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IL-4 Is a Potent Modulator of Ion Transport in the Human Bronchial Epithelium In Vitro[1]

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Recent data show that proinflammatory stimuli may modify significantly ion transport in the airway epithelium and therefore the properties of the airway surface fluid. We have studied the effect of IL-4, a cytokine involved in the pathogenesis of asthma, on transepithelial ion transport in the human bronchial epithelium in vitro. Incubation of polarized bronchial epithelial cells with IL-4 for 6–48 h causes a marked inhibition of the amiloride-sensitive Na^+ channel as measured in short circuit current experiments. On the other hand, IL-4 evokes a 2-fold increase in the current activated by a cAMP analog, which reflects the activity of the cystic fibrosis transmembrane conductance regulator (CFTR). Similarly, IL-4 enhances the response to apical UTP, an agonist that activates Ca^{2+}-dependent Cl^- channels. These effects are mimicked by IL-13 and blocked by an antagonist of IL-4Rα. RT-PCR experiments show that IL-4 elicits a 7-fold decrease in the level of the γ amiloride-sensitive Na^+ channel mRNA, one of the subunits of the amiloride-sensitive Na^+ channel, and an increase in CFTR mRNA. Our data suggest that IL-4 may favor the hydration of the airway surface by decreasing Na^+ absorption and increasing Cl^- secretion. This could be required to fluidify the mucus, which is hypersecreted during inflammatory conditions. On the other hand, the modifications of ion transport could also affect the ion composition of airway surface fluid. ***The Journal of Immunology,*** **2002, 168: 839–845.**

Airway epithelium controls the thickness and ion composition of airways surface fluid through the activity of ion channels and transporters (1). The amiloride-sensitive Na^+ channel (ENaC),[3] which is composed of at least three subunits (α, β, and γ), is responsible for Na^+ and consequently fluid absorption. The important physiological role of ENaC is demonstrated by the finding that α-ENaC knockout mice die at birth because they are unable to reabsorb the fluid that fills the airways (2). Furthermore, patients affected by pseudohypoaldosteronism, due to mutations impairing ENaC function, are affected by poor fluid absorption in the airways (3). In the presence of a favorable gradient, e.g., when ENaC is blocked with amiloride, Cl^- may be secreted through anion channels such as cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+}-dependent Cl^- channels. Mutations to CFTR, which occur in cystic fibrosis (CF) patients, result in defective Cl^- transport and increased Na^+ absorption (4, 5). These abnormalities cause impaired mucociliary clearance and eventually lead to chronic colonization of the airways by bacteria resistant to antibiotic treatment. The pathogenetic mechanism is controversial and could involve excessive fluid absorption or increased salt concentration in the airways surface fluid (6–9).

Recent studies indicate that inflammatory cytokines may affect transepithelial ion transport. We showed that IFN-γ causes marked changes in Na^+, Cl^-, and fluid transport in the bronchial epithelium (10). Furthermore, the expression of CFTR is modulated by IFN-γ, TNF-α, and IL-1β in intestinal epithelial cells (11–13). These findings suggest that the inflammatory state might affect the airway ion transport in vivo. IL-4 has a particular pathogenetic role in the lung. Actually, the pathology associated with asthma seems to be mediated by $CD4^+$ T lymphocytes, which produce the type 2 cytokines IL-4 and IL-5. IL-4 is increased in the airways of asthmatic subjects (14, 15), and mice deficient for Stat-6, the transcription factor that mediates IL-4 effects, fail to develop airway hyper-responsiveness upon Ag sensitization and challenge (16). These animals also lack Th2 response and mucus hypersecretion, all features found in asthma. In addition, the mucus hypersecretion characteristic of the Th2 response requires an intact IL-4Rα, the receptor for IL-4 and IL-13 (17). Finally, IL-4 directly induces mucin gene expression and goblet cell metaplasia (18, 19). Given its importance in lung pathology, we asked whether IL-4 is also able to modify transepithelial ion transport as found for IFN-γ. Indeed, in other studies it has been shown that these two cytokines may have synergistic effects on important processes in the airways (20, 21). In this study we show for the first time that IL-4 markedly alters the ion transport properties of the cultured bronchial epithelium. The effects only partially resemble those produced by IFN-γ and suggest a role for IL-4 in increasing Cl^- and fluid secretion.

Materials and Methods

Cell culture

Human bronchial and nasal epithelial cells were subcultured as previously described (22, 23). To obtain polarized epithelia, cells were plated at high density on Transwell-CLEAR or Snapwell permeable supports (Corning

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[3] Abbreviations used in this paper: ENaC, amiloride-sensitive Na^+ channel; CF, cystic fibrosis; CPT, 8-(4-chlorophenylthio)adenosine; CFTR, cystic fibrosis transmembrane conductance regulator; I_{cAMP}, cAMP-dependent current; TF IID, transcription factor IID.

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Costar, Cambridge, MA) that had diameters of 24 and 12 mm, respectively. The medium under these conditions contained 2% FBS (Fetal Clone II; HyClone, Logan, UT) and various hormones and supplements as indicated previously (22, 23). With respect to previous studies we kept the cells on permeable supports for more days (8–12 days) before performing ion transport measurements. This allowed a more complete differentiation as indicated by the development of larger short circuit currents and lower resistance (1205 ± 31 Ω/cm^2; $n = 49$) and by the presence of cilia (not shown). Experiments were performed on bronchial cells obtained from two non-CF subjects and from one F508del homozygous CF patient. Epithelial monolayers were also prepared from nasal polyps of two non-CF subjects and one F508del homozygous CF patient. Furthermore, we also conducted a conventional primary culture (23) by directly plating the cells obtained from a non-CF nasal polyp on Snapwell filters without subculturing.

Ussing chamber experiments

The permeable supports were mounted in Ussing chamber-like systems: Trans-24 miniperfusion system (World Precision Instruments, Sarasota, FL) for Transwell cups and Vertical Diffusion Chamber (Corning Costar) for Snapwell inserts. The apical and basolateral chambers were filled with Krebs bicarbonate solution that contained: 126 mM NaCl, 0.38 mM KH_2PO_4, 2.13 mM K_2HPO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 24 mM $NaHCO_3$, and 10 mM glucose. The solution was bubbled in each chamber with 5% CO_2-95% air. Experiments were performed at 37°C. The transepithelial potential difference was short circuited with a voltage clamp (558-C5; Department of Bioengineering, University of Iowa, Iowa City, IA) connected to the apical and basolateral chambers via Ag-AgCl electrodes and agar bridges. The potential difference and fluid resistance between potential-sensing electrodes were compensated.

The data presented to show the effect of cytokines on short circuit current were essentially obtained using Snapwell inserts. Given their large surface, Transwell inserts were used to provide enough cells for RNA extraction. However, Ussing chamber experiments were also performed with Transwell filters to ensure that the effect of cytokine was the same as in Snapwell.

RNA extraction

Total RNA was isolated by lysing the cells onto filters with the TRIzol reagent (Life Technologies, Gaithersburg, MD) following the manufacturer's instructions. RNA purity was checked by spectrophotometer ($OD_{260}/OD_{280} \geq 1.7$) and electrophoresis and stored at −80°C until used. The RNA was processed at the Gaslini Institute for the semiquantitative assay of ENaC subunits or was sent to the other laboratories for the quantitative RT-PCR determinations of CFTR (Créteil, France) or γENaC (Verona, Italy).

Quantitation of ENaC subunits by semiquantitative RT-PCR

Total RNA was retrotranscribed using the Advantage RT for PCR kit (Clontech, Palo Alto, CA). Two microliters of each cDNA were serially diluted 1/1 in buffer to obtain dilutions down to 1/64. Then 2 μl of each dilution was amplified in a 25-μl reaction containing 0.625 U AmpliTaq Gold, 2 mM $MgCl_2$, 15 pmol of each primer, and 200 μM dNTPs. The forward primer was labeled with fluorescent amidite (6FAM). The amplification conditions were 45 s at 95°C, 45 s at 55°C, and 45 s at 72°C. The numbers of cycles were 27, 30, 35, and 25 for αENaC, βENaC, γENaC, and β-actin, respectively. The primers used are shown in Table I.

PCR products obtained from each cDNA dilution were loaded on a 2% agarose gel to verify that the reaction was in its linear range. Then each reaction was diluted 1/10, 3 μl was loaded on an ABI 373A DNA sequencer gel (PE Applied Biosystems, Foster City, CA), and the peak areas corresponding to amplification products were calculated using GeneScan Analysis 3.1 software (PE Applied Biosystems). These values were plotted vs the amount of cDNA used for the RT-PCR. The data in the linear range of the reaction were fitted with a line to obtain a slope that correlates with the amount of the template. The slopes obtained for the three ENaC subunits were normalized for the slope of the β-actin obtained for each sample.

Quantitation of γENaC mRNA by competitive RT-PCR

The single-tube competitive RT-PCR technique previously described (24) was applied by using oligonucleotide primers designed from the human γENaC gene sequence (25), as reported in Table I. The homologous competitor for human γENaC mRNA (cγEnaC) was obtained by RT of total RNA from human bronchial epithelial cells with primer γENaC-R1, followed by amplification with primers γENaC-D1 and γENaCD*, which generated a 7-bp deletion with respect to the wild-type sequence.

RT of 200 ng total RNA was performed with primer γENaC-R1, followed by two amplification runs in the presence of known amounts of cγEnaC. Primers for PCR-1 were γENAC-D2 and γENaC-R1 for PCR-2 γENaC-D2 and γENaC-R2. The amplification conditions for PCR-1 and PCR-2 were 85°C for 5 min, then 30 cycles at 94, 56, and 72°C for 30 s. The run-off reaction (26) was conducted with the fluorescent primer γENaC-R3-TAMRA (one cycle of 94, 56, and 72°C for 30 s each). Run-off reaction product (2 μl) was run in a PE Biosystems 373A DNA sequencer. The peak area of fluorescent PCR fragments corresponding to competitor and endogenous γENaC mRNA was measured using GeneScan 672 software (PE Applied Biosystems). The ratio of competitor over endogenous areas was plotted vs the number of copies of competitor and fitted to a quadratic equation. γENaC mRNA copies were taken as those corresponding to the equivalence between the areas of the competitor and the endogenous transcript.

Quantitative determination of CFTR transcript

The expression of CFTR was determined using the competitive multiplex RT-PCR employing the transcription factor IID (TF IID) as the endogenous mRNA. Total RNA (1 μg) was reverse transcribed using Superscript II reverse transcriptase and random hexamers (Life Technologies, Cergy-Pontoise, France). The internal standards (competitors) for both CFTR and TF IID mRNA, were generated by the QuikChange site-directed mutagenesis technique using the full-length CFTR and TF IID cDNA cloned in the pBluescript phagemid vector (Stratagene, Amsterdam Zwidoost, The Netherlands), as template. The competitor for CFTR (pCFTRIS) had a 21-bp

Table I. *Sequence of oligonucleotide primers*

Primer Name	Oligonucleotide Sequence	Exon	Position
ENaC subunits (semiquantitative assay)			
αENaC-F	FAM-5′-GTTGAGAACCTTTACCCTTCA-3′	7	1306–1326
αENaC-R	5′-TAGCTGGTCACGCTGCATG-3′	9–10	1550–1568
βENaC-F	FAM-5′-CAACACGACCTACTCCATCC-3′	7	1258–1277
βENaC-R	5′-AGACGTGGAAAATCCAGTCC-3′	13	1550–1569
γENaC-F	FAM-5′-AAGAGTGGACACTAACCACA-3′		1379–1398
γENaC-R	5′-GTGATCGTCTATATCCAGG-3′		1803–1821
γENaC subunit (competitive assay)			
γENaC-D1	5′-GTAGCATCATTCACAAGGC-3′	3	551–569
γENaC-D2	5′-AATGTCATGCACATCGAGTC-3′	3	564–583
γENaC-R1	5′-AATGACTTGCAGCCCATATT-3′	5/6	927–928
γENaC-R2	5′-GCTCCTCAGCAGAATAGCTC-3′	4	766–747
γENaC-D*	5′-AATGTCATGCACATCGAGTC(Δ7)GTGGTGGG-3′	3	564–608
γENaC-R3-TAMRA	TAMRA-5′-CCTGAATGGCATTGATTC-3′	4	682–665
CFTR (competitive assay)			
CFTR 5′	5′-(6-FAM)CGGATAACAAGGAGGAACGC-3′		
CFTR 3′	5′-AGGTAATCACAAGTCTTTCAC-3′		
TF IID 5′	5′-(TET)ACAGGAGCCAAGAGTGAAGAA-3′		
TF IID 3′	5′-CCAGAAACAAAAATAAGGAGA-3′		

deletion, whereas that for TF IID (pTF IIDIS) contained a 12-bp insertion with respect to the wild-type sequences. RNA competitors were produced by in vitro transcription of these mutant plasmids with T7 RNA polymerase (Megascript kit, Ambion, Austin, TX). The competitive PCR reactions were performed with two pairs of 21-mer oligonucleotide primers purchased from Genset (Paris, France). CFTR and TF IID 5′ primers were labeled with 6-carboxyfluorescein and tetrachloro-6-carboxyfluorescein, respectively. The CFTR primer set generated exon products of 457 and 436 bp in length using target and competitor CFTR cDNA as template, respectively. The primer set for TF IID generated exon products of 260 and 272 bp from target and competitor TF IID cDNA.

An aliquot (1/20 vol) of RT reaction was amplified by competitive multiplex PCR in a 25-μl reaction volume of master mix PCR containing 0.2 μM of both CFTR and TF IID primers, 10 μl of serial dilutions (1/10) of a mixture of the competitors pCFTRIS (2×10^{-3} ng/μl) and pTF IIDIS (2×10^{-2} ng/μl), and *Taq* DNA polymerase (Life Technologies). PCR conditions were 94°C for 4 min; 34 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1.5 min; and a final extension cycle of 94°C for 30 s, 55°C for 45 s, and 72°C for 5 min. To determine the amounts of amplified products, assays were performed in triplicate using six serial dilutions of competitors against a constant amount of reverse transcribed RNA. Triplicates were pooled, and the fluorescent dye-labeled PCR products were analyzed on a 377A ABI DNA sequencer for peak color, fragment size, and peak area using GeneScan 672 Fragment Analysis software (PE Applied Biosystems). The ratio of CFTR area to its competitor provided the basis of CFTR mRNA quantification. The amount of competitor corresponding to a 1/1 ratio is equivalent to the amount of target mRNA. TF IID mRNA content was simultaneously measured in the same way. Quantification of CFTR message was expressed as the CFTR/TF IID ratio. For each RNA sample three or four independent RT reactions were performed, and two independent PCR reactions were performed for each RT.

Statistics

Results are presented as the raw data or the arithmetic mean ± SEM. Statistical significance was determined with Student *t* test for unpaired data.

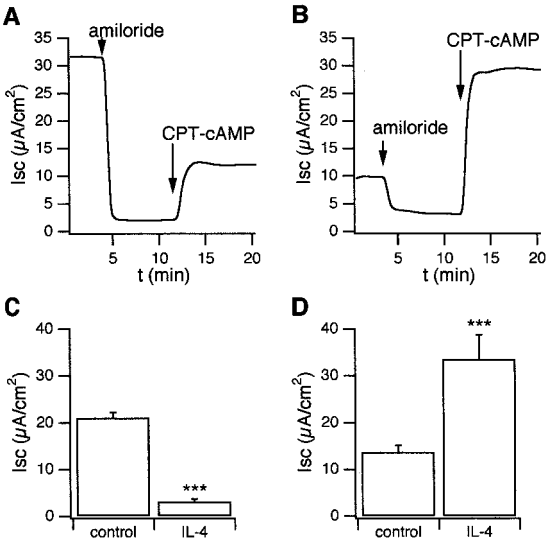

FIGURE 1. Effect of chronic stimulation with IL-4 on amiloride-sensitive and cAMP-activated currents in human bronchial epithelia. *A* and *B*, Representative traces of two Ussing chamber experiments performed on untreated and treated cells, respectively. *C*, Amiloride-sensitive current in control ($n = 39$) and treated cells ($n = 12$). *D*, Currents activated by CPT-cAMP in control ($n = 28$) and IL-4-treated cells ($n = 9$). IL-4 (10 ng/ml) was applied for 48 h. The asterisks indicate that the difference was statistically significant ($p < 0.001$).

Results

Airway epithelial cells obtained from two non-CF human bronchi were exposed for 48 h to IL-4 (10 ng/ml) and then mounted in a Ussing chamber. Short circuit current measurements showed that IL-4 treatment leads to a marked decrease in the basal electrogenic ion transport (Fig. 1). Under resting conditions, this process is largely accounted for by the activity of ENaC. Therefore, we applied 10 μM amiloride in the apical solution to evaluate the activity of the Na^+ channel in control and cytokine-treated cells. As expected, amiloride caused a fast reduction of short circuit current. However, there was a clear quantitative difference between control and treated cells (Fig. 1, *A* and *B*). Indeed, the amiloride-sensitive current (I_{amil}) underwent a 84% decrease upon cell incubation with IL-4 (from 21.0 ± 1.2 ($n = 39$) to 3.1 ± 0.6 μA/cm^2 ($n = 12$); $p <$ 0.001). Amiloride-treated airway epithelia can secrete Cl^- if stimulated with cAMP- or Ca^{2+}-elevating agents. We asked whether Cl^- transport was also affected by IL-4 incubation. Therefore, we acutely stimulated bronchial epithelia with 8-(4-chlorophenylthio) adenosine (CPT)-cAMP, a membrane-permeable cAMP analog that activates CFTR. CPT-cAMP was applied simultaneously in the apical and basolateral solutions at 500 μM. Previous studies showed that this is the concentration that produces maximal CFTR activation. In control cells CPT-cAMP elicited a stable increase in short circuit current that reached a value of 13.7 ± 1.5 μA/cm^2 ($n = 28$; Fig. 1, *A* and *D*). Cells treated with IL-4 (10 ng/ml) for 48 h showed a cAMP-dependent current (I_{cAMP}) that was more than twice that measured in untreated cells (33.5 ± 5.2 μA/cm^2; $n = 9$; $p < 0.001$; Fig. 1, *B* and *D*). This finding suggested that IL-4 leads to increased CFTR activity. However, we wondered whether IL-4 was eliciting the appearance of another cAMP-dependent ion transport distinct from CFTR. Therefore, we used IL-4 to treat bronchial cells obtained from a CF patient homozygote for F508del, a mutation that prevents most of CFTR protein to be correctly targeted to the plasma membrane. As expected for a process dependent on functional CFTR, the I_{cAMP} measured in CF cells was very small even after IL-4 treatment (compare Fig. 1 and Fig. 2). Cytokine-treated CF cells showed an I_{cAMP} of only 1.02 ± 0.21 μA/cm^2 ($n = 9$). Although this value was small, it was significantly higher ($p < 0.01$) than the current measured in untreated CF cells (0.18 ± 0.03 μA/cm^2; $n = 8$). IL-4 also inhibited Na^+ transport in CF cells; the I_{amil} was only 12.6 ± 4.9% of that in untreated cells ($n =$ 12; $p < 0.01$).

We also stimulated bronchial epithelial cells with apical UTP to elicit Ca^{2+}-dependent Cl^- secretion. In control cells the resulting effect was a transient increase in short circuit current that had a maximum value of 6.7 ± 1.3 μA/cm^2 ($n = 26$; Fig. 3). This current (I_{UTP}) peaked after 1 min of UTP application and then returned to prestimulation levels in 15–20 min. Cells treated with IL-4 (10 ng/ml for 48 h) responded to UTP with a higher current amplitude (Fig. 3); the peak was 31.1 ± 1.8 μA/cm^2 ($n = 8$; $p <$ 0.001). Despite this large response, the current deactivated in a way similar to that of untreated cells. CF cells also showed an increased response to UTP when treated with IL-4; the amplitude of I_{UTP} was up-regulated by >10-fold (1245 ± 151% with respect to untreated cells; $n = 9$; $p < 0.001$). It is interesting to note that I_{UTP} up-regulation was significantly stronger than that in non-CF cells ($p < 0.05$). A similar difference was previously observed for IFN-γ (10).

To confirm the significance of data obtained with bronchial cells, we performed Ussing chamber experiments on epithelia prepared from nasal polyps. The amiloride-sensitive current was reduced by IL-4 treatment (10 ng/ml) to 25.2 ± 7.2% of the control value ($n = 8$; $p < 0.05$). The I_{cAMP} was instead up-regulated (235.3 ± 8.5% of control; $n = 5$; $p < 0.01$) as in bronchial epithelia. Nasal epithelial cells from one CF patient were also evaluated. The amiloride-sensitive current was significantly inhibited (3.1 ± 0.9% of control; $n = 3$; $p < 0.01$), whereas the small response to the cAMP analog was up-regulated (from 0.10 ± 0.05

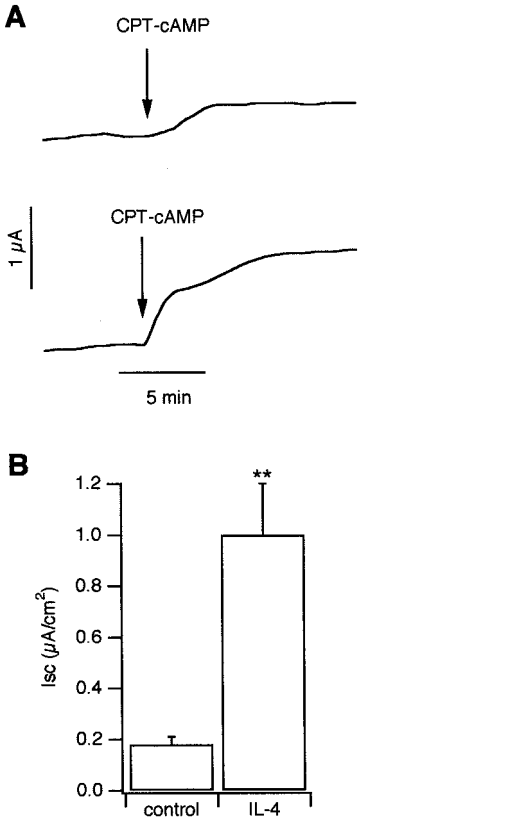

FIGURE 2. Effect of IL-4 on the cAMP-dependent current in cystic fibrosis cells. *A*, Representative traces from an untreated (*upper trace*) and a treated (*lower trace*) epithelium. The cAMP analog was applied in the presence of amiloride. *B*, Summary of results obtained from similar experiments ($n = 8$ and 9 for control and treated cells, respectively). IL-4 was applied at 10 ng/ml for 48 h. The difference was statistically significant ($p < 0.001$).

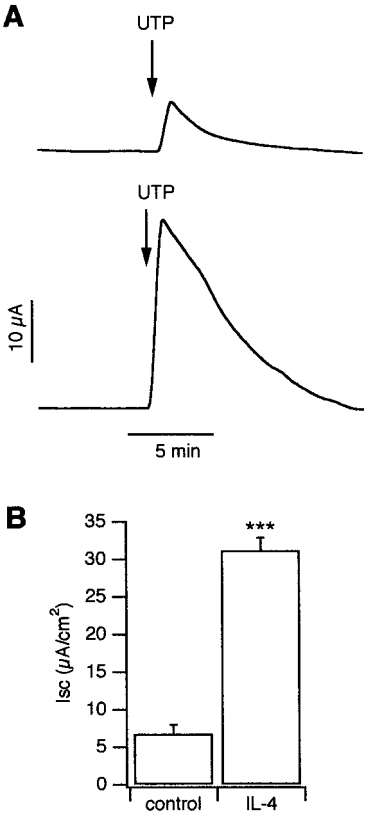

FIGURE 3. Up-regulation of UTP-dependent currents by IL-4. *A*, Representative traces obtained from untreated (*upper trace*) and treated (*lower trace*) cells. UTP was applied in the presence of amiloride. *B*, Average of the peak currents activated by UTP in control ($n = 26$) and cytokine-treated cells ($n = 8$). IL-4 significantly increased the UTP-dependent effect ($p < 0.001$).

to 1.85 ± 0.02 μA/cm^2; $n = 3$ for treated and untreated cells; $p < 0.01$), thus confirming the findings obtained with the CF bronchus. The response to apical UTP in non-CF and CF nasal epithelia was also up-regulated by IL-4 (243 ± 14 and 567 ± 26%, respectively; $n = 3$ for all conditions; $p < 0.01$). As in bronchial cells, we found that I_{UTP} up-regulation by IL-4 was significantly higher in CF cells ($p < 0.05$).

We asked whether the effects of IL-4 were a result of the subculture procedure that we used for nasal and bronchial cells. Therefore, we studied nasal epithelial cells using a conventional primary culture protocol in which the cells are directly plated on permeable supports after proteolytic dissociation (23). Although this procedure usually gives short circuit currents larger than those in subcultured cells, IL-4 had the same effect in relative terms. I_{amil} decreased from 40.3 ± 8.3 to 2.5 ± 0.85 μA/cm^2 ($p < 0.01$), I_{cAMP} increased from 52.5 ± 2.3 to 94.5 ± 3.5 μA/cm^2 ($p < 0.01$), and I_{UTP} increased from 3.9 ± 1.1 to 17.5 ± 1.4 μA/cm^2 ($p < 0.01$; $n = 3$ for all conditions).

We tested IL-4 at different concentrations. The usual concentrations that have been used in vitro range from 10 to 100 ng/ml (18, 20, 21). Our results show that 1 ng/ml was sufficient to produce maximal effects on I_{amil}, I_{cAMP}, and I_{UTP} (Fig. 4). A steep change was observed at concentrations between 0.1 and 1 ng/ml. This behavior of the dose-response curve could reflect positive cooperativity of IL-4. We also determined the time dependence. Interestingly, at 6 h, I_{amil} and I_{UTP}, but not I_{cAMP}, showed a significant change. However, nearly maximal effects were observed for the three types of currents after 24 h of treatment and remained stable for 48 h (Fig. 5).

To assess whether IL-4 acts by directly changing the expression of ENaC and CFTR, we used RT-PCR-based assays to determine the levels of the corresponding mRNAs. Regarding ENaC, we used a semiquantitative approach to assess the possible changes in α, β, and γ subunit expression. IL-4 acts mainly on the γENaC, causing a 7-fold decrease in its mRNA (Fig. 6). The amount of β subunit is also significantly decreased by 50%. On the contrary, α subunit expression is not affected by IL-4. To confirm the effect of IL-4 on γENaC we performed a quantitative competitive RT-PCR. In control cells we found 5040 ± 226 copies of γENaC mRNA/μg total RNA ($n = 3$). In IL-4-treated cells the content of this mRNA decreased to 700 ± 141 copies/μg ($n = 3$; $p < 0.01$). We also determined the effect of IFN-γ, since we previously showed that this cytokine inhibits ENaC activity in bronchial epithelial cells (10). In contrast to IL-4, IFN-γ does not significantly affect the expression of any of the ENaC subunits (Fig. 6).

The in vitro effect of IL-4 on CFTR expression of bronchial epithelial cells was evaluated by an RT-competitive multiplex PCR approach. Results for CFTR were normalized for TF IID expression, which we determined to be unaffected by cytokine treatment. We found that IL-4 doubles the CFTR mRNA content (Fig. 7), which is consistent with the increase in I_{cAMP} measured in treated cells (Fig. 1). The effect of TNF-α, another cytokine found to markedly increase CFTR protein expression (10), was also evaluated. Our RT-PCR data confirm previous results obtained at the protein level by showing a 12-fold increase in CFTR mRNA in TNF-α-treated cells (Fig. 7).

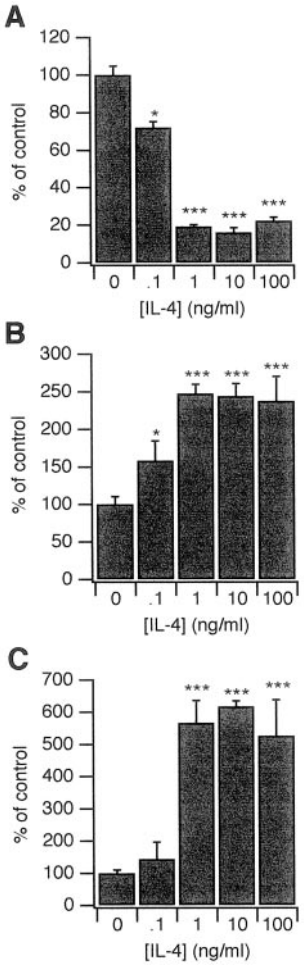

FIGURE 4. Dose-dependence of IL-4 effects. The cytokine was applied at the indicated concentrations for 48 h. *A–C*, Summary of data obtained for amiloride-sensitive, cAMP-dependent, and UTP-activated currents, respectively ($n = 4–6$ for each condition). Data are presented as a percentage of the control value. Asterisks indicate significant differences (*, $p < 0.05$; ***, $p < 0.001$).

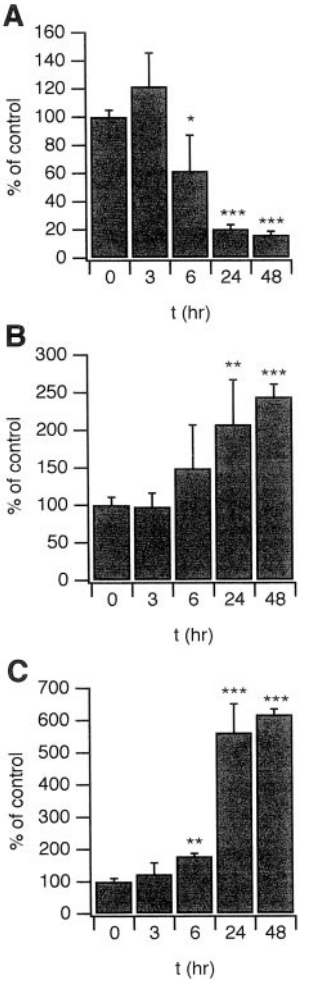

FIGURE 5. Time-course of IL-4 effects. The cytokine was applied for the indicated duration at the concentration of 10 ng/ml. *A–C*, Summary of data obtained on amiloride-sensitive, cAMP-dependent, and UTP-activated currents, respectively ($n = 4–6$ for each condition). Data are presented as a percentage of the control value. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

IL-13 shows many of the biological activities of IL-4 in various cells (17, 27). We asked whether IL-13 can mimic the effects of IL-4 on ion transport in human bronchial epithelial cells. We treated these cells for 48 h with 10 ng/ml IL-13. Short circuit current measurements showed that this cytokine inhibited I_{amil} and up-regulated I_{cAMP} and I_{UTP} in way very similar to IL-4 (Fig. 8*A*). The receptor for IL-4 has a subunit (IL-4Rα) that is also shared by a receptor for IL-13 (27). We tested the involvement of IL-4Rα using a mutated IL-4 (R121D, Y124D), which retains binding to the receptor but has lost its ability to induce the signal (28). This antagonist was used at 1 μg/ml in the presence of 1 ng/ml IL-4. As shown in Fig. 8*B*, the activity of IL-4 was significantly inhibited by the IL-4Rα antagonist.

Discussion

There is increasing evidence that proinflammatory stimuli lead to significant changes in transepithelial ion transport (10–13). These responses may play a role in the innate immune response and in the barrier function of epithelial cells. In the airways, changes in the balance between absorption and secretion of fluids and electrolytes can lead to modifications in the thickness, ion composition, and fluidity of the periciliary fluid. Depending on the net effect, this may favor or impair mucociliary clearance. We have found recently that IFN-γ down-regulates CFTR and ENaC activity in human bronchial epithelial cells, whereas, on the other hand, it significantly up-regulates Ca^{2+}-dependent Cl^- secretion (10).

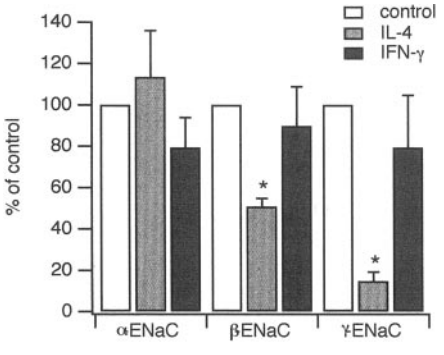

FIGURE 6. Expression of ENaC subunits in control and cytokine-treated cells assessed with the semiquantitative RT-PCR. Cells were treated with IL-4 (10 ng/ml) or IFN-γ (1000 U/ml) for 48 h. Data are presented as a percentage of the control value. Asterisks indicate a significant decrease in expression with respect to the control value ($n = 3–4$ for each condition; $p < 0.05$).

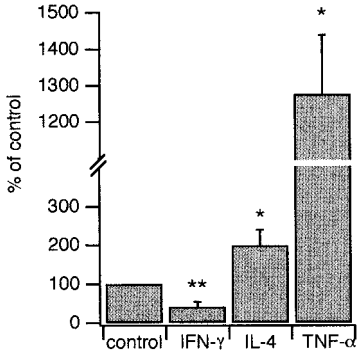

FIGURE 7. Quantification of CFTR mRNA with the RT-PCR assay. Cells were treated with IL-4 (10 ng/ml), IFN-γ (1000 U/ml), or TNF-α (40 ng/ml) for 48 h. Data are presented as a percentage of the control value. The differences were statistically significant ($n = 3$ for each condition; *, $p < 0.05$; **, $p < 0.01$).

Down-regulation of Na^+ absorption may eventually favor hydration of mucous secretions. However, the physiological meaning of CFTR inhibition is less clear. In this study we have investigated the effects of another cytokine, IL-4, which is involved in the humoral Th2 immune response. In a way similar to IFN-γ, IL-4 also decreases ENaC activity. However, the mechanism seems markedly different. Indeed, IL-4 strongly decreases the mRNA levels of γENaC and, to a lesser extent, that of the β subunit. On the contrary, IFN-γ does not seem to affect ENaC expression. Therefore, its inhibitory effect on Na^+ absorption has to involve another mechanism. ENaC activity is controlled by several mechanisms, such as the ubiquitination by Nedd4 (29), carboxylmethylation (30), and proteolysis (31). Any of these processes could be the one that is affected by IFN-γ. It is not surprising to find that only one of the ENaC subunits is markedly down-regulated by IL-4. There are already examples of independent regulation of ENaC subunits in a tissue-specific way. For example, in mouse kidney aldosterone increases by 2- to 3-fold the expression of αENaC, while changes in β and γ subunits are small (32). On the contrary, in rat distal colon aldosterone enhances the expression of β- and γENaC, whereas the α subunit is unchanged or decreased (33).

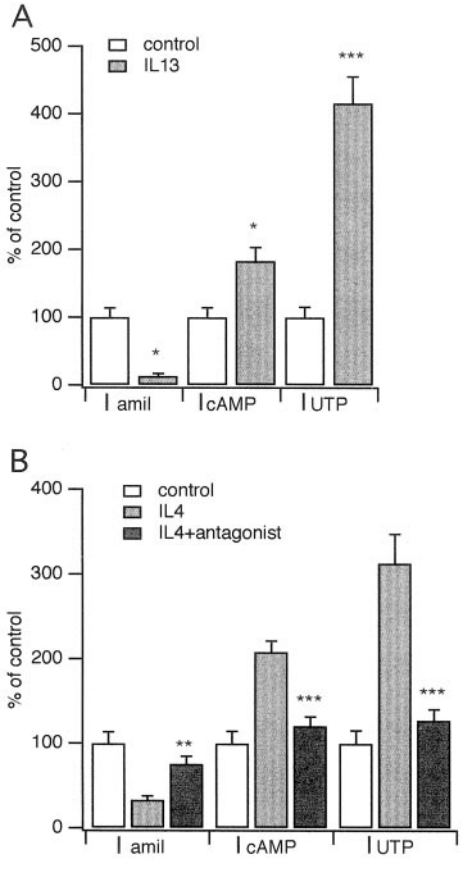

FIGURE 8. Effect of IL-13 and blockade of IL-4 activity by an IL-4Rα antagonist. *A*, Summary of effects of IL-13 (10 ng/ml; 48 h) on amiloride-sensitive, cAMP-dependent, and UTP-activated current ($n = 4–6$ for each condition). *B*, Effect of IL-4 in the presence and the absence of the IL-4Rα antagonist ($n = 7$ for each condition). Data are presented as a percentage of the control value for both *A* and *B*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

In contrast to IFN-γ, IL-4 up-regulates the cAMP-dependent current. According to RT-PCR data this effect can be explained by an increase in the level of CFTR mRNA. The involvement of CFTR in the IL-4 effect is also confirmed by the fact that cells obtained from a F508del CF patient do not show a similar increase in cAMP-dependent current in absolute terms. Therefore, we can exclude an up-regulation of an alternative cAMP-activated channel. However, on a relative basis, CF cells showed a more than a doubling of the cAMP-dependent current upon treatment with IL-4. It is possible that this effect is due to the increase in the mutant CFTR that reaches the membrane. Actually, F508del is a mutation that causes CFTR to be trapped in the endoplasmic reticulum, but it is believed that a small fraction of the mutant protein may escape cell quality control systems (34, 35). Although statistically significant, the CFTR-dependent current measured in IL-4-treated CF cells is quite small and at the limit of the threshold estimated to be required for a normal phenotype (i.e., 5–10% of wild-type function) (36). However, elucidation of regulatory mechanisms underlying the IL-4 effect may reveal novel ways to up-regulate CFTR in CF cells to therapeutic levels. In this respect it is worth noting that TNF-α promotes a larger increase in CFTR mRNA levels. This effect is consistent with the 9-fold increase in CFTR protein observed previously in our laboratory (10). However, for reasons that are currently unclear, this dramatic increase is not paralleled by a significant up-regulation of chloride transport. One may speculate that TNF-α, besides increasing CFTR levels, is also evoking post-transcriptional or post-translational changes that limit its activity. We have no data to determine whether the lack of increased Cl^- transport is due to altered CFTR channel at the plasma membrane or to intracellular localization of the channels. Future subcellular localization studies could help to clarify this point.

As also observed for IFN-γ, IL-4 up-regulates the activity of Ca^{2+}-dependent Cl^- channels, as revealed by the strong increase in the current elicited by apical UTP. However, there are important differences between the two cytokines. Besides increasing the peak, IFN-γ also prolongs the UTP-evoked current (10). This effect is not observed in IL-4-treated cells. Furthermore, 48 h of IFN-γ treatment are required to observe the increase in the UTP-dependent response, whereas IL-4 begins to be effective after only 6 h. The molecular basis of UTP-dependent current up-regulation is unknown and could involve the hyperexpression of Ca^{2+}-activated Cl^- channels, changes in channel regulators, or up-regulation of purinergic receptors.

We found that IL-13 has the same effects as IL-4 on Na^+ and Cl^- transport in bronchial epithelium. This is not surprising, since these two cytokines often share signaling cascades and receptor subunits such as IL-4Rα (27). Indeed, this chain combines with the common γ-chain protein to form a specific IL-4 receptor or with IL-13Rα1 to generate a receptor common to IL-4 and IL-13. The

inhibitory effect of a specific antagonist (28) suggests that the IL-4Rα subunit is involved in the modulation of ion transport by IL-4 and possibly IL-13 in airway epithelial cells. However, we cannot exclude that IL-13 could also be acting through a specific IL-13R (27). The regulatory steps downstream from the receptor have not been elucidated. This cascade could be restricted to the intracellular milieu and involve protein kinases, transcription factors, and other regulatory proteins. Additionally, it could be based on the release of soluble factors (e.g., chemokines) that would act in an autocrine fashion.

Our study shows that IL-4 is able in vitro to down-regulate Na^+ transport and, on the other hand, to increase the activity of Cl^- channels. However, it has to be considered that the response in vivo can be modified by the presence of other cytokines and soluble mediators and by the interactions of epithelial cells with recruited leukocytes. We can speculate that in vivo IL-4 may favor Cl^- and fluid secretion, thus leading to increased hydration of mucous secretions and improved clearance of airway surface. It is worth noting that IL-4 is responsible for mucus hypersecretion from goblet cells (18, 19). Therefore, the changes in ion transport observed in our study could be instrumental to help mucus clearance. However, it has to be considered that another model proposed to explain the role of ion transport in the airway epithelium postulates that a low salt concentration is required to maintain the defensin activity (8). Considering this model, IL-4 could alter the bactericidal properties of periciliary fluid.

The elucidation of the mechanisms underlying cytokine-dependent ion transport modification may help to design novel strategies for the fluidification of mucus secretions and the treatment of chronic pulmonary diseases.

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