Enhancement of P2Y₆-Induced Cl⁻ Secretion by IL-13 and Modulation of SK4 Channels Activity in Human Bronchial Cells

Sandrine Dulong, Karen Bernard and Jordi Ehrenfeld

Laboratoire de Physiologie Cellulaire et Moléculaire, Université de Nice-Sophia Antipolis

Key Words

IL-13 • P2Y₆ receptors • Bronchial cells • SK4 • CFTR • CaCC • Differentiation

Abstract

Expression of functional P2Y₆ receptors was demonstrated in primary cultures of human bronchial cells (NHBE cells). P2Y₆ receptors were located only on the apical membranes of NHBE cells. Their stimulation by UDP induced a chloride secretion (short-circuit current) reflected by the development of two I_{sc} components (I_{fast} and I_{late}). A pharmacological characterization of those two I_{sc} components showed the involvement of CaCC and CFTR channel activity in I_{fast} and I_{late} respectively. I_{fast} was also found to be under control of basolateral SK4 channels. Indeed, inhibition of SK4 channels opening by clotrimazole dramatically reduced I_{fast} amplitude. The epithelial ion transporting phenotype depends on the cellular state of differentiation. As previously reported, we observed that Ultrosor G increased the epithelial tightness and Na⁺-transport capacity while IL-13 switch the epithelial ion transport phenotype from a Na⁺-absorbing to a Cl⁻-secreting one. In our study, we report for the first time a change in the K⁺ cell permeability associated

to IL-13-induced cell differentiation. IL-13 treatment increased the-resting K⁺ permeability as well as the Ca²⁺-dependent K⁺ permeability stimulated by UDP or ionomycin. SK4 channels activity, underlying the Ca²⁺-dependent K⁺ permeability was in particular increased by IL-13. The on/off effect of IL-13 on P2Y₆-induced Cl⁻ secretion may help to identify the molecular determinants responsible for the CaCC channel activity.

Copyright © 2007 S. Karger AG, Basel

Introduction

A calcium-activated chloride conductance (CaCC), regulated by increases in intracellular free Ca²⁺ (Ca_i), has been reported in normal [1-3] and CF airway epithelia [2, 4, 5]. CaCC was shown to be upregulated in the CFTR (-/-) knockout mouse [6] as well as in human CF airway epithelia [2, 7]. Therefore, it has been suggested that CaCC could compensate for the loss of functional CFTR in Cystic Fibrosis airways [6].

In native airway epithelial cells [8-11] as well as in airway cell lines [12, 13], K⁺ channels are involved in Cl⁻ secretion by generating a favorable electrochemical

gradient for Cl⁻ exit through opened Cl⁻ channels.

SK4 channels, mainly expressed at the basolateral membranes, were shown to play a major role in the control of Ca²⁺-activated Cl⁻ secretion in airway epithelia [11-13]. The membrane potential hyperpolarisation subsequent to the SK4 channels opening establishes the driving force for Cl⁻ secretion.

In airway cells, Ca²⁺-activation of Cl⁻ and K⁺ conductances are under control of multiple agonists (nucleotides, histamine, bradykinin, isoproterenol, neuropeptides) [14-20]. Extracellular nucleotides favor fluid secretion [4, 21, 22], mucociliary clearance [23-25] and ciliary beat frequency [26] by exerting autocrine and/or paracrine effects on airway epithelial cells. Effects of nucleotides are mediated through the metabotropic P2Y receptor family, including P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₃ subtypes which are expressed in human lungs [27, 28]. P2Y receptors stimulation leads to the activation of the Gq/PLC- β signaling pathway and subsequent activation of Ca²⁺-dependent ion channels [29, 30]. Among P2Y receptors, the P2Y₂ subtype (P_{2U}-purinergic), activated by the purine ATP and the pyrimidine UTP, has received most of the attention in the autocrine/paracrine effects reported above. P2Y₂ key role in Cl⁻ secretion has been outlined by the P2Y₂ knockout mouse model, which presents a reduction of 85 to 95% of the nucleotide-stimulated Cl⁻ secretion [31]. The role of the other P2Y receptors subtypes in the airways remains to be determined. The stimulation of P2Y₆ receptors by their most potent agonist (UDP) [32], has also been reported to stimulate ion secretion in human nasal epithelial cells [33] and in the mouse trachea [34]. This effect was maintained in the nasal and tracheal epithelia of the P2Y₂ (-/-) mouse [31]. However, another study using porcine distal bronchi failed to demonstrate stimulation of ion transport in this tissue [35] following P2Y₆ receptors stimulation. P2Y₆ receptors have been proposed as a target for therapies design to correct defective Cl⁻ transport in CF since the P2Y₆-induced Cl⁻ secretion was reported to be maintained in the (CF) gallbladder epithelium [36]. In addition, the stability of UDP at the airway surface and the apical expression of P2Y₆ receptors strengthen the potential therapeutic value of these receptors.

The respiratory epithelium, in interaction with the immune system, also acts as a barrier to protect the lung from inhaled substances and pathogens. Inflammatory stimuli produced in respiratory diseases have been found to regulate human airway epithelium ion transport characteristics [37-39]. In particular, IL-13 and IL-4 have

been reported to modify the human bronchial epithelium ion transport phenotype by reducing Na⁺ absorption and enhancing anion conductance activity following UTP or ionomycin application. Interestingly, the reported link between P2Y receptors and interleukin production in airway epithelial cells [40], suggests that a mutual interaction could exist.

In our study, we looked at the mechanism responsible for the enhancement of nucleotide-induced anion secretion by IL-13 in primary cultures of human bronchial epithelial cells (NHBE). As P2Y₆ receptor-associated signaling pathway has been suggested as a target of interest in the development of new Cl⁻ secretion agonists, we choose to induce Ca²⁺-dependent Cl⁻ secretion by UDP. The functional expression of P2Y₆ receptors in NHBE cells was assessed and two UDP-induced Cl⁻ secretion components (likely mediated by CaCC and CFTR) were identified. The role of K⁺ channels (in particular of SK4 channels) in favoring the driving force for Cl⁻ exit was found to be determinant. The state of differentiation of cells, modulated by Ultrosor G (a commonly used serum substitute) or IL-13, will be shown to modulate (1) Cl⁻ and Na⁺-transports (2) UDP-stimulated Ca²⁺-dependent Cl⁻-secretion amplitude (3) resting and UDP-stimulated ⁸⁶Rb effluxes. The regulation of epithelial K⁺ permeability by Ultrosor G and IL-13 reported in this study is of importance considering the key role of K⁺ channels in Cl⁻ secretion and research of possible targets to stimulate this function in airways of CF patients.

Materials and Methods

Cell culture

NHBE cells (Cambrex, East Rutherford, NJ) were grown on 75 cm²-plastic flasks in bronchial epithelial cell growth medium (BEGM; Clonetics) supplemented with bovine pituitary extract (52 μ g/ml), hydrocortisone (0.5 μ g/ml), human recombinant epidermal growth factor (0.5 ng/ml), epinephrine (0.5 μ g/ml), transferrin (10 μ g/ml), insulin (5 μ g/ml), retinoic acid (0.1 μ g/ml), triiodothyronine (6.5 μ g/ml), gentamicin (50 μ g/ml) and amphotericin B (50 μ g/ml). The medium was changed every 48 h until cells were 90% confluent. Cells were then dissociated with trypsin/EDTA and cultured in a differentiation medium (CM) containing 50% DMEM in BEGM containing the same supplements as above but with the exception of amphotericin B and triiodothyronine and with a final retinoic acid concentration of 50 nM (all-*trans* retinoic acid). Cells grown on inserts (Transwell-Clear, Corning Inc, NY, USA) were kept submerged on both the apical and basolateral sides for the first 7 days of culture, after which they were submitted to an apical air/liquid interface for the remainder of the culture period. Cells

were used between 14 and 21 days of air/liquid interface. For all steps, cells were maintained at 37°C in a 5% CO₂ atmosphere. When indicated, Ultrosor G (2%) was added to the CM medium during the 21 days of cell growth and differentiation under an air/liquid interface and 10 ng/ml IL-13 were applied or not 48 h before experimentation.

Measurements of short-circuit current and epithelial resistance

Cell monolayers were mounted in home made Ussing chambers as described previously [13]. Bath solutions were gassed with 5% CO₂ to adjust the pH to 7.4 and all experiments were performed at 37°C in a humidified atmosphere of 5% CO₂/95% air. The Ussing chamber was connected to an automatic voltage clamp (Physiologic Instruments, VCC-600, TX, USA) and for short-circuit current (I_{sc}) measurements cells were bathed in a Ringer solution containing (in mM): 120 NaCl, 5 KCl, 24.8 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, 2.5 CaCl₂ and 11.1 D-glucose. HCO₃⁻ containing solutions were equilibrated with a gas mixture of 5% CO₂/95% air. Once mounted in the Ussing chamber cell monolayers were allowed an equilibration period of 10 min for the stabilization of the resting I_{sc} . The transepithelial resistance (Rt) was measured by applying (1 s) bipolar 1 mV voltage pulse every 60 s and was calculated according to the Ohm's law ($Rt = \Delta Vt / \Delta I$).

⁸⁶Rb effluxes

Cell monolayers grown on permeable supports were loaded with ⁸⁶Rb (37 kBq/ml) for three hours from the basal side in a humidified atmosphere of 5% CO₂/95% air at 37°C. After three quick washing steps with the non radioactive Ringer solution, ⁸⁶Rb effluxes were followed in open circuit conditions by sampling the basal bathing medium every 30 s. The apical bathing solution was sampled at the end of each experiment and cell monolayers were lysed with NaOH (1N) in order to determine the remaining intracellular radioactivity. The sampled radioactivity was counted using a liquid scintillation counter (Packard Instruments, USA) after the addition of 4 ml liquid scintillation fluid (ACS, Amersham Corporation, IL, USA) to each sample. Efflux rates were calculated as the percentage (%) per min of ⁸⁶Rb loss into the medium relative to total ⁸⁶Rb contained in the monolayer at the beginning of the time period measured ($[\text{cpm}_x - \text{cpm}_{(x+1)}] / \text{cpm}_x \times 100$), where x and x+1 represent successive time points) and plotted as a function of time. Due to the large cellular K⁺ pool, reliable ⁸⁶Rb efflux measurements could be measured during long time periods; after 10 min of ⁸⁶Rb efflux, 85% of the initial ⁸⁶Rb load was still present in non-treated cells. Medium radioactivity at the end of the experiment was at least 20 times larger than the background level. With a rapid time sampling (30 s), only basolateral ⁸⁶Rb effluxes were measured. In experiments reported below, the basolateral ⁸⁶Rb loss was 8 to 9 times larger than the apical one consistent with our previous study using a human bronchial cell line (16HBE14o-) as a model [13]. Nevertheless, the apical ⁸⁶Rb loss was taken into account to calculate efflux rates.

For some of our experiments, we measured the apical ⁸⁶Rb efflux. For technical reasons a sampling of 3 min was chosen. Apical ⁸⁶Rb effluxes were calculated and expressed as the

percentage (%) per min of ⁸⁶Rb loss into the medium relative to the total ⁸⁶Rb contained in the monolayer, taking into account the ⁸⁶Rb loss through basolateral membranes.

Chemicals

DIDS (4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid, Disodium salt), clotrimazole (1-(oChloro α , α diphenylbenzyl)imidazole), ionomycin, UDP and hexokinase were purchased from Sigma (Sigma Chemical Co., MO, USA). To insure that no UTP was contaminated UDP, we pre-incubated the UDP-stock solution with hexokinase (10 U/ml) and glucose (25 mM) for 1h prior experimentation, as described by Lazarowski *et al.*, [33]. The CFTR inhibitor, CFTR_{inh-1723} was a gift of Dr Verkman (San Francisco, USA) and chromanol 293B was a generous gift from Dr J. Pünter (Aventis Pharma Deutschland GmbH, Germany). All reagents were prepared as > 1000 fold stock solutions in DMSO.

Data analysis

All data are represented as mean $\pm$ S.E.M. where *n* indicates the number of experiments. Paired or unpaired Student's *t*-test was used and a *p* value < 0.05 was chosen to indicate statistical significance. Statistical analysis was done by the GraphPAD software version 1.13 (Christiane Mo, University of Montreal, Canada).

Results

UDP-induced I_{sc} response and cell differentiation

Several studies have outlined the key role of supplements in the culture medium, in the differentiation of airway cells [39, 41, 42] into a polarized epithelium presenting an ion transporting phenotype. Effects of two supplements (or serum substitutes) added in the culture medium (Ultrosor G and IL-13) were studied on Na⁺ and Cl⁻ transport capacities of a reconstituted epithelia of NHBE cells. Effect of UDP, a P2Y₆ receptor agonist, was in particular followed since this nucleotide was reported to stimulate Cl⁻ secretion in other airway epithelial models [33, 34]. As reported in the "Materials and Methods" section, cells were first grown for one week submerged on their apical and basolateral sides with CM medium and then differentiated for another two weeks under an air/liquid interface (condition 1). Ultrosor G (2%) was added to the CM medium during the first 21 days of cell differentiation without (condition 2) or with (condition 3) addition of IL-13 (10 ng/ml) for the last 48 hours of cell differentiation. Ion transport experiments were performed in a Ringer solution. As illustrated in Fig. 1A and resumed in Fig. 1D, cells differentiated in CM alone did not exhibit a significant amiloride-sensitive current (sodium transport) ($0.5 \pm 0.2 \mu\text{A} \cdot \text{cm}^{-2}$, *n*=7). Application of UDP (100 μM) on the apical side induced an I_{sc}

Fig. 1. Effect of Ultroser G and IL-13 on amiloride- and UDP-sensitive I_{sc} in NHBE cells: Typical I_{sc} recordings of cells cultured in CM (A), in CM plus Ultroser G (2%) (B) and in CM supplemented with Ultroser G and IL-13 (10 ng/ml) IL-13 was applied 48 hours before experiments. (C). Amiloride (50 μ M) and UDP (100 μ M) were applied on the apical side of the epithelium. In (D) are compared the amiloride sensitive currents (I_{Na}), the fast and transient I_{sc} increase (I_{fast}) and the delayed I_{sc} (I_{late}) occurring after UDP application, in the three culture and differentiation conditions tested. Student's t-test; $n=7$ for CM; $n=8$ for CM plus Ultroser G and $n=8$ for Ultroser G plus IL-13; * $p<0.05$; ** $p<0.01$; *** $p<0.005$ and **** $p<0.0001$ compared to CM; n.s. indicates not significant. §§§ $p<0.005$; §§§§ $p<0.0001$ compared CM plus Ultroser G with or without IL-13.

increase which developed (maximum at $5.3 \pm 2.4 \mu A \cdot cm^{-2}$, $n=7$) and decreased progressively with time. The presence of Ultroser G (Fig. 1B) in the CM medium resulted in the appearance of an amiloride-sensitive current ($5.9 \pm 1.6 \mu A \cdot cm^{-2}$, $n=8$) and in the reduction of the UDP-induced component ($1.4 \pm 0.4 \mu A \cdot cm^{-2}$, $n=8$) in comparison to the control condition (CM medium without Ultroser G) (Fig. 1D). The presence of both Ultroser G and IL-13 in the CM medium (Fig. 1C and 1D) resulted in a decrease by two fold of the amiloride-sensitive current amplitude ($1.9 \pm 0.3 \mu A \cdot cm^{-2}$, $n=8$). However, the main change associated with the presence of IL-13 in the cell culture medium was the development of a new transient current component (I_{fast}) ($33.0 \pm 1.1 \mu A \cdot cm^{-2}$, $n=8$) in addition to the sustained one described above following addition of UDP. After re-examination of the UDP-stimulated current recordings we also noticed the presence of I_{fast} , while considerably reduced, for cells differentiated in the CM medium without any supplement ($1.7 \pm 0.4 \mu A \cdot cm^{-2}$, $n=7$) and for cells differentiated in

CM medium plus Ultroser G ($3.9 \pm 1.0 \mu A \cdot cm^{-2}$, $n=8$) (arrows Fig. 1A and 1B). The second UDP-stimulated current component (I_{late}) was also observed with cells treated with Ultroser G and IL-13 ($6.0 \pm 1.1 \mu A \cdot cm^{-2}$, $n=8$). Fig. 1D gives a comparison between amplitudes of the amiloride-sensitive and the UDP-induced currents (I_{fast} and I_{late}) for each differentiation condition. For cells treated with both Ultroser G and IL-13, basolateral application of UDP (100 μ M) had minor effects on I_{sc} (ΔI_{sc} : $0.3 \pm 0.1 \mu A \cdot cm^{-2}$, $n=4$). A subsequent apical UDP application (after 10 min) resulted in the development of the two UDP-induced I_{sc} components already described (I_{fast} was of $43.0 \pm 2.5 \mu A \cdot cm^{-2}$ and I_{late} was of $4.8 \pm 0.8 \mu A \cdot cm^{-2}$, $n=4$).

It has to be noticed that the transepithelial resistance was higher after differentiation of NHBE cells in CM supplemented with Ultroser G ($1948 \pm 367 \Omega \cdot cm^2$, $n=14$) or with Ultroser G plus IL-13 ($2076 \pm 320 \Omega \cdot cm^2$, $n=22$) than in CM alone ($528 \pm 446 \Omega \cdot cm^2$, $n=7$).

Fig. 2. Pharmacological characterization of the two UDP-induced I_{sc} components: (A) Effect of CFTR_{inh-172} (5 μ M) on the UDP-induced I_{sc} stimulation (I_{fast} and I_{late}) in NHBE cells differentiated in CM plus Ultrosor G and IL-13 (n=4). (B) Effect of DIDS (300 μ M) on I_{fast} and I_{late} (n=4). Drugs were added on the apical side of the epithelium 10 min before 100 μ M UDP application (apical side). Experiments in presence of 50 μ M amiloride. Significance by Student's t-test: *p<0.05 and ***p<0.001 compared with control; n.s. indicates not significant.

Characterization of I_{fast} and I_{late}

We intended to better characterize the two UDP-induced I_{sc} components (I_{fast} and I_{late}) present in NHBE cells treated with IL-13 and Ultrosor G. Since the Na^+ transport was blocked by amiloride it was likely that the UDP-stimulated I_{sc} was associated to the development of an anion conductance as previously reported in nasal

Fig. 3. Effect of clotrimazole on the two UDP-induced I_{sc} components: (A) Typical control recording: UDP-induced I_{sc} stimulation (I_{fast} and I_{late}) was tested in NHBE cells differentiated in CM plus Ultrosor G and IL-13. (B) Typical clotrimazole recording; clotrimazole (10 μ M) was added on the basal side of the epithelium 15 min before 100 μ M UDP application (apical side). CFTR_{inh-172} was added at the end of the experiment. (C) Comparison of the effect of clotrimazole on the mean ΔI_{sc} changes (I_{fast} and I_{late}), n=7. Significance by Student's t-test: **p<0.005 compared with control; n.s. indicates not significant.

cells [33], in the 16HBE14o- bronchial cell line [13] or in the mouse trachea [34]. We therefore studied effects of two anion blockers, DIDS and CFTR_{inh-172}, (Fig. 2) on the UDP-stimulated I_{sc} . CFTR_{inh-172} (5 μ M), a specific CFTR inhibitor [43] added 10 min before UDP application had no effect on I_{fast} while it almost completely blocked I_{late} ($79.2 \pm 4.8\%$ inhibition, n=4, p<0.001); the inhibitory

Fig. 4. UDP stimulates ^{86}Rb fluxes in NHBE cells through SK4 channel opening: ^{86}Rb effluxes were measured through basolateral membranes of NHBE cells differentiated in CM plus Ultrosor G and IL-13 (n=7) in presence or absence of clotrimazole (10 μM basolateral application). UDP (100 μM , apical side) was added after 270 s (at 30 s, the high ^{86}Rb rate correspond to unwashed extracellular medium).

effect of $\text{CFTR}_{\text{inh-172}}$ was also observed when added on the decay of I_{late} (see Fig. 3B). DIDS (300 μM), a non-specific Cl^- channel blocker reported to inhibit Ca^{2+} -activated Cl^- channels [44, 45] induced a $64.1 \pm 5.9\%$ inhibition (n=4, $p < 0.001$) of I_{fast} but was ineffective on I_{late} when added 10 min before UDP application. These results confirm the anionic nature of the UDP-stimulated I_{sc} , likely corresponding to a Cl^- transport, and are indicative of the stimulation of two different components by UDP.

The role of K channels in the UDP-induced anion secretion

In epithelial cells, K^+ channels are involved in Cl^- secretion by generating a favorable electrochemical gradient for Cl^- exit [8, 9]. In native airway cells [10, 11] as in airway cell lines [12, 13, 19], the small conductance Ca^{2+} -activated K^+ channels, SK4, were shown to play a major role in the control of the Ca^{2+} -activated Cl^- secretion. Effects of clotrimazole, a specific SK4 channel inhibitor [46] was therefore investigated on the UDP-stimulated I_{sc} in NHBE cells treated with IL-13 for 48 h. Basolateral application of clotrimazole (10 μM) 15 min before UDP application inhibited I_{fast} by $61.1 \pm 6.2\%$ (n=7, $p < 0.001$) but was ineffective on I_{late} (the difference between control and clotrimazole-treated cells was of $2.8 \pm 1.2 \mu\text{A} \cdot \text{cm}^{-2}$, n=7; N.S.; Fig. 3).

Involvement of Ca^{2+} -activated K^+ channels in the stimulation of anion secretion following apical UDP application was further investigated by following ^{86}Rb effluxes through NHBE cell membranes. Our preliminary experiments demonstrated that $88 \pm 1\%$ (n=24) of ^{86}Rb effluxes (including effluxes through Ca^{2+} -activated K^+ channels) were occurring through basolateral membranes with a minimal transport through apical ones. For this reason, we used a 30 s sampling time period to follow as

a function of time ^{86}Rb effluxes through basolateral membranes of NHBE epithelia. As illustrated by Fig. 4, after a 270 s control period, apical application of UDP (100 μM) induced a transient stimulation of ^{86}Rb effluxes (1.6 fold maximal increase, $p < 0.001$, n=7) which was not observed when epithelia were treated with 10 μM clotrimazole (n=7). Altogether these experiments showed that UDP-induced anion secretion in IL-13-treated NHBE cells is associated with the stimulation of SK4 channels. This observation is consistent with the already reported increase of Ca_i following UDP application [47].

Ionomycin-induced anion transport is enhanced by IL-13

NHBE cells not treated with IL-13 did not exhibit a robust transient I_{sc} component (I_{fast}) following UDP stimulation (Fig. 1). As this may have been resulted from an altered intracellular calcium signaling, we studied effects of IL-13 treatment on I_{sc} and ^{86}Rb effluxes stimulated by the calcium ionophore, ionomycin. Ionomycin has been previously used to stimulate calcium-dependent anion transport in a human bronchial cell line [13, 15] as in primary cultures of human bronchial cells [45]. In agreement with these previous reports, we observed an I_{sc} stimulation by ionomycin (0.2 μM) (Fig. 5A) for IL-13-treated NHBE cells. The ionomycin-induced current presented a biphasic profile as described for the UDP-stimulated I_{sc} (a fast and transient phase followed by a delayed and sustained one). Comparison of the peak values for both phases (Fig. 5C) showed that I_{fast} was 7 fold larger ($p < 0.0001$, n=5) and I_{late} 2.8 fold larger ($p < 0.001$, n=5) for IL-13-treated NHBE cells versus non-treated cells (Fig. 5A, Fig. 5B). The enhancement effect of IL-13 on the Ca^{2+} -dependent Cl^- secretion is therefore observed for receptor-mediated and receptor-independent anion secretion.

Fig. 5. The ionomycin-induced anion transport is stimulated by IL-13: Typical I_{sc} recordings of cells cultured in CM plus Ultrosor G (2%) and IL-13 (10 ng/ml) (A) and in CM supplemented only with Ultrosor G (B). Amiloride (50 μM) and ionomycin (0.2 μM) were applied on the apical side of the epithelium. (C) Comparison of the fast- and delayed- I_{sc} changes following ionomycin application (peak measured) in both culture conditions. Significance by Student's t-test: **** $p < 0.0001$ and ** $p < 0.01$, $n = 5$, compared with cells treated without IL-13.

Ultrosor G and IL-13 favor K^+ permeability in NHBE cells through activation of basolateral SK4 channels

Considering the key role of SK4 channels in the Ca^{2+} -activated anion Cl^- transport, we investigated their possible modulation by the supplements (Ultrosor G or IL-13) added to the cell culture medium. We first assessed SK4 channels activation by ionomycin in cells treated with Ultrosor G (plus or minus IL-13) and in cells maintained in the non-supplemented CM medium (Fig. 6). Treatment of NHBE cells with Ultrosor G, with ($n = 11$) or without IL-13 ($n = 11$), increased ^{86}Rb effluxes in the control period (first 240 s without ionomycin) compared to cells maintained in non-supplemented CM medium ($n = 8$). ^{86}Rb effluxes were of 0.60 ± 0.06 %/min, 1.03 ± 0.12 %/min and 1.34 ± 0.13 %/min, in CM, CM plus Ultrosor G and CM plus Ultrosor G and IL-13, respectively. No significant difference was observed between IL-13-treated or non-treated cells suggesting that Ultrosor G by itself was at the origin of the ^{86}Rb effluxes increase and that IL-13

may not affect K^+ permeabilities responsible for the resting ^{86}Rb effluxes. Ionomycin (0.2 μM) stimulated ^{86}Rb effluxes through NHBE basolateral membranes for each of the three experimental conditions tested. However, ionomycin stimulation was larger for IL-13 and Ultrosor G-treated cells than for cells treated only with Ultrosor G (differences in fluxes becoming significant after the first minute of ionomycin application). Cells cultured in non-supplemented CM medium presented the smallest ionomycin-induced ^{86}Rb efflux. We also observed that ^{86}Rb effluxes were stimulated by UDP independently of IL-13 treatment. However, the difference in UDP-induced ^{86}Rb effluxes amplitude between IL-13-treated (1.29 ± 0.13 %/min) and non-treated cells (0.77 ± 0.11 %/min) was found significant ($p < 0.03$, $n = 18$) (Fig. 7). The marked effect of IL-13 treatment to stimulate I_{fast} upon UDP application could have been therefore due to changes in K_{Ca} transport through basolateral membranes of NHBE cells.

Fig. 6. Effect of Ultrosor G and IL-13 on the ionomycin stimulation of ^{86}Rb effluxes in NHBE cells: (A) In less than 30 sec, ionomycin ($0.2\ \mu\text{M}$, apical side) induces an increase of ^{86}Rb effluxes measured through basolateral membranes of NHBE cells differentiated in CM ($n=8$), in CM plus Ultrosor G (2%) ($n=11$) and in CM supplemented with Ultrosor G and IL-13 ($10\ \text{ng/ml}$) ($n=11$). (B) Comparison of ^{86}Rb efflux rate (%/min) in the three culture conditions plotting the means of the five ^{86}Rb efflux rates before and after ionomycin application. Significance by Student's t-test: * $p<0.05$; *** $p<0.0001$ compared with cells differentiated in CM.

Are apical K channels involved in UDP and ionomycin-stimulated currents?

We already mentioned that ^{86}Rb effluxes through apical membranes of NHBE epithelia were lower by 8 to 9 times than ^{86}Rb effluxes through basolateral membranes (see above). We nevertheless investigated a possible effect of UDP or ionomycin on the apical ^{86}Rb permeability. For technical reasons, a 3 min sampling time period was chosen for these experiments. Apical application of UDP ($100\ \mu\text{M}$) or ionomycin ($0.2\ \mu\text{M}$) induced a small increase in apical ^{86}Rb effluxes in IL-13-treated cells (control efflux: $0.12 \pm 0.04\ \%/ \text{min}$ compare to UDP-induced efflux: $0.52 \pm 0.08\ \%/ \text{min}$, $p<0.0001$, $n=4$; control efflux: $0.07 \pm 0.01\ \%/ \text{min}$ compare to ionomycin-induced efflux $0.93 \pm 0.05\ \%/ \text{min}$, $n=4$, $p<0.001$). No significant differences were found between

Fig. 7. Effect of IL-13 on the UDP-stimulated ^{86}Rb effluxes in NHBE cells: UDP ($100\ \mu\text{M}$, apical side) induces an increase of ^{86}Rb effluxes measured through basolateral membranes of NHBE cells differentiated in CM plus Ultrosor G (2%) ($n=10$) and in CM supplemented with Ultrosor G and IL-13 ($10\ \text{ng/ml}$) ($n=18$). ^{86}Rb efflux rate (%/min) in both culture conditions are represented as the means of ^{86}Rb efflux rates calculated 2.5 min (five 30 s samples) just before and after UDP application.

IL-13-treated and non-treated cells for UDP- or ionomycin-induced apical ^{86}Rb effluxes (data not shown). Therefore, apical Ca^{2+} -activated K^+ channels may contribute to the membrane potential hyperpolarization following UDP or ionomycin application strengthening the key role of basolateral K_{Ca} channels. It may also correspond to different calcium-activated K^+ channels from those present on the basolateral membranes since their activity did not appear to be affected by IL-13 treatment.

Are K_{cAMP} channels involved in the UDP-induced I_{sc} stimulation?

K_{cAMP} channels have been described as another pathway for K^+ transport in secreting epithelia [8]. We therefore investigated the possible implication of K_{cAMP} channels in the UDP-mediated I_{sc} stimulation. In IL-13-treated NHBE cells, chromanol 293B ($100\ \mu\text{M}$), a specific inhibitor of the KCNQ1 channel, part of the K_{cAMP} channel [12, 48-50] did not affect the amplitude of the two UDP-stimulated I_{sc} components (I_{fast} was of $24.8 \pm 2.9\ \mu\text{A} \cdot \text{cm}^{-2}$ and $22.2 \pm 4.8\ \mu\text{A} \cdot \text{cm}^{-2}$ and I_{late} was of $4.4 \pm 0.9\ \mu\text{A} \cdot \text{cm}^{-2}$ and $3.4 \pm 0.7\ \mu\text{A} \cdot \text{cm}^{-2}$ in absence or presence of chromanol respectively, $n=6$). Chromanol effect on basolateral ^{86}Rb effluxes was also investigated upon UDP application. As illustrated in Fig. 8A, this compound had no effect on UDP-induced ^{86}Rb effluxes. However, to

Fig. 8. Effects of chromanol 293B and forskolin/IBMX on ^{86}Rb effluxes in NHBE cells: (A) UDP (100 μM , apical side) induces an increase of ^{86}Rb effluxes measured through basolateral membranes of NHBE cells in control and chromanol 293B treated cells ($n=7$). Chromanol 293B (100 μM) was added to the basolateral side of the monolayers 10 min before and during the experiment (B) Forskolin (5 μM) and IBMX (500 μM) are added on the apical side when indicated; $n=4$.

assess more directly for the expression of K_{cAMP} channels, we used forskolin (5 μM) plus IBMX (100 μM) to increase the intracellular cAMP content of NHBE cells. This cocktail induced a modest but significant stimulation of basolateral ^{86}Rb effluxes (Fig. 8B). This effect was partially blocked (66% inhibition) by chromanol 293B. After 3.0 min of forskolin plus IBMX application, NHBE cells lost 5.46 ± 0.16 % of their initial ^{86}Rb content ($n=4$) and 4.51 ± 0.21 % in presence of chromanol 293B ($n=4$, $p<0.005$).

Discussion

This study outlines the primordial role of supplements added in the culture medium of airway primary cultures in determining their ion transport phenotype. Using short-

circuit current and ^{86}Rb efflux assays we shown in particular the importance of Ultrosor G and IL-13 in modulating the activity of small conductance Ca^{2+} -activated K^{+} channels, SK4, which play a key role in Ca^{2+} -induced Cl^{-} secretion.

Effects of Ultrosor G and IL-13 on the ion transport phenotype of NHBE cells

The presence of Ultrosor G in the differentiation medium of NHBE cells was shown to be essential in the appearance of an amiloride-sensitive sodium transport as well as in the differentiation of a tight epithelium. A similar effect of Ultrosor G was already reported for primary cultures of the human tracheal epithelium which presented high I_{sc} levels (I_{Na}) following differentiation in presence of this serum substitute [41, 51].

In presence of both Ultrosor G and IL-13, NHBE cells ion transport phenotype was considerably modified. This condition increased (twelve times for I_{fast}) the UDP-dependent anion transport while reducing by twice the Na^{+} transport activity switching the epithelium Na^{+} absorbing phenotype to a Cl^{-} secreting phenotype. Therefore our experiments confirmed the inhibitory effect of IL-13 on Na^{+} transport activity and the enhancement effect of this interleukin on the nucleotide-induced Cl^{-} secretion reported in human bronchial cells by Danahay *et al*, [39]. This effect of IL-13 on sodium transport has been linked to a reduction of $\beta\text{-ENaC}$ and $\gamma\text{-ENaC}$ subunits mRNA levels by Galietta *et al*, [38].

Stimulation of NHBE cells P2Y_6 receptors by their more potent agonist UDP induced a biphasic increase in I_{sc} (I_{fast} and I_{late}) as described after P2Y_2 receptors stimulation by UTP in the same cellular model [39]. The pharmacological characterization of these two UDP-induced I_{sc} components suggests that two different anion transport pathways are responsible for I_{fast} and I_{late} . Ca^{2+} -activated channels could participate in I_{fast} since this phase is inhibited by DIDS, which was previously reported as a CaCC channels inhibitor [44, 45]. The inhibition of I_{late} by the CFTR chloride channel blocker CFTR_{inh172} supports the hypothesis of the involvement of CFTR channels in that phase. Stimulation of this channel by cAMP or by an increase of Ca^{2+}_i which in turn stimulates PKC activity [52] is likely implicated. In addition this study showed a different sensitivity of I_{fast} and I_{late} to IL-13 treatment of NHBE cells. Indeed, I_{fast} was increased by twelve times while I_{late} was increased by two times after treatment of cells with IL-13. This different interleukin sensitivity of I_{fast} and I_{late} also argues in favor of the stimulation of two different anion pathways stimulated by UDP.

The key role of SK4 channels in the UDP-stimulated I_{sc}

The role of K^+ channels in the genesis of a favorable electrochemical gradient to Cl^- exit through apical membranes of airway epithelia has been outlined by numerous studies [8, 9, 53]. In native airway cells [10, 11] as in airway cell lines [12, 13, 19], SK4 channels were shown to play a major role in the control of Ca^{2+} -activated Cl^- secretion. I_{fast} inhibition of by clotrimazole confirmed the relation between I_{fast} and Ca^{2+} -induced Cl^- secretion and showed the stimulation of SK4 channels following $P2Y_6$ receptors activation. I_{fast} occurred simultaneously to the transient increase of ^{86}Rb effluxes rate through basolateral membranes of NHBE cells, i.e. in less than 30 s after UDP application (see Fig. 4). Conversely to I_{fast} , clotrimazole had no effect on I_{late} , suggesting that SK4 channels did not participate to the electrochemical gradient for Cl^- secretion during that second UDP-induced I_{sc} phase. In agreement with these observations, it can be noticed that I_{late} occurred between 2 and 4 min following UDP application when the SK4 channel activity, assessed by measuring ^{86}Rb efflux, had returned to its basal level.

UDP has been shown to elevate intracellular calcium as well as intracellular cAMP in colonic epithelia [47]. Since SK4 channels were not involved in I_{late} we investigated for the role of K_{cAMP} channels in this UDP-induced I_{sc} component. A modest and progressive stimulation of basolateral ^{86}Rb effluxes was observed after applying forskolin plus IBMX, a cocktail known to increase the intracellular cAMP content. This effect was partially inhibited by chromanol 293B which was consistent with the presence of functional K_{cAMP} channels in NHBE cells. In addition, we identified KCNQ1 subunit mRNA by RT-PCR in this cellular model (unpublished data). As UDP-induced I_{fast} and I_{late} as well as UDP-induced ^{86}Rb effluxes were not sensitive to chromanol 293B, the stimulation of K_{cAMP} channels following $P2Y_6$ receptors is unlikely. Therefore, the lack of effect of SK4 and KCNQ1 channels inhibitors on I_{late} may suggest the involvement of other K^+ channels in establishing the electrochemical gradient necessary to Cl^- exit through CFTR channels. While we quantified ^{86}Rb efflux through apical membranes being 10 times smaller than the basolateral efflux, we showed the stimulation of apical ^{86}Rb permeability following $P2Y_6$ receptors activation by UDP or ionomycin application. Apical K^+ channels could therefore strengthen the driving force for Cl^- during Ca^{2+} -dependent Cl^- secretion. The molecular identity of these

apical calcium-activated channels remains to be determined.

Cell differentiation by IL-13 affects K^+ permeability

We showed that Ultrosor G treatment of NHBE cells stimulated resting and ionomycin-stimulated basolateral ^{86}Rb effluxes (see Fig. 6). IL-13 treatment following Ultrosor G led to a further increase of resting and ionomycin-stimulated ^{86}Rb effluxes.

The lack of effect of clotrimazole and chromanol 293B on resting ^{86}Rb effluxes suggested the implication of other K^+ channels in this resting basolateral K^+ permeability. We mentioned earlier that transepithelial Na^+ transport (absorption) was increased by Ultrosor G. According to Ussing model [54] and related to the "crosstalk" phenomena [55] a concomitant basolateral K^+ permeability stimulation associated to Na^+ absorption is expected. The identity of Ultrosor G-stimulated K^+ channels is still not yet determined. In agreement with our study, in H441 human distal airway epithelial cells SK4(KCNN4) did not participate to the membrane potential nor to the transepithelial Na^+ transport in resting conditions while SK4 was suggested to be activated upon a hormone-induced cell calcium increase [56].

Ionomycin-induced basolateral ^{86}Rb effluxes (through SK4 channels) were larger for Ultrosor G versus non-Ultrosor G differentiated cells. IL-13 further enhanced this difference. This observation may explain the increase of I_{fast} amplitude since the membrane hyperpolarization and the resulting driving force for chloride exit are expected to correlate with SK4 channels activity. However it has to be noticed that the UDP-stimulated chloride secretion likely mediated by CaCC (I_{fast}), was 10 times larger in cells treated with IL-13 than in non-treated cells, while the UDP-stimulated ^{86}Rb effluxes were only increased by two fold in IL-13-treated NHBE cells. In addition, while Ultrosor G alone enhanced ionomycin or UDP-stimulated ^{86}Rb effluxes I_{fast} was not enhanced by Ultrosor G alone (see Fig. 1). These findings lead us to postulate that a transcriptional effect of IL-13 on CaCC (or associated proteins) is probably also involved in the stimulation of the UDP-induced chloride secretion (I_{fast}). The concomitant enhancement of SK4 channels activity together with an enhanced activity of CaCC would strengthen the IL-13 induced stimulation of chloride secretion. Our study is to our knowledge the first report of SK4 activity modulation by factors involved in airway epithelium differentiation.

Apical expression of P2Y₆ receptors in NHBE cells

The stimulation of Cl⁻ secretion through CaCC and CFTR channels following UDP application showed the functional expression of P2Y₆ receptors since UDP as been described as their most potent agonist. As described previously for the human nasal epithelium [33, 57], we only observed functional expression of P2Y₆ receptors on apical membranes of NHBE cells. However in the human bronchial epithelial cell line 16HBE14o-, we observed apical and basolateral expression of P2Y₆ receptors (unpublished data) as it was reported for the mouse tracheal epithelia [34]. The stimulation of bronchial Cl⁻ secretion through CaCC channels following apical P2Y₆ receptors activation may represents a target

signaling pathway in the development of drugs aiming to restore Cl⁻ transport in CF airways [33]. The identification of CaCC channels at a molecular level may also provide insights in the development of new Cl⁻ secretion agonist. In this context, Il-13, because of its modulating effect on the ion transport phenotype of human bronchial cells might be used as a tool to identify proteins participating to Cl⁻ secretion.

Acknowledgements

This study was supported by CNRS, UNSA and the CF association (VLM).

References

- Al-Bazzaz F, Jayaram T: Ion transport by canine tracheal mucosa: effect of elevation of cellular calcium. *Exp Lung Res* 1981;2:121-130.
- Boucher RC, Cheng EH, Paradiso AM, Stutts MJ, Knowles MR, Earp HS: Chloride secretory response of cystic fibrosis human airway epithelia. Preservation of calcium but not protein kinase C- and A-dependent mechanisms. *J Clin Invest* 1989;84:1424-1431.
- Hartmann T, Kondo M, Mochizuki H, Verkman AS, Widdicombe JH: Calcium-dependent regulation of Cl secretion in tracheal epithelium. *Am J Physiol* 1992;262:163-168.
- Knowles MR, Clarke LL, Boucher RC: Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. *N Engl J Med* 1991;325:533-538.
- Clarke LL, Boucher RC: Chloride secretory response to extracellular ATP in human normal and cystic fibrosis nasal epithelia. *Am J Physiol* 1992;263:348-356.
- Grubb BR, Boucher RC: Pathophysiology of gene-targeted mouse models for cystic fibrosis. *Physiol Rev* 1999;79:S193-214.
- Willumsen NJ, Davis CW, Boucher RC: Cellular Cl⁻ transport in cultured cystic fibrosis airway epithelium. *Am J Physiol* 1989;256:1045-1053.
- Mc Cann JD, Welsh MJ: Basolateral K⁺ channels in airway epithelia. II. Role in Cl⁻ secretion and evidence for two types of K⁺ channel. *Am J Physiol* 1990;258:343-348.
- Devor DC, Frizzell RA: Calcium-mediated agonists activate an inwardly rectified K⁺ channel in colonic secretory cells. *Am J Physiol* 1993;265:1271-1280.
- Devor DC, Bridge RJ, Pilewski, JM: Pharmacological modulation of ion transport across wild type and ?F508 CFTR-expressing human bronchial epithelia. *Am J Physiol Cell Physiol* 2000;279:461-479.
- Mall M, Gonska T, Thomas J, Schrieder R, Seydewitz HH, Kuehr J, Brandis M, Kunzelmann K: Modulation of Ca²⁺ - activated Cl⁻ secretion by basolateral K⁺ channels in human normal and cystic fibrosis airway epithelia. *Pediatr Res* 2003;53:608-618.
- Cowley EA, Linsdell P: Characterization of basolateral K⁺ channels underlying anion secretion in the human airway cell line Calu-3. *J Physiol* 2002;538:747-757.
- Bernard K, Bogliolo S, Soriani O, Ehrenfeld, J: Modulation of calcium-dependent chloride secretion by basolateral SK4-like channels in a human bronchial cell line. *J Membr Biol* 2003;196:15-31.
- Cozens AL, Yezzi MJ, Kunzelmann K, Ohru T, Chin L, Eng K, Finkbeiner WE, Widdicombe JH, Gruener DC: CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1994;10:38-47.
- Koslowsky T, Hug T, Ecke D, Klein P, Greger R, Gruenert DC, Kunzelmann K: Ca(2+)- and swelling-induced activation of ion conductances in bronchial epithelial cells. *Pflugers Arch* 1994;428:597-603.
- Walsh DE, Harvey BJ, Urbach V: CFTR regulation of intracellular calcium in normal and cystic fibrosis human airway epithelia. *J Membr Biol* 2000;177:209-19.
- Mall M, Wissner A, Shreiber R, Kuehr J, Seydewitz HH, Brandis M, Greger R, Kunzelmann K: Role of K(V)LQT1 in cyclic adenosine monophosphate-mediated Cl(-) secretion in human airway epithelia. *Am J Respir Cell Mol Biol* 2000;23:283-289.
- Abraham WM, Bourdelais AJ, Sabater JR, Ahmed A, Lee TA, Serebriakov I, Baden DG: Airway responses to aerosolized brevetoxins in an animal model of asthma. *Am J Respir Crit Care Med* 2005;171:26-34.
- Bernard K, Bogliolo S, Ehrenfeld J: Vasotocin and vasopressin stimulation of the chloride secretion in the human bronchial epithelial cell line, 16HBE14o-. *Br J Pharmacol* 2005;144:1037-1050.
- Derand R, Montoni A, Bulteau-Pignoux L, Janet T, Moreau B, Muller JM, Becq F: Activation of VPAC1 receptors by VIP and PACAP-27 in human bronchial epithelial cells induces CFTR-dependent chloride secretion. *Br J Pharmacol* 2004;141:698-708.2004.
- Stutts MJ, Fitz JG, Paradiso AM, Boucher RC: Multiple modes of regulation of airway epithelial chloride secretion by extracellular ATP. *Am J Physiol* 1994;267:1442-1451.
- Hwang TH, Schwiebert EM, Guggino WB: Apical and basolateral ATP stimulates tracheal epithelial chloride secretion via multiple purinergic receptors. *Am J Physiol* 1996;270:1611-1623.

- 23 Kim CH, Kim SS, Choi JY, Shin JH, Kim JY, Namkung W, Lee JG, Lee MG, Yoon JH: Membrane-specific expression of functional purinergic receptors in normal human nasal epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2004;287:835-842.
- 24 Mason SJ, Paradiso AM, Boucher RC: Regulation of transepithelial ion transport and intracellular calcium by extracellular ATP in human normal and cystic fibrosis airway epithelium. *Br J Pharmacol* 1991;103:1649-1656.
- 25 Davis CW, Dowell ML, Lethem M, Van Scott M: Goblet cell degranulation in isolated canine tracheal epithelium: response to exogenous ATP, ADP, and adenosine. *Am J Physiol* 1992;262:C1313-323.
- 26 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;280:C1485-497.
- 27 Ralevic V, Burnstock G: Receptors for purines and pyrimidines. *Pharmacol Rev* 1998;50:413-492. Review.
- 28 Zhang FL, Luo L, Gustafson E, Palmer K, Qiao X, Fan X, Yang S, Laz TM, Bayne M, Monsma F Jr: P2Y₁₃: identification and characterization of a novel Galphai-coupled ADP receptor from human and mouse. *J Pharmacol Exp Ther* 2002;301:705-713.
- 29 Leipziger J: Control of epithelial transport via luminal P2 receptors. *Am J Physiol Renal Physiol* 2003;284:419-432. Review.
- 30 Bucheimer RE, Linden J: Purinergic regulation of epithelial transport. *J Physiol* 2004;555:311-21.
- 31 Cressman VL, Lazarowski E, Homolya L, Boucher RC, Koller BH, Grubb BR: Effect of loss of P2Y₂ receptor gene expression on nucleotide regulation of murine epithelial Cl⁻ transport. *J Biol Chem* 1999;274:26461-26468.
- 32 Nicholas RA, Watt WC, Lazarowski 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;50:224-229.
- 33 Lazarowski ER, Paradiso AM, Watt WC, Harden TK, Boucher RC: UDP activates a mucosal-restricted receptor on human nasal epithelial cells that is distinct from the P2Y₂ receptor. *Proc Natl Acad Sci U S A* 1997;94:2599-603.
- 34 Schreiber R, Kunzelmann K: Purinergic P2Y₆ receptors induce Ca²⁺ and CFTR dependent Cl⁻ secretion in mouse trachea. *Cell Physiol Biochem* 2005;16:99-108.
- 35 Inglis SK, Collett A, McAlroy HL, Wilson SM, Olver RE: Effect of luminal nucleotides on Cl⁻ secretion and Na⁺ absorption in distal bronchi. *Pflugers Arch* 1999;438:621-627.
- 36 Lazarowski ER, Rochelle LG, O'Neil WK, Ribeiro CM, Grubb BR, Zhang V, Harden TK, Boucher RC: Cloning and functional characterization of two murine uridine nucleotide receptors reveal a potential target for correcting ion transport deficiency in cystic fibrosis gallbladder. *J Pharmacol Exp Ther* 2001;297:43-49.
- 37 Galletta LJ, Folli C, Marchetti C, Romano L, Carpani D, Conese M, Zegarar-Moran O: Modification of transepithelial ion transport in human cultured bronchial epithelial cells by interferon-gamma. *Am J Physiol Lung Cell Mol Physiol* 2000;278:1186-1194.
- 38 Galletta LJ, Pagesy P, Folli C, Caci E, Romio L, Costes B, Nicolis E, Cabrini G, Goossens M, Ravazzolo R, Zegarar-Moran O: IL-4 is a potent modulator of ion transport in the human bronchial epithelium in vitro. *J Immunol* 2002;168:839-45.
- 39 Danahay H, Atherton H, Jones G, Bridges RJ, Poll CT: Interleukin-13 induces a hypersecretory ion transport phenotype in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2002;282:226-36.
- 40 Muller T, Bayer H, Myrtek D, Ferrari D, Sorichter S, Ziegeehagen MW, Zissel G, Virchow JC Jr, Luttmann W, Norgauer J, Di Virgilio F, Idzko M: The P2Y₁₄ receptor of airway epithelial cells: coupling to intracellular Ca²⁺ and IL-8 secretion. *Am J Respir Cell Mol Biol* 2005;33:601-609.
- 41 Sachs LA, Finkbeiner WE, Widdicombe JH: Effects of media on differentiation of cultured human tracheal epithelium. *In Vitro Cell Dev Biol Anim* 2003;39:56-62.
- 42 Dimova S, Brewster ME, Noppe M, Jorissen M, Augustijns P: The use of human nasal in vitro cell systems during drug discovery and development. *Toxicol In Vitro* 2005;19:107-122.
- 43 Ma T, Thiagarajah JR, Yang H, Sonawane ND, Folli C, Galletta LJ, Verkman AS: Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J Clin Invest* 2002;110:1651-8.
- 44 Ji HL, DuVall MD, Patton HK, Satterfield CL, Fuller CM, Benos DJ: Functional expression of a truncated Ca²⁺-activated Cl⁻ channel and activation by phorbol ester. *Am J Physiol* 1998;274:C455-464.
- 45 Atherton HC, Jones G, Danahay H: IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. *Am J Physiol Lung Cell Mol Physiol* 2003;285:730-739.
- 46 Ishii Y, Yang H, Sakamoto T, Nomura A, Hasegawa S, Hirata F, Bassett DJ: Rat alveolar macrophage cytokine production and regulation of neutrophil recruitment following acute ozone exposure. *Toxicol Appl Pharmacol* 1997;147:214-223.
- 47 Kottgen M, Loffler T, Jacobi C, Nitschke R, Pavenstadt H, Schreiber R, Frische S, Nielsen S, Leipziger J: P2Y₆ receptor mediates colonic NaCl secretion via differential activation of cAMP-mediated transport. *J Clin Invest* 2003;111:371-379.
- 48 Bleich M, Briel M, Busch AE, Lang HJ, Gerlach U, Gogelein H, Greger R, Kunzelmann K: KVLQT channels are inhibited by the K⁺ channel blocker 293B. *Pflugers Arch* 1997;434:499-501.
- 49 Honore E, Attali B, Romey G, Heurteaux C, Ricard P, Lesage F, Lazdunski M, Barhanin J: Cloning, expression, pharmacology and regulation of a delayed rectifier K⁺ channel in mouse heart. *EMBO J* 1991;10:2805-2811.
- 50 Warth R, Bleich M: K⁺ channels and colonic function. *Rev Physiol Biochem Pharmacol* 2000;140:1-62. Review.
- 51 Yamaya M, Finkbeiner WE, Widdicombe JH: Ion transport by cultures of human tracheobronchial submucosal glands. *Am J Physiol Lung Cell Mol Physiol* 1991;261:485-490.
- 52 Tabcharani JA, Chang XB, Riordan JR, Hanrahan JW: Phosphorylation-regulated Cl⁻ channel in CHO cells stably expressing the cystic fibrosis gene. *Nature* 1991;352:628-631.
- 53 Davis KA, Cowley EA: Two-pore-domain potassium channels support anion secretion from human airway Calu-3 epithelial cells. *Pflugers Arch* 2006;451:631-641.
- 54 Koefoed-Johnsen V, Ussing HH: The nature of the frog skin potential. *Acta Physiol Scand* 1958;42:298-308.
- 55 McRobbie EAC, Ussing HH: Osmotic behaviour of the epithelial cells of frog skin. *Acta Phys Scand* 1961;53:348-365.
- 56 Wilson SM, Brown SG, McTavish N, McNeill RP, Husband EM, Inglis SK, Olver RE, Clunes MT: Expression of intermediate-conductance, Ca²⁺-activated K⁺ channel (KCNN4) in H441 human distal airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2006;291:957-965.
- 57 Kim KC, Zheng QX, Van-Seuning I: Involvement of a signal transduction mechanism in ATP-induced mucin release from cultured airway goblet cells. *Am J Respir Cell Mol Biol* 1993;8:121-125.