Interleukin-1β Differentially Regulates β2 Adrenoreceptor and Prostaglandin E₂-mediated cAMP Accumulation and Chloride Efflux from Calu-3 Bronchial Epithelial Cells

ROLE OF RECEPTOR CHANGES, ADENYLYL CYCLASE, CYCLO-OXYGENASE 2, AND PROTEIN KINASE A*

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Here we tested the effect of interleukin-1β, a pro-inflammatory cytokine, on cAMP accumulation and chloride efflux in Calu-3 airway epithelial cells in response to ligands binding to adenylyl cyclase-coupled receptors such as the β2 adrenoreceptor and EP prostaglandin receptors. Interleukin-1β significantly increased isoprenaline-induced cAMP accumulation by increasing β2 adrenoreceptor numbers via a protein kinase A-dependent mechanism. In contrast, interleukin-1β significantly impaired prostaglandin E₂-induced cAMP accumulation by induction of cyclo-oxygenase-2, prostaglandin E₂ production, and a resulting down-regulation of adenylyl cyclase. The cAMP changes were all mirrored by alterations in chloride efflux assessed using the fluorescent chloride probe (N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide) with interleukin-1β increasing chloride efflux in response to isoprenaline and reducing the response to prostaglandin E₂. Studies with glibenclamide confirmed that chloride efflux was via the cystic fibrosis transmembrane conductance regulator. Calu-3 expresses EP₄ receptors, but not EP₂, and receptor expression is reduced by interleukin-1β. Collectively, these results provide mechanistic insight into how interleukin-1β can differentially regulate cAMP generation and chloride efflux in response to different adenylyl cyclase-coupled ligands in the same cell. These findings have important implications for diseases involving inflammation and abnormal ion flux such as cystic fibrosis.

Cystic fibrosis (CF)¹ is a multisystem disorder and the most commonly occurring lethal autosomal recessive hereditary disorder in Caucasian populations. It is caused by mutations in a 230-kb gene located on chromosome 7 that codes for CFTR (1). CFTR functions in the main as a cAMP-regulated channel but also regulates the activity of other ion channels and transporters such as apical ENaC, basolateral Na/K ATPase (2, 3), and the outwardly rectifying chloride channel (4), and can transport bicarbonate (5). Progression of lung disease due to chronic infection is the main cause of morbidity and mortality in CF (6). Alterations in ion flux in cystic fibrosis lungs is thought to be central in the pathogenesis of lung disease, although the mechanism is debated. Several hypotheses have been suggested with the biochemical defect being linked to impaired antibacterial activity of β-defensins (7), mucociliary clearance (8), and altered sialisation of bacterial receptors (9).

Although defective CFTR predisposes to inflammation, the reverse situation, namely whether inflammation can impair CFTR function, has not been extensively studied. This would be important as it would set up a cycle that would further damage the defense properties of the bronchial epithelium. Several pieces of evidence would be consistent with this hypothesis.

Residual chloride efflux declines as lung function falls (10), there is considerable variation in clinical outcome among patients with the same genotype (11), and variable chloride conductance in rectal epithelium has been observed between monozygotic and dizygotic twins (12). Collectively, these studies raise the intriguing possibility that the airway environment can modify the underlying biochemistry.

The airways in CF are characterized by neutrophil-dominated inflammation with high levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and tumor necrosis factor α in sputum, serum, and bronchial lavage samples (13). IL-1β is particularly interesting as studies from our own group and others have shown that it can reduce cAMP accumulation in response to ligands that bind to adenylyl cyclase-linked receptors in other biological systems (14). Few studies have looked at the functional consequences of these changes. Here we have shown that IL-1β alters cAMP-mediated chloride efflux in Calu-3 airway epithelial cells. IL-1β had differential effects depending on whether cells were stimulated with an EP prostaglandin or β adrenoreceptor receptor ligand. IL-1β reduced chloride efflux in response to PGE₂, but enhanced the response to isoprenaline, a β₂ adrenoreceptor agonist.

Mechanistic studies showed that the effect was mediated by IL-1β having differential effects on cAMP accumulation to the two agents. The down-regulation of cAMP accumulation and chloride efflux in response to prostaglandin E₂ (PGE₂) was mediated by an autocrine loop involving induction of cyclo-oxygenase 2 (COX-2) and endogenous PGE₂ production.

In contrast, the up-regulation of isoprenaline-induced cAMP accumulation was prostanooid-independent, was at least in part mediated by protein kinase A, and involved increased expression of β₂ adrenoreceptors. These studies provide important mechanistic and functional insights into how different adenylyl cyclase-coupled receptors can be differentially regulated by cytokines in the same cell type. These effects may play an
**MATERIALS AND METHODS**

Experiments were performed on cultured Calu-3 cells, a transformed bronchial epithelial cell line originally derived from a 25-year-old male in 1975 (obtained from LGC Ltd., Teddington, UK). This cell line shows cAMP-dependent chloride flux through CFTR that is expressed in high levels (15, 16). Cells were incubated in 24-well plates (at passage 3–7) in 10% fetal calf serum-Eagle’s minimum essential medium, supplemented with non-essential amino acids and sodium pyruvate, in humidified 5% CO₂-95% air at 37 °C, and the medium was changed every 2–3 days. Cells were cultured to confluence (typically 7–10 days) and growth arrested in fetal calf serum-free Eagle’s minimum essential medium containing 1 mM isobutylmethylxanthine to prevent cAMP breakdown by phosphodiesterases. Isoprenaline, PGE₂ (ONO-AE1 329) agonist was then added to control and treated cells and incubated for 10 min. This time was chosen based on our previous studies. Experiments were terminated by the addition of 100 μl of ice-cold 30% trichloroacetic acid. The resulting solution was transferred to centrifuge tubes kept at 4 °C, and the trichloroacetic acid removed by amine-freeon extraction. The cAMP content of the extract was determined using a protein binding assay (17). The bound ³H cAMP was measured using the Tri-Carb 2100TR liquid scintillation analyzer (Packard Bioscience LTD., Pangbourne, Berkshire, UK).

**Cyclic AMP Accumulation**—Immediately before each experiment, medium was removed and the cells washed twice with sterile phosphate-buffered saline. This was then replaced by 500 μl of serum-free Eagle’s minimum essential medium containing 1 mM isobutylmethylxanthine to prevent cAMP breakdown by phosphodiesterases. Isoprenaline, PGE₂, forskolin, and the selective EP₃ (ONO-AE1 259) or EP₂ (ONO-AE1 329) agonist was then added to control and treated cells and incubated for 24 h. Appropriate controls were performed in all experiments; cells were treated with vehicle-only for the same times. Inhibitors were added to the treated controls were performed in all experiments; cells were treated with PGE₂ (B). In contrast cAMP accumulation in response to ISO was significantly enhanced (A). Data expressed as mean ± S.E. (n = 12 for each group). ***, p < 0.001; *, p < 0.05.

**β₂ Adrenoreceptor Assay**—β₂ adrenoreceptor numbers were measured using a radioligand binding assay (18). Cells were incubated with increasing concentrations of [³H]CGP12177 (doubling concentrations from 0.0625 to 8 nM), a β₂ adrenoreceptor ligand, in serum-free medium for 1 h. The at the end of this time supernatant was removed and the cells washed twice in sterile phosphate-buffered saline. Cells were then lysed with 0.5 M sodium hydroxide, and the bound [³H]CGP12177 was measured using the Tri-Carb 2100TR liquid scintillation analyzer. These results were taken to indicate the total binding of [³H]CGP12177. The procedure was repeated with 0.1 μM ICI 118551, a potent specific β₂ adrenoreceptor antagonist, added to all the ascending concentrations of [³H]CGP12177 to specifically occupy all available β₂ adrenoreceptor sites. The resulting measurements of [³H]CGP12177 binding are therefore representative of nonspecific binding. Specific binding was calculated from the difference between the total and nonspecific binding at each concentration of [³H]CGP12177. Protein content was measured using the method of Bradford (19).

**Assay of Chloride Flux**—Chloride flux was measured using the fluorescent chloride probe MQAE (20). Cells were grown to confluence in 96-well flat-bottomed and black-walled plates (Costar, UK). Once confluent, half the cells were treated with serum-free medium plus IL-1β and half with serum-free medium plus vehicle alone.

After 4 h, 5 μl of 8 mM MQAE was added to each well to bring the total well volume to 100 μl. 20 h later the medium was removed, and each well was washed three times with chloride-containing buffer, tipping onto blotting paper as before. The plates were incubated with 100 μl of chloride-containing buffer (to induce chloride channel activation before initiating chloride efflux) for 10 min at room temperature. During experiments with glibenclamide (200 μM), glibenclamide was added to selected wells during this period. The plates were then read on a Wallac Victor fluorescence plate reader (LKB Wallac, Turku, Finland).
IL-1β, cAMP Production, Chloride Efflux in Airway Epithelium

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A

![Graph A](image1)

B

![Graph B](image2)

C

![Graph C](image3)

FIG. 2. Treatment with IL-1β causes COX-2 induction and PGE₂ production. 200 μM IL-1β causes a time-dependent increase in PGE₂ production (A). This increase is abolished by the broad spectrum COX inhibitor indomethacin and the COX-2-specific inhibitor NS-398 (B). IL-1β causes COX-2 induction from 4 h of treatment, maintained to 24 h (C). Data expressed as mean ± S.E. (n = 4–8 for each group). ***, p < 0.0001; **, p < 0.001; *, p < 0.05.

FIG. 3. The effects of the broad spectrum COX inhibitor indomethacin and the COX-2-selective inhibitor NS-398 on changes in cAMP accumulation induced by IL-1β. A, COX inhibitors have no effect on the increase in cAMP accumulation in response to isoprenaline. B, the reduction in PGE₂-induced cAMP accumulation is abolished by both agents. These results suggest that the reduced cAMP response to PGE₂ is caused by induction of COX-2 and production of endogenous PGE₂. In contrast, the enhanced response to ISO appears to be COX independent. Data expressed as mean ± S.E. (n = 8–16 for each group). ***, p < 0.0001; **, p < 0.001.

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction—Cells were grown to confluence in 6-well plates, and growth was arrested for 24 h and then treated for 0, 1, 2, 4, 8, and 24 h with 200 μM IL-1β. Total RNA was isolated using the RNeasy mini kit (Qiagen, West Sussex, UK) following the manufacturer’s protocol; 2 μg of total RNA was reverse transcribed in a total volume of 25 μl, including 200 units of M-MLV reverse transcriptase (Promega, Madison, WI), 25 units of RNase inhibitor (Promega), 200 units of oligo(dT)₁₅ primer, 0.5 mM of each dNTPs and 1× first-strand buffer provided by Promega. The reaction was incubated at 42 °C for 90 min. PCR was carried out by adding 2 μl of cDNA to a total reaction volume of 50 μl, giving final concentrations of 1.5 mM magnesium chloride, 1× PCR buffer, 200 μM of each dNTPs (Promega), and 0.5 μM of both the upstream and downstream primers (Sigma). The primer sequences were as follows: EP₂-R sense, 5'-TCC AAT GAC TCC CAG TCT GAG GA-3';EP₂-R antisense, 5'-TCA AAG GTC AGC CTG TTT AC-3';EP₄-R sense, 5'-TCT GAC CTC GGT GTC CAA AAA TCG-3';EP₄-R antisense, 5'-TGG GTA CTG CAG CCG CGA GCT A-3'; glyceraldehyde-3-phosphate dehydrogenase sense, 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'; glyceraldehyde-3-phosphate dehydrogenase antisense, 5'-CTG AAT GAC CTC CAG GAA GCA-3'. 2.5 μl of RED Taq DNA polymerase (Sigma) was then added to each reaction mixture. Amplification was carried out with a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA) after an initial denaturation at 95 °C for 3 min. This was followed by 35 cycles of PCR using the following times and temperatures: denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s, primer extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products were electrophoresed on 1% agarose gel in 0.5× TBE buffer (89 mM Tris borate, 2 mM EDTA, pH 8.3) containing 0.5...
Fig. 4. The effect of exogenously applied PGE2 (1 μM) for 24 h on cAMP accumulation in response to PGE2 and isoprenaline. A, cAMP accumulation is reduced in response to PGE2, mimicking the effect of IL-1β. B, cAMP accumulation is reduced in response to isoprenaline. These data provide further evidence that the effect of IL-1β on PGE2-induced cAMP accumulation is mediated by induction of COX-2 and production of endogenous PGE2. Data expressed as mean ± S.E. (n = 4 for each group). ***, p < 0.001; **, p < 0.01.

μg/ml ethidium bromide and visualized using ultraviolet illumination and the GeneGenius gel documentation and analysis system (Syngene).

Western Blot Analysis—Western blotting for COX-2 and β2 adrenoceptor was performed as previously described (17).

Materials—Recombinant human IL-1β, PGE2, isoprenaline, indomethacin, forskolin, isobutylmethylxanthine, cAMP-dependent protein kinase, cAMP, and ICI 118551 were all obtained from Sigma. NS-398 was obtained from Cayman Chemical (Ann Arbor, MD). [3H]CGP12177 and [3H]cAMP were obtained from Amersham Biosciences, and MQAE was obtained from Cambridge Biosciences (Cambridge, UK). Gilbenclamide was obtained from Tocris Cookson (Bristol, UK). ONO-AE1 259 and ONO-AE1 329 were obtained from ONO Pharmaceuticals (Osaka, Japan). Goat anti-mouse horseradish peroxidase-conjugated secondary antibody was purchased from BD Biosciences. H-89 was obtained from Merck Biosciences (Nottingham, UK); COX-2 and β2 adrenoceptor antibodies were obtained from Santa Cruz Biotechnology.

Statistical Analyses—Data are expressed as means and S.E. from n determinations. Statistical analysis was performed with the software program GraphPad Prism 4. For experiments measuring cAMP and PGE2 release, unpaired Student’s t test was used to determine the significance of differences between means. For experiments measuring chloride efflux, representative experiments are shown. Three time points were chosen for subsequent analysis as summary statistics. Statistical significance was determined by efflux for each condition at these time points. Unpaired two-tailed Student’s t tests were used to determine significance between these sets of observations. In assays measuring β2 adrenoceptor numbers, Bmax (a measure of receptor number) and the dissociation constant Kd were calculated using non-linear regression (GraphPad Prism 4). In all experiments p values <0.05 were accepted as statistically significant.

RESULTS

IL-1β Increases cAMP Formation in Response to Isoprenaline (ISO) but Reduces the Response to PGE2—Basal cAMP levels were low in both control cells and cells pretreated with IL-1β. Isoprenaline caused a concentration-dependent increase in cAMP accumulation, significant in control cells from 0.1 μM. Prolonged treatment with IL-1β (200 pM) for 24 h increased cAMP synthesis in response to all concentrations of ISO tested (Fig. 1A).

PGE2 also caused a concentration-dependent increase in cAMP production in control cells significant from 0.01 μM. IL-1β (200 pM) pretreatment for 24 h markedly attenuated the response to all PGE2 concentrations (Fig. 1B). We next went on to determine the role of prostanoids in the effects of IL-1β.

IL-1β Reduces COX-2 and Increases PGE2 Production in Calu-3 Cells—IL-1β (200 pM) caused a marked increase in PGE2 production that was time dependent and significant (Fig. 2A). The increase in PGE2 was abolished when cells were pretreated with the broad spectrum COX inhibitor indomethacin (1 μM) or the COX-2-selective agent NS-398 (1 μM) (Fig. 2B). We have previously shown that this concentration of NS-398 abolishes COX-2-mediated PGE2 production with no effect on COX-1 in airway cells. Western blotting showed that IL-1β induced COX-2 with protein levels that increased from 4 h and remained increased at 24 h (Fig. 2C).

Inhibitors of Prostanoid Synthesis Inhibit the Effects of IL-1β—To determine whether prostanoids were responsible for the effects of IL-1β on ISO- and PGE2-induced cAMP accumulation, we studied the effect of the broad spectrum COX inhibitor indomethacin and selective COX-2 inhibitor NS-398 on IL-1β-induced changes in cAMP production. These agents (both at 1 μM) had no effect on the increase in ISO-stimulated cAMP production seen after IL-1β
IL-1β Increases ISO-induced cAMP Accumulation by Increasing β₂ Adrenoceptor Numbers—To determine the mechanism whereby IL-1β increases ISO-induced cAMP accumulation, we studied the effects of IL-1β on β₂ adrenoceptor numbers. There was a consistent 3–4-fold increase in receptor number after incubation with IL-1β for 24 h. The mean $B_{\text{max}}$ for untreated cells was 26.5 (± 7.2) fmol/μg protein. This rose to 76.1 (± 10.5) fmol/μg protein after 24 h of treatment with IL-1β. Representative whole cell binding data are shown in Fig. 6, A and B. Fig. 6C shows the receptor density for each condition. These observations were confirmed by Western blotting. Fig. 6D shows the time-dependent increase in β₂ adrenoceptor protein.

Calu-3 Cells Express EP₂ but Not EP₄ Receptors; EP₄ Expression Is Reduced by IL-1β—PGE₂-stimulated cAMP accumulation occurs via stimulation of the Gₛ-coupled prostanoid receptors EP₂ and EP₄. We therefore examined the effects of agonists and antagonists at these receptors on PGE₂-stimulated cAMP accumulation. Stimulation with EP₄ agonist (10 nM) led to a marked increase in cAMP accumulation after 10 min that was equivalent to that seen in response to PGE₂ stimulation. Exposure to the same concentration of EP₄ agonist did not lead to an increase in cAMP accumulation. Pretreatment of cells for 1 h with a selective EP₂ antagonist abolished cAMP accumulation in response to both PGE₂ and EP₄ agonist (Fig. 7A). These experiments were repeated using chloride efflux as the end point. The same effects were observed (data not shown). We then examined the expression of EP₂ and EP₄ receptor messenger RNA in these cells using reverse transcription PCR. Cells were treated for between 0 and 24 h with IL-1β prior to RNA
EP$_2$ receptor mRNA was not detected at any time point. EP$_4$ receptor mRNA was present at all time points and was reduced by IL-1$eta$/H9252 treatment after 24 h (Fig. 7B). Fig. 7C shows densitometry data from three separate experiments.

The PKA Inhibitor H-89 Abolishes the Effect of IL-1$eta$ on ISO-induced cAMP Accumulation but Has No Effect on That Caused by PGE$_2$—Experiments were performed to determine whether the effects of IL-1$eta$ on cAMP accumulation were me-
diated by protein kinase A. We used the inhibitor H-89 at a concentration of 10 μM and found that it abolished the increase in cAMP accumulation due to IL-1β when cells were stimulated with ISO (Fig. 8A). In contrast, H-89 had no effect on the attenuated response to PGE₂ (Fig. 8B). These results suggest IL-1β enhances cAMP accumulation to β₂ adrenoreceptor ago-

Fig. 7. PGE₂ (1 μM) and the highly selective EP₄ receptor agonist ONO-AE1 329 (10 nM) cause a marked increase in cAMP accumulation. A, the selective EP₂ receptor agonist ONO-AE1 259 (10 nM) has no effect on cAMP accumulation. The selective EP₂ receptor antagonist abolishes PGE₂ and EP₂ receptor agonist-induced cAMP accumulation. Data expressed as mean ± S.E. (n = 4–16 for each point). B, Calu-3 cells express only the EP₄ receptor whose expression is reduced by treatment with IL-1β. A representative blot of three experiments is shown. C, densitometry from three experiments shows the reduction in EP₄ receptor expression caused by IL-1β treatment (C). Collectively, these experiments show that Calu-3 cells express only the EP₄ prostanoid receptor subtype, and its expression is reduced by IL-1β.
nists via PKA but that the attenuated response to prostanoids is PKA independent.

**IL-1β Increases Chloride Efflux from Cells in Response to ISO but Reduces Efflux in Response to PGE₂**—We next went on to determine whether the effects of IL-1β on cAMP were mirrored by effects on cAMP-mediated chloride flux. Isoproterenol and PGE₂ both caused concentration-dependent increases in chloride efflux (Fig. 9, A and B). From these time course experiments, time points of 3, 5, and 16 min were chosen for summary analysis. Chloride efflux was blocked at all summary time points by 200 μM glibenclamide, confirming that it was CFTR-mediated (data not shown). IL-1β (200 pM) pretreatment for 24 h increased 10 μM ISO-induced chloride efflux but reduced 1 μM PGE₂-mediated chloride efflux. These changes mirrored those seen in experiments where cAMP production was measured. The effects of IL-1β on ISO- and PGE₂-mediated chloride efflux are shown at these points (Figs. 10 and 11).

**COX Inhibitors Abolish IL-1β-induced Changes in Chloride Efflux in Response to PGE₂**—Experiments were performed to determine whether the mechanism of the effects of IL-1β on chloride efflux in response to PGE₂ were the same as those seen on cAMP accumulation. The COX-2-specific inhibitor NS-398 was able to completely abolish the attenuated response at all summary time points (Fig. 12). The broad spectrum COX inhibitor indomethacin had the same effect (data not shown). Furthermore, PGE₂ pretreatment mimicked the effect of IL-1β (Fig. 13).

**IL-1β Has No Effect on Cell Proliferation**—To make sure that none of the effects in these studies was an artifact due to alterations in cell numbers, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays of cells after 24 h of treatment with IL-1β at the concentration used in other experiments. There was no significant difference between treated and control cell populations (data not shown).

**DISCUSSION**

The main findings of these studies are that IL-1β has differential effects on ISO- and PGE₂-mediated cAMP accumulation and chloride efflux in Calu-3 cells. IL-1β enhanced ISO-mediated cAMP accumulation via an increase in β₂ adrenoreceptor numbers. In contrast, IL-1β reduced PGE₂-mediated cAMP accumulation via an increase in β₂ adrenoreceptor numbers. These effects of IL-1β can differentially effect the responses to different adenylyl cyclase-coupled receptor ligands in the same cell. Furthermore, these findings have important functional consequences. The effects of IL-1β on ISO- and PGE₂-mediated chloride flux mirrored its effects on cAMP accumulation with the response to ISO being enhanced and the response to PGE₂ being attenuated. These findings have implications for the understanding of the pathophysiology of cystic fibrosis lung disease and may also be relevant for disease processes at other mucosal surfaces such as the gut. Our results suggest that pro-inflammatory cytokines such as IL-1β present in CF airways may reduce PGE₂-mediated chloride flux. This might further impair defective chloride flux in cystic fibrosis and exacerbate the abnormalities in airway surface liquid composition that occur as a result of CFTR dysfunction. This effect could also cause abnormal fluid movement in non-cystic fibro-
sis bronchiectasis that might worsen the disease.

It is well established that CF lungs contain high levels of pro-inflammatory cytokines such as IL-1β (13). As IL-1β can regulate adenyl cyclase function in some biological systems, we hypothesized that pro-inflammatory cytokines such as IL-1β would alter cAMP accumulation in response to ligands binding to adenyl cyclase-coupled receptors and, therefore, CFTR function. The CFTR channel is controlled by receptors that couple to guanine nucleotide-binding proteins and adenyl cyclase leading to production of cAMP (15, 16). Cyclic AMP then activates protein kinase A, which phosphorylates the regulatory domain of CFTR. Airway epithelial cells express several adenyl cyclase-coupled receptors, including β2 adrenoreceptors and EP prostanoid receptors whose activation promotes CFTR-mediated chloride flux (21). Although there is some evidence that cytokines can affect the function of components of this signaling pathway in airway cells, including epithelial cells (22) and airway smooth muscle cells (14), little is known about the functional consequences of these effects, particularly on CFTR-mediated chloride efflux in airway epithelial cells.

It is generally accepted that the main physiological regulator of CFTR is cAMP. However, surprisingly little information exists about the pathways that lead to physiologically important cAMP production in airway epithelial cells. Prostanoid receptors and adrenoreceptors are likely to be the main candidate sources of signal transduction as stimulation of these receptors causes marked changes in cAMP. Adrenergic innervation of the lung is relatively sparse (23), suggesting that...
circulating catecholamines may activate $\beta_2$ adrenoreceptors on epithelial cells. As PGE$_2$ is produced locally in large quantities in the lung (24), it is likely that its actions at EP$_2$ and EP$_4$ prostanoid receptors are important in increasing cAMP-mediated chloride flux.

We chose Calu-3 cells for our studies as they have previously been shown to possess both CFTR-mediated chloride flux and $\beta_2$ adrenoreceptors (15, 16). Our experiments have shown that these cells express EP$_4$ prostanoid receptors. These cells thus provide a useful model system to study regulation of these pathways in airway epithelial cells. Available CF airway epithelial cell lines were not used, as they do not have sufficient cAMP-mediated chloride flux to be able to measure a further reduction by IL-1$.\beta$. This is a property of these cell lines and does not represent the properties of CF airway epithelial cells in vivo, which do show residual chloride flux. Cells responded to both isoprenaline and PGE$_2$ with rapid accumulation of cAMP. We found that IL-1$\beta$ enhanced ISO-induced cAMP accumulation but paradoxically reduced PGE$_2$-induced cAMP accumulation. The magnitude of these effects differed, with the attenuation of PGE$_2$-induced cAMP accumulation being the more marked. We then performed mechanistic studies to characterize these effects further. Recent studies in human airway smooth muscle (25) and airway epithelial (26) cells have shown that the effects of IL-1$\beta$ on the release of other cytokines and mediators can occur via the action of protein kinase A. We therefore went on to see whether a similar mechanism could underlie the effects of IL-1$\beta$ on Calu-3 cells. Studies with the PKA inhibitor H-89 suggested that PKA plays an important role in the effect of IL-1$\beta$ on ISO-induced cAMP accumulation but that its effect on prostanoid-induced cAMP accumulation is PKA independent. We measured $\beta_2$ adrenoreceptor numbers using a ligand binding assay. We found a large increase in $\beta_2$ adrenoreceptor numbers after 24 h of pretreatment with IL-1$\beta$, suggesting that this was at least partly responsible for the increased ISO-induced cAMP accumulation. These findings were then confirmed by Western blotting. This result is similar to studies in BEAS2B human airway epithelial cells where IL-1$\beta$ caused a 2.5-fold increase in receptor number (22) and to a recent study in fetal guinea pig lung homogenates (27). Our studies extend previous studies to determine whether the up-regulation of $\beta_2$ adrenoreceptor numbers and cAMP accumulation has any functional significance. To do this we used MQAE, a fluorescent chloride probe. We found that ISO caused a marked dose-dependent increase in chloride efflux from the cells. Studies with glibenclamide, a CFTR inhibitor, showed that ISO-induced chloride efflux was CFTR-mediated.

Treatment with drugs that act at the $\beta_2$ adrenoreceptor (e.g. Salbutamol and Salmeterol) is common in cystic fibrosis, largely in patients who show symptoms of reversible airways obstruction. Use of these drugs has been shown to lead to both short and long term improvements in lung function (28, 29). This improvement is mediated by increases in cellular cAMP, which in turn excerpts beneficial effects via relaxation of airway smooth muscle and possibly increased activation of residual CFTR function in epithelial cells. The effects of IL-1$\beta$ on $\beta_2$ adrenoreceptor number and adenyl cyclase function would be likely to increase the effectiveness of these agents on residual CFTR function in CF.

In marked contrast to the effects of IL-1$\beta$ on ISO-induced cAMP accumulation, it attenuated PGE$_2$-induced cAMP accumulation and chloride efflux. We hypothesized that endogenous prostanoids produced in response to IL-1$\beta$ might be responsible for this effect. Cyclo-oxygenase is the enzyme that converts arachidonic acid into PGH$_2$, which is subsequently converted to prostanoids by specific synthases. COX exists in three isoforms (30). COX-1 produces physiological levels of prostanoids (31), whereas COX-2 is induced in inflammatory conditions (32). The recently identified COX-3 is a splice variant of COX-1, whose function is unclear (33). We found that IL-1$\beta$ induced COX-2 and caused an increase in PGE$_2$ release. We measured PGE$_2$ as it is the most abundant arachidonic acid metabolite in human airway epithelium (24). Interestingly, recent research has demonstrated that CF patients have abnormally high levels of arachidonic acid and reduced docosahexanoic acid, a fatty acid with anti-inflammatory properties (34). To determine whether IL-1$\beta$-induced PGE$_2$ production was responsible for down-regulation of PGE$_2$-induced cAMP accumulation by chronic desensitization, we used indomethacin, a broad spectrum COX inhibitor, and NS-398, a specific COX-2 inhibitor. We have previously shown that the concentration of NS-398 used inhibits 90% of COX-2-mediated prostanoid production, with no effect on COX-1 (35). Consistent
with a major role for COX-2 in this process, both agents abolished the effects of IL-1β on PGE₂-induced cAMP production and chloride efflux.

To assess the possible site of the desensitization, we performed studies using forskolin, a direct activator of adenyl cyclase, to stimulate cAMP accumulation. We found that IL-1β reduced forskolin-induced cAMP accumulation, suggesting that IL-1β was causing down-regulation of adenyl cyclase. Consistent with this, exogenous PGE₂ also reduced forskolin-induced cAMP accumulation. In addition to its effects on adenyl cyclase, we have also shown that IL-1β reduces the expression of EP₄ prostanoid receptors. Presumably the reason that IL-1β did not cause a similar reduction in ISO-induced cAMP accumulation but paradoxically increased it was that the increased β₂ adrenoreceptor numbers were sufficient to overcome the reduced adenyl cyclase activity.

Data regarding the effect of other cytokines on CFTR-mediated chloride flux in epithelial cells are sparse. Interferon-γ reduced cAMP-dependent chloride efflux in primary cultures of human bronchial epithelial cells stimulated with the cAMP analogue 8-(4-chlorophenylthio) (CPT)-cAMP (36). In bile duct epithelium, a combination of IL-6, interferon-γ, 23461
**FIG. 13.** The effect of 24 h of pre-treatment with exogenous PGE$_2$ on subsequent PGE$_2$-induced chloride efflux. Exogenous PGE$_2$ reduced subsequent PGE$_2$-induced chloride efflux at all time points: 3 min (A), 5 min (B), and 16 min (C), mimicking the effect of IL-1β. Data expressed as mean ± S.E. ($n = 18$ for each group). ***, $p < 0.0001$.

**FIG. 14.** A schematic diagram summarizing an important conclusion of this study. IL-1β sets up a positive feedback loop whereby autocrine production of PGE$_2$ down-regulates subsequent cAMP production and chloride efflux in response to endogenous PGE$_2$. It is likely that this would lead to further abnormalities in airway surface liquid composition and thus predispose the airway to further infection and production of pro-inflammatory cytokines.
tumor necrosis factor α, and IL-1β reduced chloride efflux in response to forskolin, but individual cytokines had no effect (37). It is likely that the actions of IL-1β in attenuating PGE2-mediated cAMP accumulation and chloride flux would play a role in the pathogenesis of cystic fibrosis lung disease. The abnormal airway surface liquid due to CFTR dysfunction in the CF lung leads to infection and inflammation. This inflammatory response with increased levels of IL-1β would down-regulate adenylyl cyclase and the action of endogenous PGE2 to increase cAMP and activate CFTR. This would further impair electrolyte abnormalities, predisposing to further infection and inflammation by setting up a positive feedback mechanism (Fig. 14). Strategies that break this loop would be useful therapeutically. Interestingly, clinical trials have shown the broad spectrum COX inhibitor ibuprofen can delay CF lung disease progression (38), consistent with these observations. The effect of IL-1β in regulating chloride flux may also be important in other mucosal sites. For example, CFTR is important in electrolyte secretion in the gut, and similar effects on colonic mucosa may be relevant to the pathogenesis of inflammatory and infective bowel disorders.

In conclusion, we have shown that IL-1β differentially regulates cAMP accumulation and cAMP-mediated chloride efflux in response to adrenoreceptor or EP receptor ligands in Calu-3 human bronchial epithelial cells. This is likely to be an important phenomenon relevant to the pathophysiology and treatment of cystic fibrosis lung disease.

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