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Isoprostane-Mediated Secretion from Human Airway Epithelial Cells

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ABSTRACT

Isoprostanes are liberated when reactive oxygen species (ROS) mediate the peroxidation of arachidonic acid or other polyunsaturated fatty acids. Because exposure to ROS is associated with tissue damage in the lung, we examined whether exposure to isoprostanes elicited a response in airway epithelial cells, potentially implicating isoprostane production in the epithelial response to oxidant stress. Application of the isoprostane 8-iso-prostaglandin E_2 (8-iso-PGE_2) produced an increase in transepithelial anion secretion across monolayers of the human airway epithelial cell line Calu-3, measured as an increase in short circuit current (I_{sc}). This increase in I_{sc} was greater when 8-iso-PGE_2 was applied to the basolateral rather than the apical face of the Calu-3 monolayers and was almost entirely abolished by the addition of diphenylamine-2-carboxylate, implicating the cystic fibrosis transmembrane conductance regulator Cl^- channel in the response. Experiments with electrically isolated apical and basolateral membrane preparations revealed that 8-iso-PGE_2 stimulated both apical Cl^- and basolateral K^+ conductances. Using reverse transcription-polymerase chain reaction, we found that Calu-3 cells express the TPα, but not the TPβ, isoform of the receptor, and that these cells secrete in response to the thromboxane A_2 (TP) receptor agonist 9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$ (U-46619). However, although part of the response seems to mediated via TP receptors, there are significant non–TP receptor-mediated effects on both the apical and basolateral membranes of Calu-3 cells. This is the first report of an isoprostane eliciting an effect in airway epithelial cells and suggests a potential role for this class of molecules in pulmonary host defense.

Oxidative stress has been implicated in the pathogenesis of a number of respiratory diseases, including asthma (Dworski 2000), adult respiratory distress syndrome (Lamb et al., 1999), cystic fibrosis (CF; van der Vliet et al., 1997) and chronic obstructive pulmonary disease (Repine et al., 1997). The recruitment of activated inflammatory cells to the airways results in the generation of highly cytotoxic reactive oxygen species (ROS), including superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), agents that may damage tissue via direct oxidation of proteins, DNA, or lipids. In addition to the potential for direct oxidative damage, exposure to ROS such as H_2O_2 also results in the production of a large class of prostaglandin-like compounds termed isoprostanes. Isoprostanes are generated when oxygen-centered free radicals such as H_2O_2 and superoxide react with the unsaturated bonds of fatty acids such as membrane-bound arachidonic acid (Morrow and Roberts, 1996) or other polyunsaturated fatty acids such as docosahexaenoic acid (Fam et al., 2002). Although structurally similar to the prostaglandins, differing in the *cis*-orientation of their side chains compared with the *trans*-orientation of prostaglandins, isoprostanes are believed to be produced independently of the cyclooxygenase pathway (Morrow et al., 1990; Morrow and Roberts, 1996). Isoprostanes have been described as markers of oxidative stress and lipid peroxidation in an enormous number of pathologies, including several pulmonary conditions. To date, markedly elevated levels of isoprostanes have been reported in exhaled breath condensates from patients with asthma (Montuschi et al., 1999), chronic obstructive pulmonary disease (Montuschi et al., 2000a), and CF (Montuschi et al., 2000b) and in the bronchoalveolar lavage fluid from patients with various interstitial lung diseases (Montuschi et al., 1998). Thus, they clearly can be viewed as markers of oxidant stress associated with a wide variety of inflammatory lung conditions. However, isoprostanes themselves are biologically active molecules, and there are increasing reports of their eliciting a wide variety of responses in a tissues including vascular

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ABBREVIATIONS: CF, cystic fibrosis; ROS, reactive oxygen species; CFTR, cystic fibrosis transmembrane conductance regulator; PCR, polymerase chain reaction; SQ 29,548, [1*S*-[1a,2a(*Z*),3a,4a]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo(2.2.1)hept-2-yl]-5-heptenoic acid; U-46619, 9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$; DPC, diphenylamine-2-carboxylate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PKA, protein kinase A; PG, prostaglandin; PKC, protein kinase C; RT, reverse transcription.

(Janssen et al., 2001) and airway smooth muscle (Janssen et al., 2000; Catalli et al., 2002), platelets (Kobzar et al., 1997; Leitinger et al., 1997), and endothelial (Fukunaga et al., 1992; Zahler and Becker, 1999) and inflammatory cells (Zahler and Becker, 1999).

To date, no studies have been performed investigating the effects of isoprostanes on airway epithelial cells, although these cells will be exposed to incidents of oxidant stress in vivo. We have recently reported that H_2O_2 stimulates transepithelial anion secretion across intact monolayers of the human airway epithelial cell line Calu-3 (Cowley and Linsdell, 2002a). Calu-3 cells have become a widely used model of human airway submucosal gland serous cells (Shen et al., 1994; Moon et al., 1997; Cowley and Linsdell, 2002a,b). In vivo submucosal gland serous cells play a crucial role in maintaining a sterile environment in the airways because they secrete a number of antimicrobial compounds, such as lysozyme, lactoferrin, secretory protease inhibitor, and secretory IgA (Basbaum et al., 1990). Essentially, they also maintain effective mucociliary clearance, because they are responsible for the glandular secretion of salt and water (Pilewski and Frizzell, 1999), hydrating mucus as it is secreted from mucous cells, and regulating the volume of the airway surface liquid lining the respiratory epithelial cells. In vitro, Calu-3 cells retain several of the markers of serous cell function, secreting antimicrobials and demonstrating active transepithelial anion secretion in response to a number of pharmacological stimuli that raise intracellular cAMP or Ca^{2+} concentrations (Shen et al., 1994; Moon et al., 1997). Serous cells also express the highest levels of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Engelhardt et al., 1992), mutations in which result in CF. Indeed, the high degree of CFTR expression in the serous cell in relation to other airway cell types has led to the proposal that these cells represent the primary site of CF pathology (Pilewski and Frizzell, 1999).

Thus, we now sought to investigate whether isoprostanes, potentially produced during airway inflammation when H_2O_2 acts on arachidonic acid in the airways, elicit a biological effect on Calu-3 cells, a human airway epithelial cell line, and how those effects are mediated.

Materials and Methods

Measurement of Transepithelial Short-Circuit Current (I_{sc}). Calu-3 cells (American Type Culture Collection, Manassas, VA) were maintained and plated on Snapwell inserts (Corning Costar, Cambridge, MA) as described previously (Cowley and Linsdell, 2002a). Cells were grown at an air-liquid interface with medium present only on the basolateral side and experiments performed 10 to 20 days after the establishment of this interface. Inserts were mounted in an Ussing chamber (World Precision Instruments, Sarasota, FL), and the transepithelial potential difference clamped to zero using a DVC-1000 voltage-clamp apparatus (World Precision Instruments). The transepithelial short-circuit current (I_{sc}) was recorded using Ag-AgCl electrodes in agar bridges. Apical and basolateral solutions were maintained at 37°C by heated water jackets and were separately perfused and oxygenated with a 95% O_2/5% CO_2 mixture. Bath solutions for intact monolayers were 120 mM NaCl, 25 mM $NaHCO_3$, 3.3 mM KH_2PO_4, 0.8 mM K_2HPO_4, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM glucose (basolateral) or mannitol (apical), pH 7.4 at 37°C, when gassed with 95% O_2/5% CO_2.

Permeabilized Monolayers. To investigate the activity of apical Cl^- and basolateral K^+ channels in isolation, the opposite membrane was permeabilized by addition of 180 μg/ml nystatin (Sigma, Oakville, ON, Canada) in the presence of appropriate buffers (Cowley and Linsdell, 2002a). Thus, apical Cl^- conductance was studied in the presence of a serosal to mucosal Cl^- gradient with the following bath solutions: apical, 145 mM Na-gluconate, 3.3 mM NaH_2PO_4, 0.8 mM Na_2HPO_4, 1.2 mM $Mg(gluconate)_2$, 4 mM $Ca(gluconate)_2$, 10 mM glucose, and 10 HEPES; basolateral, 145 mM NaCl, 3.3 mM NaH_2PO_4, 0.8 mM Na_2HPO_4, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mannitol, and 10 mM HEPES.

Basolateral K^+ channels were studied by permeabilization of the apical membrane in the presence of a mucosal to serosal K^+ gradient established by the following bath solutions: apical, 145 mM K-gluconate, 3 mM KH_2PO_4, 0.8 mM K_2HPO_4, 1.2 mM $Mg(gluconate)_2$, 4 mM $Ca(gluconate)_2$, 10 mM glucose, and 10 mM HEPES; basolateral, 145 mM Na-gluconate, 3.3 mM NaH_2PO_4, 0.8 mM Na_2HPO_4, 1.2 mM $Mg(gluconate)_2$, 4 mM $Ca(gluconate)_2$, 10 mM glucose, and 10 mM HEPES. All solutions were pH 7.4 at 37°C.

RNA Extraction. Total RNA was extracted from Calu-3s cells using TRIzol reagent (Invitrogen, Burlington, ON, Canada). RNA was then DNase-treated with RQ1 RNase-free DNase (Promega, Madison, WI), and the product was run on a 1% agarose gel to check integrity. DNase-treated RNA (2 μg) was then reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) in the presence of 5 mM dNTP and 1 μM oligo(dT) (Amersham Biosciences, Baie d'Urfe, PQ, Canada) to produce cDNA.

Polymerase Chain Reaction. After reverse transcription, PCR was performed to amplify DNA fragments using the primers described by Miggin and Kinsella (1998). A common primer (5′ GAGATGATGGCTCAGCTCCT 3′) was used to look for both TP isoforms, whereas different primers were used to distinguish between the TPα (5′ CCAGCCCCTGAATCCTCA 3′) and TPβ isoforms (5′ AGACTCCGTCTGGGCCG 3′). All custom primers were obtained from Invitrogen (Burlington, ON, Canada), and reactions were performed using primer pairs at 10 μM with 2.5 units of *Taq* polymerase (MBI Fermentas, Burlington, ON, Canada), 25 mM $MgCl_2$, and 5 mM dNTP in a total reaction volume of 25 μl. The amplification conditions were: denaturation at 95°C for 1 min, annealing at 58°C for 30 s, and elongation at 72°C for 3 min. PCR products were visualized by loading a 8-μl sample on a 1.5% agarose gel containing 250 μg/l ethidium bromide, alongside a 100-base pair DNA ladder (Invitrogen).

DNA Sequencing. To confirm the identity of the amplified PCR fragments, the product was isolated from the gel using the QIAquick gel extraction kit (QIAGEN, Mississauga, ON, Canada). DNA was then ligated into the pGEM vector (Promega), propagated in *Escherichia coli* strain JM109 and sequenced using the Sequenase DNA sequencing kit (U.S. Biochemical, Cleveland, OH).

Chemicals. 8-iso-Prostaglandin E_2, 8-iso-prostaglandin $F2_\alpha$, SQ 29,548 and U-46619 were obtained from Caymen Chemical (Ann Arbor, MI). Diphenylamine-2-carboxylate (DPC), nystatin, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), clotrimazole, and clofilium tosylate were obtained from Sigma Aldrich (Oakville, ON, Canada). The cell-permeable protein kinase A inhibitor 14–22 amide, myristoylated, and chelerythrine chloride and BAPTA-AM were from Calbiochem (San Diego, CA). Stock solutions of the isoprostanes, DPC, and clotrimazole were made up in ethanol, whereas nystatin, DIDS, SQ 29,548, U-46619, clofilium, and the protein kinase A (PKA) inhibitor were made up in dimethyl sulfoxide so that the final bath concentration of solvent was ≤0.1%. DIDS was made up in buffer. Application of dimethyl sulfoxide or ethanol alone had no effects upon the monolayers.

Statistics. All data are presented as means ± S.E.M. Differences between groups were tested for statistical significance using the Student's t test or one-way analysis of variance followed by a Bonferroni t test where appropriate. Significance was determined as $p < 0.05$.

Results

Effect of Isoprostanes on I_{sc} across Intact Calu-3 Monolayers. The basal I_{sc} was increased in a dose-dependent manner by the addition of 8-iso-PGE_2 to either the apical or the basolateral face of monolayers of Calu-3 cells (Fig. 1). Such an increase in I_{sc} is consistent with increased transepithelial anion secretion across the cells. However, a larger increase in I_{sc} was consistently seen when 8-iso-PGE_2 was applied to the basolateral side of the cells (Fig. 1, A and D) rather than the apical (Fig. 1, B and D). In either situation however, application of 8-iso-PGE_2 initially produced a large, transient increase in I_{sc} that was followed by a sustained component (Fig. 1). This response is similar in magnitude and duration to that seen with the addition of forskolin (Fig. 1C), an agent known to elevate intracellular cAMP that we reported previously to produce an increase in I_{sc} of 23.2 ± 2.6 $\mu A/cm^2$ (Cowley and Linsdell, 2002b).

The addition of 8-iso-$PGF_{2\alpha}$ to the basolateral aspect of cells did increase I_{sc}, although to a much lesser extent than 8-iso-PGE_2 (Fig 1E). When 8-iso-$PGF_{2\alpha}$was added apically, the increase in I_{sc} was also minor compared with 8-iso-PGE_2. Because 8-iso-PGE_2 produced a much larger increase in I_{sc} than 8-iso-$PGF_{2\alpha}$ (Fig. 1) we went on to investigate the mechanism of the 8-iso-PGE_2 response in more detail. 300 nM 8-iso-PGE_2 was chosen as the dose for all subsequent investigations. The increase in I_{sc} recorded when 300 nM 8-iso-PGE_2 was applied to the basolateral side of Calu-3 cells was 47.2 ± 2.5 $\mu A/cm^2$ (n = 9) compared with 24.4 ± 1.7 $\mu A/cm^2$ (n = 4) for apical application. For comparison, 300 nM 8-iso-$PGF_{2\alpha}$ applied basolaterally produced an increase in I_{sc} of 4.7 ± 1.1 $\mu A/cm^2$ (n = 3), whereas no increase could be detected for the same dose applied apically.

EC_{50} values were determined as 53 and 160 nM for 8-iso-PGE_2 applied to the basolateral and apical sides, respectively, whereas 8-iso-$PGF_{2\alpha}$had an EC_{50} of 300 nM for basolateral application. The dose-response curves in Fig. 1, D and E, show a supramaximal decline to the agonist. This may well reflect some degree of desensitization, or it may be that at the higher concentrations, additional receptor types are recruited in a nonspecific manner, some of which act to inhibit secretion in these cells.

Effect of Chloride Channel Inhibitors on the 8-iso-PGE_2–Mediated Increase in I_{sc} across Calu-3 Monolayers. Both basal and stimulated anion secretion from Calu-3 cells has previously been shown to be dependent upon the activity of CFTR Cl^- channels (Shen et al., 1994; Singh et al., 1997; Moon et al., 1997; Cowley and Linsdell 2002a). To investigate whether the 8-iso-PGE_2–mediated increase in I_{sc} seen across monolayers of Calu-3 cells was dependent upon CFTR, we examined the effect of the Cl^- channel inhibitors DPC and DIDS (Schultz et al., 1999). When 0.5 mM DPC was applied to the apical side of the monolayers after 300 nM 8-iso-PGE_2, the secretory response was immediately inhibited (Fig. 2A). Furthermore, when 0.5 mM DPC was applied before the isoprostane (Fig. 2B), the basal I_{sc} was initially inhibited, and the subsequent increase in I_{sc} to 300 nM 8-iso-PGE_2 was significantly reduced to 6.5 ± 1.2 $\mu A/cm^2$ (n = 3) or 13% of the control response (Fig. 2C). In contrast, prior application of the disulfonic stilbene DIDS (250 μM) had no effect on the magnitude of the response to 300 nM 8-iso-PGE_2 (48.1 ± 5.2 $\mu A/cm^2$, n = 6, Fig. 2C). Although DIDS inhibits a variety of Cl^- channels (Schultz et al., 1999), CFTR is insensitive to extracellularly applied DIDS. This combination of sensitivity to DPC and insensitivity to DIDS suggests that the anion secretion in response to 8-iso-PGE_2 may be mediated via CFTR, and a further series of experiments was performed to investigate the PKA-dependence of the channel mediating the 8-iso-PGE_2 response.

Effect of 8-iso-PGE_2 on Apical Cl^- Conductance. To further examine the mechanism of the increase in transepithelial anion secretion in response to this isoprostane, we isolated the apical membrane Cl^- conductance by permeabilizing the basolateral membrane with the pore-forming antibiotic nystatin in the presence of a serosal-to-mucosal Cl^- gradient. Addition of 300 nM 8-iso-PGE_2 to the apical face of the permeabilized monolayers resulted in an increase in I_{sc} of 46.4 ± 1.6 $\mu A/cm^2$, n = 5 (Fig. 3A), which under these conditions represents increased efflux of Cl^- ions across the apical membrane down their concentration gradient. Again, this increase in I_{sc} was insensitive to DIDS (250 μM) applied to the extracellular aspect of the membrane but inhibitable by DPC (0.5 mM; Fig. 3A, n = 4). To investigate the mechanism responsible for the increase in apical membrane Cl^- conductance (G_{Cl}), we used an inhibitor of protein kinase A, PKA inhibitor 14–22 amide, because the activity of CFTR is known to be regulated via the PKA/cAMP pathway (Sheppard and Welsh, 1999). Monolayers of Calu-3 cells were incubated in 10 μM PKA inhibitor 14–22 amide, myristoylated for 60 min, after which the basolateral membrane was permeabilized by the addition of nystatin. Under these conditions, the subsequent application of 300 nM 8-iso-PGE_2 to the apical aspect of the membrane did not result in an increase in the I_{sc} (Fig. 3*B*; n = 4).

An additional series of experiments was undertaken to investigate whether the response to 8-iso-PGE_2 was mediated via increases in intracellular Ca^{2+} and the protein kinase C (PKC) pathway. Preincubation of cells with 50 μM of the Ca^{2+} chelator BAPTA-AM before permeabilization of the basolateral membrane with nystatin had no effect on the magnitude of the subsequent response to 300 nM 8-iso-PGE_2 (mean increase in I_{sc} = 44.1 ± 7.7 $\mu A/cm^2$; n = 3, Fig. 3C). Furthermore, the possible involvement of PKC was investigated by preincubating cells for 60 min with the PKC inhibitor chelerythrine chloride (mean increase in I_{sc} = 10 μM; 45.2 ± 2.0 $\mu A/cm^2$; n = 3, Fig. 3D.) Thus, we are able to conclude that the 8-iso-PGE_2–mediated increase in G_{Cl} is dependent on the PKA pathway, whereas we can find no evidence that the PKC pathway is involved.

Effect of 8-iso-PGE_2 on Basolateral K^+ Conductance. To isolate basolateral K^+ conductance, the apical membrane was permeabilized with nystatin in the presence of Cl^--free buffers to establish a mucosal to serosal K^+ gradient. Under these conditions, application of 300 nM 8-iso-PGE_2 to the basolateral side of the monolayers produced a transient increase in I_{sc} of 24.2 ± 2.8 $\mu A/cm^2$, n = 5 (Fig. 4, A and B), which reflects an increase in K^+ conductance (G_K). We have previously demonstrated that agonist-stimulated secretion across Calu-3 monolayers can be inhibited by the K^+ channel inhibitors clofilium and clotrimazole, agents that probably inhibit the KvLQT1 and Ca^{2+}-activated hSK4 (or hIK) channels present in these cells and are encoded by the genes *KCNQ1* and *KCNN4*, respectively (Cowley and Linsdell, 2002b). In cell monolayers pretreated with 30 μM clotrim-

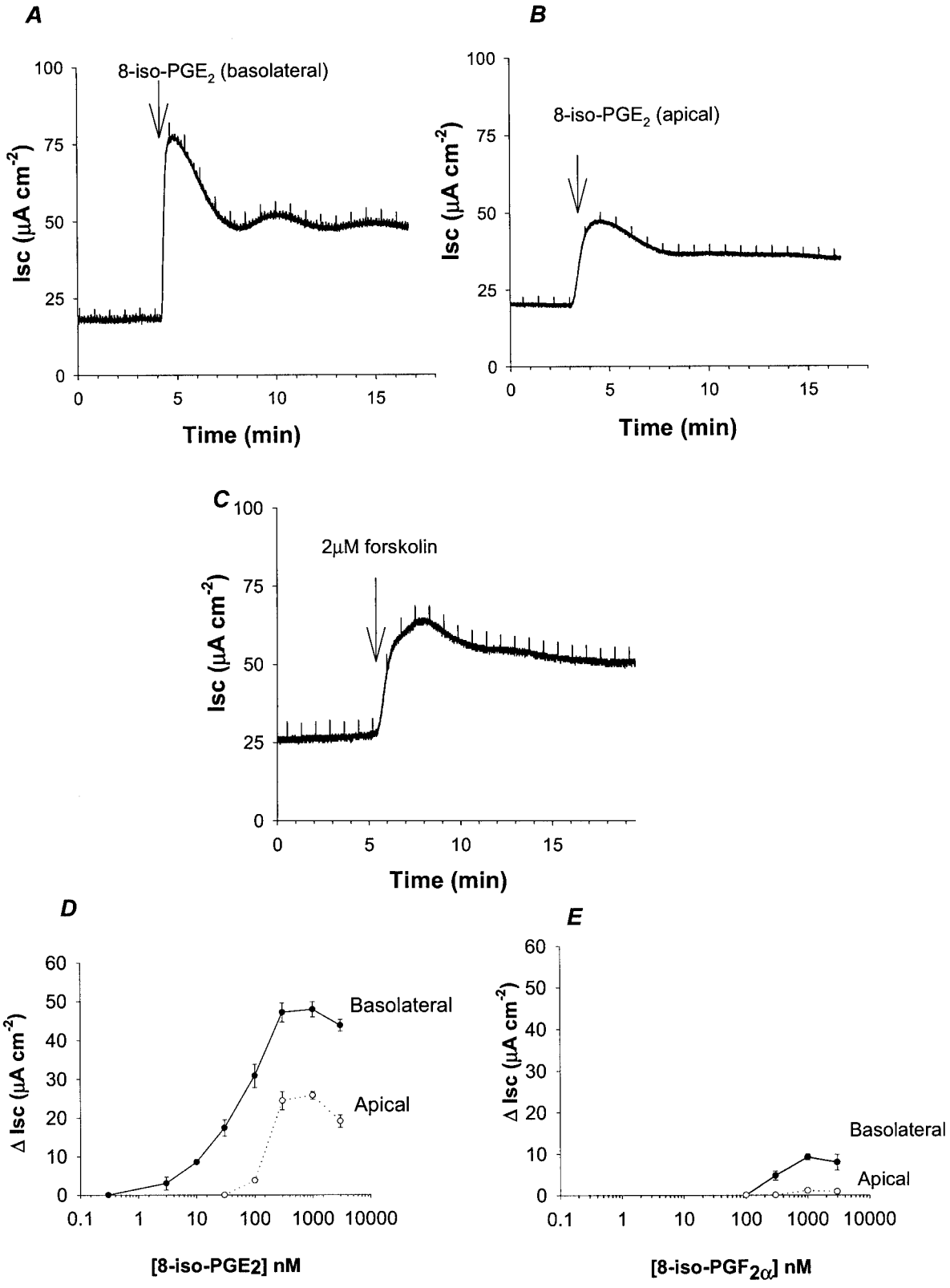

Fig. 1. Isoprostanes 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ stimulates short circuit current (I_{sc}) cross Calu-3 monolayers. Application of 8-iso-PGE_2 to either the basolateral (A) or apical (B) side of monolayers of Calu-3 cells results in a rapid increase in I_{sc}. A much larger response is seen for the same concentration (300 nM) of 8-iso-PGE_2 when applied to the basolateral rather than the apical aspect. For comparison, the response to a maximal dose of forskolin (2 μM) is shown in C. Application of 8-iso-PGE_2 (D) or 8-iso-$PGF_{2\alpha}$ (E) increased I_{sc} across Calu-3 monolayers in a dose-dependent manner. However, the response to 8-iso-$PGF_{2\alpha}$ was small and elicited only at the highest concentrations. Basolateral application of either isoprostane produces a larger response than apical application. Each point represents the mean of between three and nine experiments; when no error bar is shown, the size of the error bar was less than the size of the symbol.

azole before the addition of 300 nM 8-iso-PGE_2, the increase in I_{sc} was only 5.8 ± 0.8 $\mu A/cm^2$ (Fig. 4B; $n = 4$), or approximately 24% of the control value. The addition of 100 μM clofilium also significantly reduced the subsequent response to 8-iso-PGE_2, this time to 12.8 ± 2.8 $\mu A/cm^2$ (Fig. 4*B*, $n = 4$) or 52% of control values. It seems that the major component of the increase in G_K we see in response to 8-iso-PGE_2 is probably mediated via a clotrimazole-sensitive channel, probably Ca^{2+}-activated K^+ channel hSK4, whereas the clofilium-sensitive channels such as KvLQT1 seem less important.

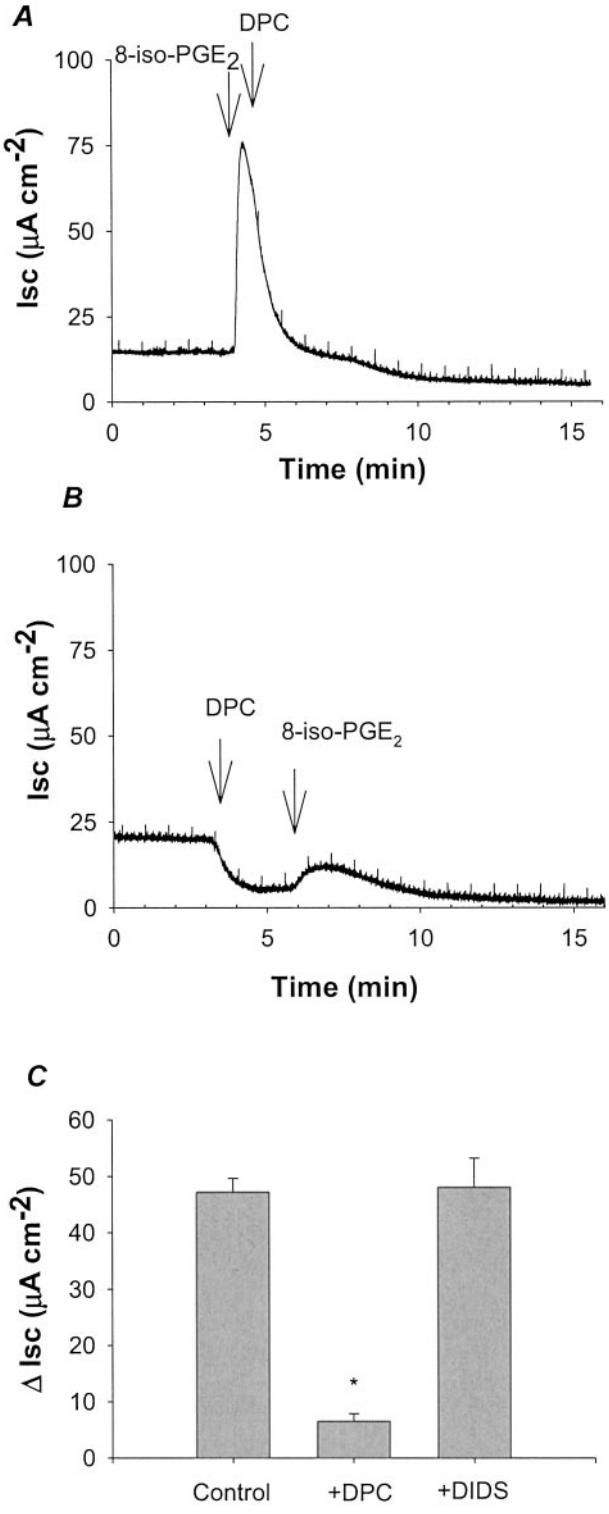

Fig. 2. Effect of chloride channel inhibitors on the 8-iso-PGE_2-mediated increase in I_{sc} across Calu-3 monolayers. The duration of this response was reduced and the sustained component inhibited when DPC (0.5 mM) was applied to the apical aspect of the monolayers after 8-iso-PGE_2 application (A; compare with Fig. 1A). When 0.5 mM DPC was applied before 8-iso-PGE_2 application (B), the subsequent response to the stimulus was significantly reduced (C). Application of 250 μM DIDS had no effect (C).

Mechanism of 8-iso-PGE_2 Action. There is evidence that 8-iso-PGE_2 (and 8-iso-$PGF_{2\alpha}$) may achieve their effects via binding to the thromboxane A_2 (TP) receptor (Elmhurst et al., 1997; Sametz et al., 2000; Janssen, 2001; Kinsella, 2001). However, there are also reports suggesting that unique isoprostane-selective receptors may exist (Longmire et al., 1994). Presently, it is unclear which receptor types could be responsible for the effects of 8-iso-PGE_2 that we see in Calu-3 cells. To investigate whether the TP receptor could be mediating this effect, we first examined whether the response to 8-iso-PGE_2 could be inhibited by SQ 29,548, a specific TP receptor antagonist (Fukunaga et al., 1993; Elmhurst et al., 1997). Application of 10 μM SQ 29,548 10 min before the addition of 300 nM 8-iso-PGE_2 significantly reduced the increase in I_{sc} when the isoprostane was applied to the apical (35% of control, $n = 3$; Fig. 5A) but not the basolateral ($n = 6$; Fig. 5*B*) side of the Calu-3 monolayers.

To establish the presence of functional TP receptors on Calu-3 cells, we next used a specific thromboxane A_2 mimetic, U-46619 (Walsh and Kinsella, 2000; Janssen and Tazzeo, 2002). Addition of 3 μM U-46619 to both sides of intact monolayers of Calu-3 cells stimulated an increase in I_{sc} (22.9 ± 2.0 $\mu A/cm^2$; Fig. 5*C*; $n = 3$), indicating that functional TP receptors are indeed present on Calu-3 cells. Application of the TP receptor agonist to either the apical or the basolateral face stimulated an increase in I_{sc}, suggesting that TP receptors are present on both sides of the monolayers. Basolateral application of 3 μM U-46619 increased I_{sc} by 11.46 ± 2.1 $\mu A/cm^2$ ($n = 4$), whereas apical application increased I_{sc} by 10.86 ± 1.7 $\mu A/cm^2$.

Two isoforms of the TP receptor have been described, TPα and TPβ, both members of the G-protein-coupled receptor superfamily that differ with regard to their C termini (Narumiya et al., 1999; Kinsella, 2001). Using specific primers designed to distinguish between these two splice variants (Miggin and Kinsella, 1998), we performed RT-PCR on total RNA extracted from confluent cultures of Calu-3 cells (Fig. 5*D*). We were able to detect a 400-base pair fragment of the TPα isoform and were unable to detect the TPβ isoform (expected fragment size was 269 base pairs). The band detected using the TPα-specific primers was excised, subcloned into the pGEM vector, and sequenced to confirm its identity. Comparison of the sequence produced with the published sequence (National Center for Biotechnology Information) confirmed 100% identity with the human TP receptor (NCBI accession number XM0476330).

To further investigate the mode of action of U-46619 in Calu-3 cells and compare this with the effects of 8-iso-PGE_2, permeabilized monolayers were used to investigate the second-messenger pathways responsible for its secretory effect. Again, monolayers were preincubated for 60 min in the presence of the either PKA inhibitor 14–22 amide (10 μM) or the PKC inhibitor chelerythrine chloride (10 μM). After incubation, the basolateral membrane was permeabilized by the

addition of nystatin, and the G_{Cl} was recorded in response to 3 μM U-46619. A typical response to U-46619 is shown in Fig 6A. Preincubation with the PKA inhibitor 14–22 amide virtually abolished the response to U-46619 (Fig. 6B; $n = 3$), as did incubation with chelerythrine chloride (Fig. 6C; $n = 3$).

Discussion

In this study, we investigated whether isoprostanes elicited a physiological response from human airway epithelial cells. Our finding, that the isoprostane 8-iso-PGE_2 stimulates transepithelial anion secretion across intact monolayers of Calu-3 cells, is the first report of the effects of an isoprostane on airway epithelial cells and has important implications for our understanding of the pulmonary host defense response to oxidant stress.

To date, there has only been one report of the effect of isoprostanes on epithelial tissue (Elmhurst et al., 1997), despite the potential importance of the epithelium in mediating responses to oxidant stress. This found application of 8-iso-PGE_2 to the serosal (basolateral) side of epithelial sheets from canine proximal colon induced dose-dependent increases in the I_{sc}. We report here that 8-iso-PGE_2 also increases I_{sc} measured across cultured monolayers of a human airway epithelial cell line, Calu-3, a widely used and accepted model of the human submucosal gland serous cell (Shen et al., 1994; Moon et al., 1997; Cowley and Linsdell, 2002a,b).

The most widely studied isoprostanes have been 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$; therefore, these are the molecules we chose to investigate. We found that 8-iso-PGE_2 stimulates anion secretion from Calu-3 cells measured as an increase in

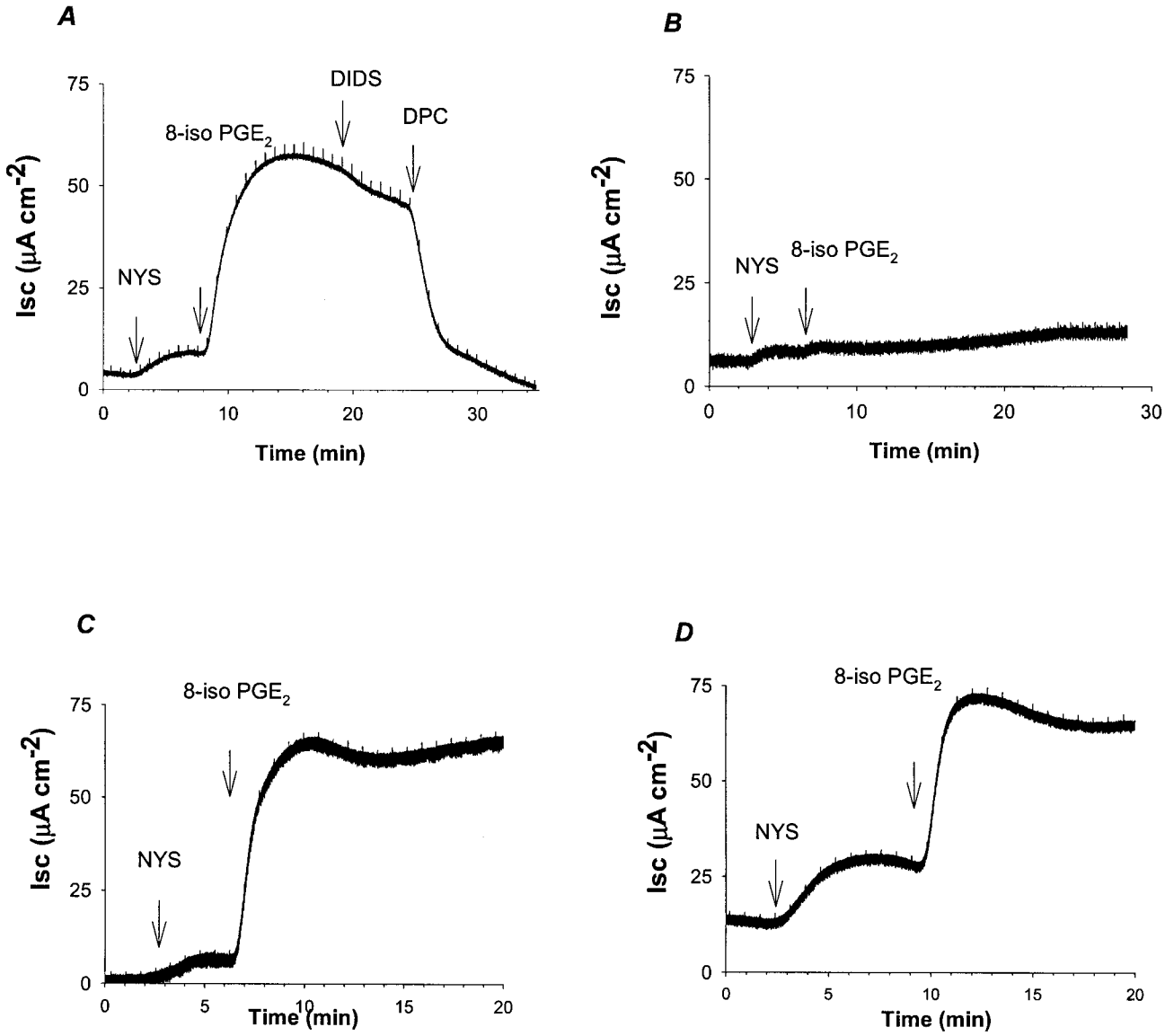

Fig. 3. 8-iso-PGE_2 increases the apical Cl^- efflux in permeabilized Calu-3 monolayers. Apical Cl^- conductance was isolated by permeabilizing the basolateral membrane with nystatin (NYS) in the presence of a serosal to mucosal Cl^- gradient, so that an increase in I_{sc} reflects Cl^- efflux. Addition of 300 nM 8-iso-PGE_2 to the apical face of the membrane induced an increase in Cl^- conductance ($n = 5$), which was insensitive to 250 μM DIDS ($n = 4$) but inhibited by 0.5 mM DPC applied apically ($n = 4$; A). Incubation with protein kinase inhibitor 14–22 amide for 60 min before permeabilizing the basolateral membrane completely abolished the increase in Cl^- conductance normally seen with 300 nM 8-iso-PGE_2 ($n = 4$; B). Preincubation with either the Ca^{2+} chelator BAPTA-AM (50 μM for 60 min, $n = 3$; *C*) or the PKC inhibitor chelerythrine chloride (10 μM for 30 min, $n = 3$; *D*) had no effect on the subsequent response to 300 nM 8-iso-PGE_2.

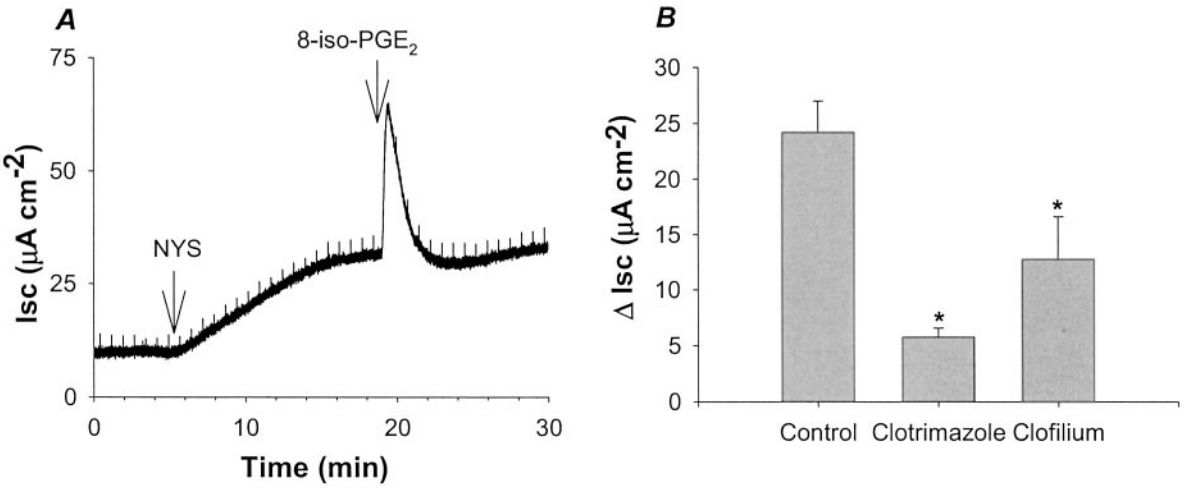

Fig. 4. Basolateral K^+ conductance in permeabilized Calu-3 cells is increased by 8-iso-PGE_2. Basolateral K^+ conductance was isolated by permeabilization of the apical membrane with nystatin in the presence of a mucosal to serosal K^+ gradient. The application of 300 nM 8-iso-PGE_2 to the basolateral membrane under these conditions caused an increase in I_{sc}, reflecting an increase in K^+ efflux (A and B). Application of the K^+ channel blockers clotrimazole (30 μM) or clofilium (100 μM) reduced the 8-iso-PGE_2–mediated response to 24% (n = 4) or 52% (n = 4), respectively, of the control value (n = 4).

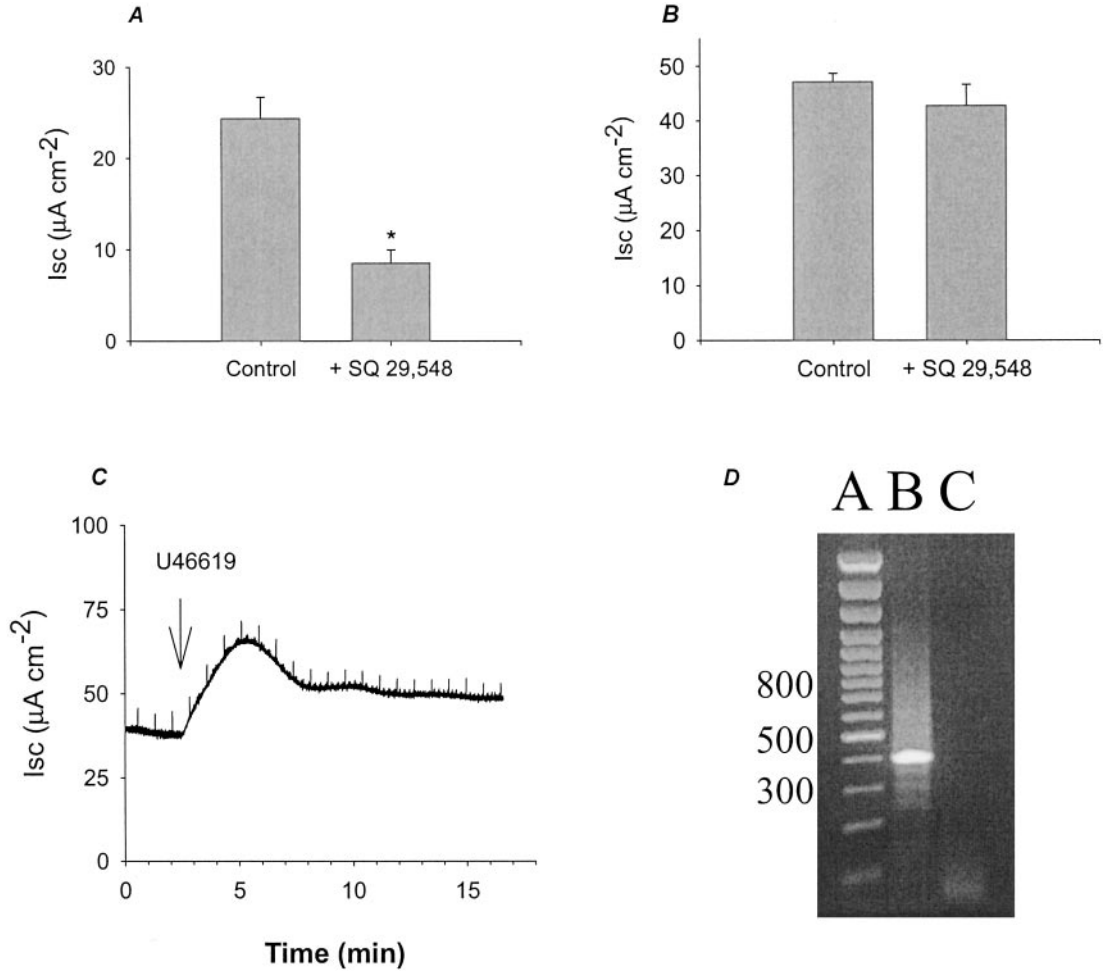

Fig. 5. Calu-3 cells express functional TP receptors and transcripts for the TP α receptor. Application of the TP receptor antagonist SQ 29,548 (10 μM) 10 min before the addition of 300 nM 8-iso-PGE_2 significantly reduced the increase in I_{sc} when the isoprostane was applied to the apical (n = 3; A) but not the basolateral (n = 6; B) side of the Calu-3 monolayers. Application of 3 μM U46619, a TP receptor agonist, resulted in an increase in the I_{sc} across monolayers of Calu-3 cells (C). Transcripts were detected for the thromboxane A_2 (TP) receptor α isoform (D; lane 2) but not β (lane 3). Lane A contains a 100-base pair marker.

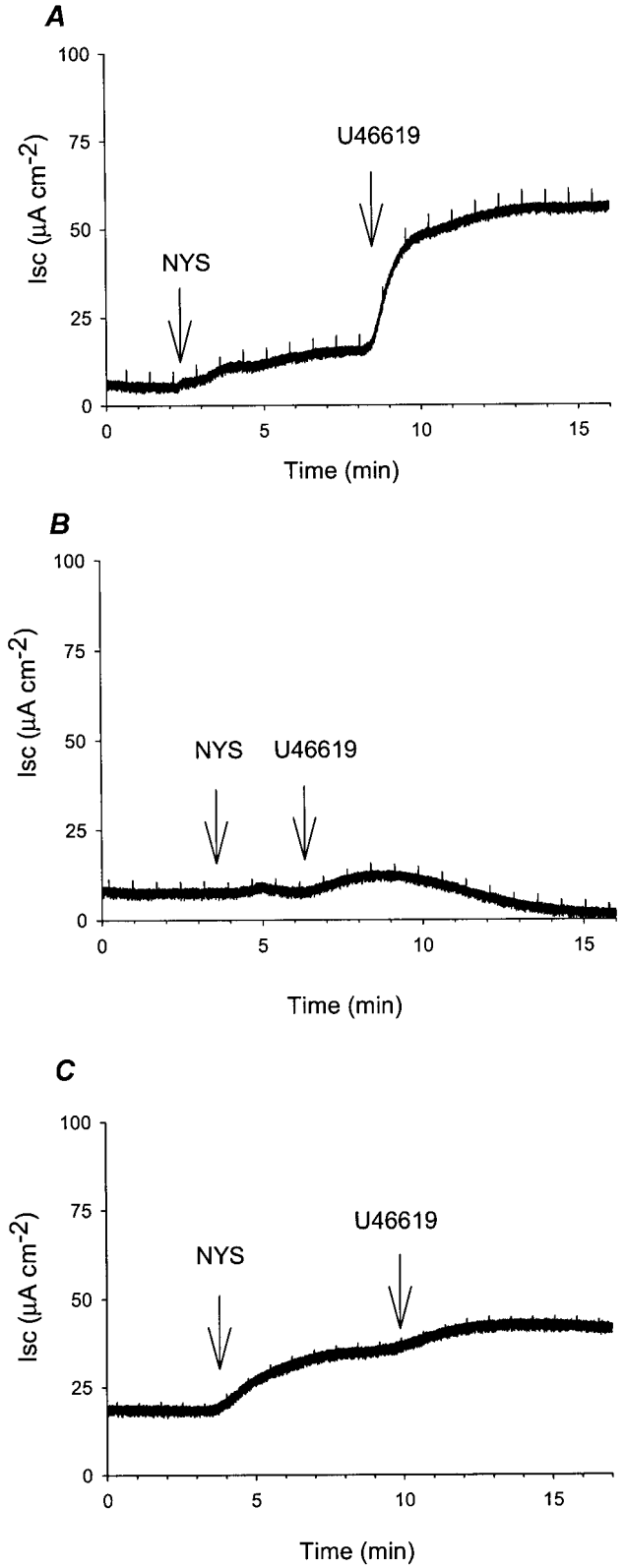

Fig. 6. The increase in apical Cl^- efflux in permeabilized Calu-3 monolayers in response to the TP receptor agonist is inhibited by inhibition of PKA and PKC. Apical Cl^- conductance was isolated by permeabilizing the basolateral membrane with nystatin (NYS) in the presence of a serosal-to-mucosal Cl^- gradient, so that an increase in I_{sc} reflects Cl^- efflux. Application of the TP receptor agonist U-44619 (3 μM) to the apical face of the membrane induced an increase in Cl^- conductance (A) that was inhibited by preincubation with protein kinase inhibitor 14–22 amide for 60 min (B; $n = 3$), and chelerythrine chloride for 30 min (C; $n = 3$).

I_{sc}. For any given concentration, the increase in I_{sc} was always larger when 8-iso-PGE_2 was applied to the basolateral compared with the apical side of the cells (Fig. 1). In contrast, application of 8-iso-$PGF_{2\alpha}$ produced only very small increases in I_{sc} even at large doses (Fig. 1E), and was not subsequently investigated further.

The increase in I_{sc} seen in response to 8-iso-PGE_2 was almost entirely inhibited by DPC (Figs. 2 and 3A) and unaffected by the disulfonic stilbene DIDS (Figs. 2C and 3A). This DPC sensitivity and DIDS insensitivity suggests that the increases we are seeing are mediated via the activation of CFTR. However, DPC is far from a specific inhibitor of CFTR, and this result does not entirely rule out the involvement of another Cl^- channel type. Therefore, we further investigated the role of PKA in mediating this response, because CFTR activity in Calu-3 cells is regulated by PKA activity (Cobb et al., 2002). Preincubation with the PKA inhibitor 14–22 amide abolished the apical membrane increase in G_{Cl} in response to 8-iso-PGE_2 (Fig. 3B) and is additional evidence that we are looking at a CFTR-mediated response. Furthermore, we could find no evidence that the PKC pathway is involved in mediating the response to 8-iso-PGE_2, because the increase in G_{Cl} was unaffected by either the Ca^{2+} chelator BAPTA-AM or the PKC inhibitor chelerythrine chloride.

In addition to stimulating apical G_{Cl}, 8-iso-PGE_2 also stimulated G_K across the basolateral membrane of Calu-3 cells, an increase that was significantly reduced by the K^+ channel blockers clofilium and clotrimazole (Fig. 4B). Halm and Halm (2001) reported that 8-iso-PGE_2 stimulated K^+ secretion across guinea pig distal colon, although the K^+ channels responsible were not investigated. Clofilium inhibits the cAMP-activated K^+ channel KvLQT1, whereas clotrimazole inhibits the Ca^{2+}-activated K^+ channel hSK4 (or hIK), both previously demonstrated to be present in Calu-3 cells (Cowley and Linsdell, 2002b.) At the dose used (100 μM), we cannot be certain that clofilium is acting exclusively to inhibit only KvLQT1; it may well be that other K^+ channel types are inhibited, because the entire complement of K^+ channels in Calu-3 cells has not been described. However, the overall effect of opening basolateral K^+ channels would be to increase anion secretion across the apical membrane, because this K^+ exit would hyperpolarize the cell, increasing the electrochemical driving force for anions to leave the cell. Thus, 8-iso-PGE_2 stimulation of basolateral K^+ channels in concert with the stimulation of apical CFTR would effectively maximize the secretory response to the isoprostane. This coordinated increase in both G_{Cl} and G_K is not unique to 8-iso-PGE_2; indeed, we have found a similar result for both cAMP and H_2O_2 (Cowley and Linsdell, 2002a,b), suggesting that this may be a common physiological mechanism to facilitate secretion from these cells.

The sidedness of the response to 8-iso-PGE_2, in which a larger response was always seen when agonist was applied basolaterally rather than apically, suggests that differences exist in the receptor complement or type available to mediate the cellular response. Although it has been proposed that some isoprostane effects may be mediated via a unique (as-yet unidentified) isoprostane receptor (Morrow and Roberts, 1996), many of their effects seem to be mediated via prostanoid receptors. To date, there is evidence that 8-iso-PGE_2 may act via the TP, FP, or EP receptors depending on the tissue type (Janssen, 2001). We examined whether 8-iso-

PGE_2 was acting via the TP receptor to mediate the secretory response from Calu-3 cells because this receptor type has been most widely implicated in mediating the 8-iso-PGE_2 response (Elmhurst et al., 1997; Sametz et al., 2000; Janssen, 2001; Kinsella, 2001), Two isoforms, TPα and TPβ, which have been described previously (Narumiya et al., 1999), differ in the length of their C termini (Narumiya et al., 1999; Kinsella, 2001). Preapplication of the TP receptor antagonist SQ 29,548 (10 μM) significantly reduced the apical response to 8-iso-PGE_2, although did not abolish it (Fig. 5A). However, the same concentration of SQ 29,548 had no effect on the basolateral response to this isoprostane (Fig. 5B). The most obvious interpretation of these results is that TP receptors are present predominately on the apical aspect of these cells. Furthermore, addition of U-46619, a TP receptor agonist, induced an increase in I_{sc} across Calu-3 cells (Fig. 5C), whereas RT-PCR revealed that Calu-3s express mRNA for the TPα isoform of the receptor (Fig. 5D). Thus, we present functional and molecular evidence that Calu-3 cells express TP receptors. However, application of the TP receptor agonist induced an approximately equivalent increase in I_{sc} when it was applied to either the apical or the basolateral side of the cells. This is in apparent contrast to the SQ 29,548 antagonist data suggesting that TP receptors are present predominately apically, because it would be expected that application of U-46619 would induce more of a response apically than basolaterally. One possibility is that U-46619 is rapidly diffusing into and across the monolayers of cells, so that the response we see is mediated predominately via apical TP receptors. Alternatively, it might be that at the concentration used (3 μM) U-46619 is not acting specifically at TP receptors, but influencing other prostanoid receptors present on these cells.

To further investigate how U-46619 mediates secretion from Calu-3 cells, we looked at how PKA and PKC inhibition affected its response. Although TP receptors are widely reported to be coupled to phospholipase C and the elevation of intracellular Ca^{2+} (Kinsella, 2001), we found that incubation with inhibitors of either PKA or PKC abolished the response to U-46619. Thus, in Calu-3 cells, it would seem that activation of TP receptors affects the PKC pathway; further downstream, however, there seems to be some complex cross-talk also leading to the activation of PKA.

Taken together, our results demonstrate that functional TP receptors are present on Calu-3 cells. However, the differences we observe in the responses to 8-iso-PGE_2 and U-46619 suggest that TP receptors are, at best, only partly responsible for mediating the effects of 8-iso-PGE_2 in Calu-3 cells and that other TP receptor-independent mechanisms, such as binding to additional receptor types, are probably also involved.

The major finding of this study, that 8-iso-PGE_2 stimulates transepithelial anion secretion via CFTR, has potentially important implications for the pathogenesis of CF lung disease, because this mechanism would not function in the CF lung. In the normal submucosal gland serous cell in vivo, stimulated anion secretion via CFTR would be coupled to fluid secretion. This fluid would assist clearing any infectious agents from the airways because it would flush out and fully hydrate mucus secreted from submucosal gland mucous cells, and it would also be rich in endogenous antimicrobial compounds that would directly attack invading organisms (Basbaum et al., 1990). Thus, the release of this fluid would assist host defense mechanisms act to rid the airways of the initial inflammatory insult that leads to ROS production and isoprostane generation. CF lung disease, however, is characterized by repeated and persistent incidents of bacterial infection and consequent inflammatory responses, which ultimately lead to tissue damage and respiratory failure. In the absence of CFTR, this response would be absent, reducing the amount of fluid secreted from submucosal gland serous cells and potentially exposing the CF lung to oxidant stress for extended periods. Thus, loss of this CFTR-mediated response could compromise the CF lung and reduce its ability to carry out effective mucociliary clearance.

In conclusion, we here make the first report of the effects of an isoprostane, 8-iso-PGE_2, on human airway epithelial cells. Isoprostanes are associated with incidents of oxidative stress, because they are produced via the action of ROS on polyunsaturated fatty acids, and elevated levels of isoprostanes have been recorded in a wide variety of inflammatory lung diseases. Because human airways are constantly exposed to instances of oxidant stress, resulting both from the inhalation of foreign material as well as the production of ROS by activated inflammatory cells, it is likely that airway epithelial cells are exposed to elevated levels of isoprostanes. 8-iso-PGE_2–stimulated transepithelial anion secretion across monolayers of Calu-3 cells via concerted effects at apical CFTR Cl^- channels and basolateral K^+ channels. Stimulation of both channel types would maximize the secretory response from the cells, which we propose would help flush the airways of the agent responsible for producing the oxidant stress. Furthermore, our finding that this process is CFTR-mediated suggests that this response to isoprostanes (and oxidant stress in general) would be absent in the CF lung, reducing the effectiveness of host defense mechanisms and potentially exposing the CF lung to extended periods of oxidant stress. This is the first report of an isoprostane eliciting an effect in airway epithelial cells, and suggests a potential role for this class of molecules in pulmonary host defense.

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