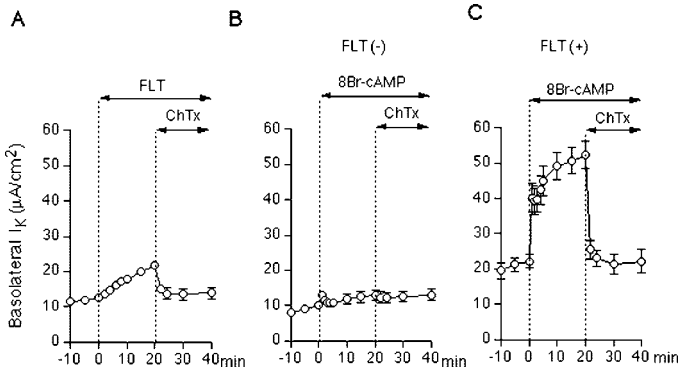

Fig. 6. Effects of FLT (100 μM, apical) and 8Br-cAMP (1 mM, basolateral) on the basolateral membrane I_K following establishment of an apical-to-basolateral K^+ gradient in the monolayer apically permeabilized with nystatin (50 μM). To estimate the involvement of the hIK-1 channel, the sensitivities of the I_K to ChTx (100 nM, basolateral) was followed after exposure to FLT (A), 8Br-cAMP (B), or FLT + 8Br-cAMP (C). 8Br-cAMP (1 mM, basolateral) had a modest effect on the ChTx-sensitive I_K in the absence of FLT [FLT (−); B]. Nevertheless, in the apical presence of fluoranthene [FLT (+)], 8Br-cAMP remarkably augmented the ChTx-sensitive I_K. Data are means ± S.E.M. ($n = 4$–7).

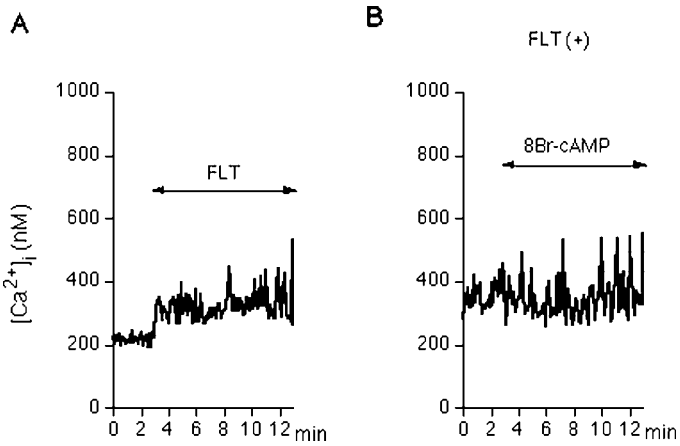

Fig. 7. Representative recordings of $[Ca^{2+}]_i$ responses to the presence of FLT. A $[Ca^{2+}]_i$ elevation in a oscillatory fashion was observed immediately after addition of fluoranthene, followed by the sustained component (A). The Ca^{2+} oscillation was still sustained 30 min after addition of fluoranthene (B). In the presence of fluoranthene [FLT (+)], additional application of 8Br-cAMP (1 mM, basolateral) resulted in no significant change.

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^- entry via NBC1 cotransporters, which electrogenically carry negative charges into the cell, is reduced, whereas that for Cl^- 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^- dominant secretion.

In the HCO_3^- free solution to estimate the NKCC1 function, the I_{sc} response to isoproterenol was potentiated by the presence of fluoranthene, in agreement with the pharmacological data obtained using bumetanide (see Figs. 4A and 5B). In the Cl^--free solution used to confirm the NBC1 function, however, basal and isoproterenol-induced I_{sc} were remarkably decreased, and exposure of the cells to isoproterenol caused only transient responses. Furthermore, the peak in the presence of fluoranthene was rather higher that that in its absence, although there was no difference between the two groups of the I_{sc} measured 4 to 30 min after addition of isoproterenol. Possibly, the absence of the major anion Cl^- produces a cytosolic charge that is negative relative to the outside, leading to a decrease in the sustained current generated by the NBC1. The fluoranthene-induced potentiation of the transient I_{sc} under the Cl^-- 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 fluoranthene.

The most important factor to activate the hIK-1 channel in Calu-3 cells is elevation of $[Ca^{2+}]_i$. Actually, fluoranthene caused a persistent increase in $[Ca^{2+}]_i$, resulting in up-regulation of the ChTx-sensitive I_K (see Figs. 6A and 7A). However, the degrees of changes in $[Ca^{2+}]_i$ (approximately 100 nM) and hIK-1-mediated K^+ current had no significant effect on the I_{sc} (see Figs. 2 and 3). This suggests that the hIK-dependent up-regulation of the NKCC1 by fluoranthene 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 fluoranthene without significant changes in $[Ca^{2+}]_i$ (see Figs. 6C and 7B). Possibly, the PKA sensitization of the hIK-1 channel produced by fluoranthene is attributable to persistent increases in $[Ca^{2+}]_i$ caused by the application of this chemical (see Fig. 7). The Ca^{2+}-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 $[Ca^{2+}]_i$ was elevated by ionomycin in *Xenopus* oocytes. Although it might be possible that fluoranthene directly modifies the sensitivity of the hIK-1 channel to PKA, mechanisms other than the synergism between Ca^{2+} and PKA have never been reported. Thus, the Ca^{2+}-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 $[Ca^{2+}]_i$ in HPB-ALL human T-lymphocytes (Krieger et al., 1994). However, $[Ca^{2+}]_i$ in mammary epithelial cells was not elevated by anthracene (Tannheimer et al., 1997), and $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ increases (unpublished data). Sustained $[Ca^{2+}]_i$ elevation induced by PAHs may be due to inhibition of sacroplasmic/endoplasmic reticulum Ca^{2+} 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+}-mobilizing effects and the chemical structures of PAHs. Furthermore, there has been no report of the effects of fluoranthene and its metabolites on sacroplasmic/endoplasmic reticulum Ca^{2+} ATPase or $[Ca^{2+}]_i$. Thus, it is difficult to clearly define why fluoranthene is the only PAH examined that mobilizes $[Ca^{2+}]_i$ and modulates anion secretion. Further investigations are necessary to clarify these points.

Yamaguchi et al. (1996) reported that fluoranthene induced apotosis in murine T cell hybridomas via Ca^{2+}-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} responses and that an PAH receptor antagonist, ellipticine, failed to counteract the effects of fluoranthene (Gasiewicz et al., 1996). Thus, the PAH receptor seems unlikely to be involved in Ca^{2+}- and cAMP-mediated ion transport in this cell line.

As seen in Figs. 2 and 3, there is a difference in I_{sc} characteristics for isoproterenol and the other cAMP-related agents, especially in the presence of fluoranthene. 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 fluoranthene-induced modulation of I_{sc}, 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 mechasnisms (Reddy and Quinton, 2001).

HCO_3^- 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 fluoranthene may affect pH homeostasis in the respiratory tract. Furthermore, cAMP-mediated Cl^- secretion potentiated by fluoranthene may produce large amounts of sputum, limiting airflow (Tamaoki et al., 1992).

In conclusion, the present study shows that a major environmental chemical, fluoranthene, which permeates the apical membrane, impaired cAMP-mediated HCO_3^- secretion and potentiated Cl^- 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 $[Ca^{2+}]_i$ 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.

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