Prostanoids secreted by alveolar macrophages enhance ionic currents in
swine tracheal submucosal gland cells

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Running title: Macrophage-secreted PGE₂ enhances airway epithelial currents

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The abbreviations used are: ACh: acetylcholine; AH6809: 6-isopropoxy-9-oxoxanthene-2-carboxylic acid; AM: alveolar macrophages; CFTR: cystic fibrosis transmembrane conductance regulator; ChTX: Charybdotoxin; COX: cyclooxygenase; DIDS: disodium 4, 4'-diisothiocyanatostilbene-2, 2'-disulfonate; DPC: diphenylamine-2-carboxylate; EP: endoprostanoid; ΔISC: changes in short circuit current; ISC: short circuit current; KCa: Ca²⁺-activated potassium channels; Mφ: macrophages; PGE₂: prostaglandin E₂; PGF₂α: prostaglandin F₂α; PKA: cAMP dependent protein kinase A; SC19220: 8-chloro-dibenzo[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-acetylhydrazide; SGC: submucosal gland cells.

Recommended section assignment: Gastrointestinal, Hepatic, Pulmonary, and Renal
Abstract

We examined the effect of substances released by swine alveolar macrophages (AM) on ionic currents in airway submucosal gland cells (SGC). AM obtained by lavage were activated by 24-hour zymosan exposure (0.1 mg/ml). Supernatant was collected and used to stimulate short circuit current changes ($\Delta I_{SC}$) in SGC monolayers in Ussing chambers. Dexamethasone (1 $\mu$M) or indomethacin (5 $\mu$M) during zymosan exposure of AM reduced or abolished the supernatant-induced $\Delta I_{SC}$. Zymosan exposure induced a 5-fold increase in cyclooxygenase (COX)-2 but not COX-1 protein levels in AM. PGE$_2$ concentration in the supernatant from zymosan-activated AM was 550 ± 10 nM (n=3) compared with 28 ± 3 nM for unstimulated AM (n=3). PGE$_2$, applied serosally, induced $\Delta I_{SC}$ with an EC$_{50}$ of 15.5 ± 1.3 nM (n=4), and 3.6 ± 1.8 $\mu$M (n=3) when applied apically. Four types of endoprostanoid receptors (EP$_1$-$4$) were detected in SGC using Western blot. PGE$_2$-induced $\Delta I_{SC}$ were inhibited by 6-isopropoxy-9-oxoxanthene-2-carboxylic acid (AH6809), but not by 8-chloro-dibenzo[b,f][1,4]oxazepine-10(11$H$)-carboxylic acid, 2-acetylhydrazide (SC19220), suggesting that EP$_2$ but not EP$_1$ receptors were activated by PGE$_2$. Pretreatment of SGC with supernatant from zymosan-activated AM, PGE$_2$, or forskolin enhanced the sensitivity to acetylcholine (ACh)-induced $\Delta I_{SC}$. PGE$_2$-induced $\Delta I_{SC}$ were blocked by charybdotoxin (ChTX), chromanol 293B or glibenclamide, ACh-induced $\Delta I_{SC}$ were only blocked by ChTX or glibenclamide. None of these blockers altered PGE$_2$ pretreatment-induced sensitization of ACh-induced $\Delta I_{SC}$. These results demonstrate that prostanoids released from activated-AM directly increase CFTR and K$^+$ channel activity. ACh-induced $\Delta I_{SC}$ are also enhanced due to enhanced activation of Ca$^{2+}$-activated K$^+$ channels ($K_{Ca}$).


Introduction

Airway surface liquid and macrophages are part of the innate defense system in the airway, and are important in the clearance of inhaled environmental particulates and pathogens. Air surface liquid, which forms a barrier to inhaled particles, is comprised of bacteriostatic fluid and mucus secreted mainly by SGC (Knowles and Boucher, 2002; Verkman, et al., 2003). Alveolar macrophages (AM) phagocytize particulates and pathogens, release substances that regulate the function of the immune system, and initiate adaptive immune responses in the lung (Sibille and Reynolds, 1990; Lohmann-Matthes, et al., 1994).

AM activated by particulates release cytokines, such as interleukin-1, -6, the chemokine macrophage inflammatory protein-1α, hematopoietic growth factor, granulocyte-macrophage colony-stimulating factor, and reactive oxygen species (Becker, et al., 1996; Dorger and Krombach, 2000; Suwa, et al., 2002). Activation of AM in the lung by particulates has been shown to induce systemic effects via the circulation by increasing leukocytosis in the bone marrow (van Eeden and Hogg, 2002). Media taken from co-cultures of macrophages and epithelial cells exposed to particulates and instilled into rabbit lung, stimulate bone marrow (Fujii, et al., 2002). AM can also alter the function of respiratory system. For example, AM-released mediators enhance the responsiveness of rat lungs to muscarinic stimulation (Padrid, et al., 1993), and particulate matter exposure of naïve mice increases airway reactivity (Walters, et al., 2001). Little is known however about the effects of AM-derived products in regulating epithelial function and airway fluid and mucus secretion.

We hypothesize that: 1) activated-AM release a substance or substances, which directly induce $\Delta I_{sc}$ across SGC monolayers; 2) the substance(s) secreted modulate ionic current changes induced by other secretagogues and neurotransmitters such as ACh. The latter
effect may contribute to hypersecretory changes during airway inflammation. We examined
the effect of substances released by zymosan-activated AM and PGE₂ on SGC, measured as
ΔI_{SC} across confluent SGC monolayers in Ussing chambers. Our results demonstrate that
zymosan induces COX-2 expression and PGE₂ release by AM. Supernatant from zymosan
activated-AM or PGE₂ induced ΔI_{SC}, mediated via activation of CFTR chloride channels and
K⁺ channels, as well as enhancing the response to ACh-induced ΔI_{SC} and K⁺ current in SGC.
These results suggest a role for the AM activated by particulates in stimulating airway fluid
secretion.
Material and Methods

Macrophage Culture and Drug Treatment. Male weanling pigs (Yorkshire) from a local vendor weighing ~30 Kg were sacrificed by exsanguination after isoflurane anesthesia. This method was approved by the local IACUC. After exsanguination macrophages were collected by bronchioalveolar lavage using 300 ml Ca$^{2+}$- and Mg$^{2+}$-free Hanks’ balanced salt solution (in mM: NaCl, 137; NaHCO$_3$, 4.2; Na$_2$HPO$_4$, 0.3; KCl, 5.4; Glucose, 5.5; EGTA, 0.5; penicillin, 100 U/ml; streptomycin, 100 µg/ml; kanamycin, 25 µg/ml, pH 7.4, 4 °C). The cells were recovered from the lavage solution by centrifugation, washed in the same lavage solution (100 g × 10 min at 4 °C) 3 times, and then resuspended in DMEM / F-12 medium with 1% heat-inactivated fetal bovine serum and plated at a density of 5 × 10$^5$ cells / cm$^2$ ml in 6-well culture dishes (4 ml culture media, 9.6 cm$^2$ surface areas). Cells were maintained in a humidified atmosphere containing 5% CO$_2$ at 37°C for 1 hour. Unattached cells were removed by washing with PBS (3 x) and culture media (1 x). The estimated final cell density was 1.7 × 10$^5$ cells / cm$^2$ ml, or 1.6 × 10$^6$ cells / ml media. Usually at least 1~2 × 10$^8$ cells were collected by a single lavage. Greater than 98% of the cells were CD14 positive as determined by immunohistochemistry. Macrophages were activated by adding zymosan A in suspension to the culture medium (0.1 mg/ml). Indomethacin and dexamethasone were dissolved in DMSO (1000 x stock solution) and added to the culture dish with or without zymosan. After 24 hours incubation, the culture medium was collected and centrifuged at 400 × g for 6 min at 0°C. The supernatant was frozen immediately and stored at -80 °C until use. The osmolarity of macrophage culture media and supernatant was measured using a vapor osmometer (model 552O, Wescor Inc., Logan, Utah).

Isolation and Culture of SGC. Unless specifically noted, isolation and culture of SGC were conducted according to Chan (Chan, et al., 1996). After AM were collected, the trachea
was quickly removed and transported to the lab in physiological saline solution containing (mM): NaCl, 140; KCl, 5.5; CaCl₂, 1; Glucose, 5.5 and Hepes, 10; pH=7.4, supplemented with penicillin and streptomycin. The epithelium was stripped off as a single layer and digested in 2 mg/ml protease and 1 mg/ml deoxyribonuclease in 15 ml physiological saline solution for 60~70 min. The isolated cells were then mixed with 5 ml fetal bovine serum to stop the digestion. The cells were centrifuged through a discontinuous Percoll® gradient that consisted of 5 layers: 10%, 20%, 30%, 40%, and 60% Percoll at 500 × g for 10 min. SGC, located at the interfaces of the 40% and 60% layers, were collected and washed twice. The SGC pellet was resuspended in BioWhittaker™ PC-1 medium containing 2 mM Glutamax, serum substitutes, and antibiotics. For Ussing chamber studies, 10⁶ SGC were plated onto each Millicell®–HA insert (12 mm diameter; Millipore, Billerica, MA) pre-coated with human placental collagen type IV (20 µg/cm²). Inserts were maintained in PC-1 medium in the cell incubator (37 °C, 5% CO₂) for 24 hours. The medium inside the inserts was then removed, allowing SGC to grow at an air interface (Chan, et al., 1996). Medium in each culture dish was changed every 48 hours and apical fluid was removed daily. Confluent SGC monolayers formed 3 to 5 days after plating. In some cases, we used collagenase 0.1 mg/ml, deoxyribonuclease 0.1 mg/ml, and bovine serum albumin 0.5 mg/ml in PC-1 media to digest minced fresh epithelium layer for 4 consecutive one-hour digestion periods at 37 °C, with each digestion stopped with fetal bovine serum. Cells were isolated using percoll gradient as mentioned above and plated in inserts. This method achieved better mucus gland cell survival ratio than that achieved by protease digestion in the final SGC suspension (Yang, et al., 1988). Resting transepithelial potentials and resistances for confluent SGC monolayers were measured using a Millicell-ERS voltohmmeter (Millipore), and currents were calculated according to Ohm’s law.
Measurement of $I_{SC}$ were performed according to previously reported methods from our lab (Chan, et al., 1996). Inserts were mounted in Lucite chambers (Costar). For transmembrane current measurement, normally both sides of chamber were filled with Krebs-Ringer buffer solution (mM): NaCl, 113; KCl, 4.8; CaCl$_2$, 2.5; NaHCO$_3$, 18; KH$_2$PO$_4$, 1.2; MgSO$_4$, 1.2; glucose, 5.5; and mannitol, 30, pH adjusted to 7.4) bubbled continuously with 95% O$_2$ / 5% CO$_2$ and circulated by a bubble lift device at 37 °C. The transmembrane potential was held at 0 mV by voltage clamp, and transmembrane current changes were measured using VCC-600 amplifiers (Physiologic Instrument) and acquired at 50 Hz by a PCI data acquisition card (DAS1602/16, Measurement Computing, Middleboro, MA) using DASYLab 6.0 (Dasytec USA). $\Delta I_{SC}$ were measured by subtracting baseline $I_{SC}$ to the peak $I_{SC}$ response after agonist stimulation. Data were analyzed using Origin 7.0 (OriginLab Corporation).

Prior to addition of agonist, 10 µM amiloride was added to the apical chamber to inhibit sodium absorption during all experiments. Compounds such as ACh, PGE$_2$, and forskolin were added to the serosal solution cumulatively from stock solutions (at least 1000 times concentrated). In some experiments, diphenylamine-2-carboxylic acid (DPC, 0.5~1 mM) or disodium 4, 4'-diisothiocyanatostilbene-2, 2'-disulfonate (DIDS, 100 µM) was added to the apical side of SGC monolayers to block chloride channel activities.

To measure the serosal membrane K$^+$ channel activity, 180 µg/ml nystatin was added to the apical chamber to permeablize the apical membrane to monovalent ions, such as Cl$^-$, K$^+$, and Na$^+$ (Hwang, et al., 1996). A high K$^+$ gradient across the serosal membrane was established and Cl$^-$ current was also minimized by replacing NaCl with potassium gluconate in the apical Ussing solution, and sodium gluconate in the serosal solution. Therefore, $\Delta I_{SC}$ reflected primarily K$^+$ channel activity in the serosal membrane. Ussing chamber solution...
components for the apical side were (mM): potassium gluconate ($\text{C}_6\text{H}_{11}\text{O}_7\text{K}$) 120, $\text{KH}_2\text{PO}_4$ 25, $\text{K}_2\text{HPO}_4$ 0.8, $\text{MgSO}_4$ 1.2, $\text{CaCl}_2$ 4, Glucose 10, and the solution for the serosal side was similar to that of apical side except that the potassium gluconate was replaced with equimolar concentration of sodium gluconate ($\text{C}_6\text{H}_{11}\text{NaO}_7$). Solutions were bicarbonate-free and bubbled continuously with 100% $\text{O}_2$.

**Measurement of Prostanoid Concentration.** Radioimmunoassay of PGE$_2$ in the supernatant was performed by Dr. Jay Westcott of National Jewish Medical and Research Center, Denver, CO. DMEM/F-12 medium was used as a blank control and supernatant from zymosan-activated AM was diluted before performing the assay.

**Protein Purification and Western Blot.** To examine the COX-1 and COX-2 protein expression levels, polyclonal antibodies for COX-1 and 2 and their respective secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After 24 hours of zymosan or vehicle treatment, supernatants from AM culture were collected, and AM were rinsed once with cold phosphate buffered saline (PBS). AM from 6-well dishes treated under the same conditions were combined and lysed in 200 µl of lysis buffer (1 x PBS, pH 7.4, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 µl/ml phenylmethanesulfonyl fluoride, 50 U/ml aprotinin, and 10 µl/ml Na$_3$VO$_4$) at 0 °C. Cell lysates were centrifuged at 10,000 x g for 10 minutes at 4 °C to yield whole-cell extracts. Protein concentration in the whole-cell extract was determined using a Comassie protein assay kit according to manufacturer’s instructions (Pierce, Rockford, IL). Whole cell extracts (10 µg protein / lane) were then denatured and electrophoresed into a 7.5% SDS-polyacrylamide running gel. Proteins were transferred from the gel to a nitrocellulose membrane (BioRad Laboratories, Hercules, CA) according to manufacturer’s instructions. Membranes were blocked for non-specific binding by incubating in 5% milk in 1 x TBS (10 mM Tris-HCl, 150
mM NaCl, pH 8.0) at 4 °C overnight. Membranes were then incubated with primary antibodies diluted with 5% milk in 1 x TBST (0.05% Tween-20, 1 x TBS) for 1 hour at room temperature. Primary antibody concentrations for COX-2 (sc-1745, goat polyclonal) and COX-1 (sc-7950, rabbit polyclonal) were optimized at 1:2,000 and 1:100 dilutions, respectively. After primary antibody incubation the membranes were washed three times with 1 x TBST for 5 minutes each, and then incubated for 45 minutes at room temperature with horseradish peroxidase (HRP) conjugated secondary antibodies. Secondary antibodies concentrations were optimized at 1:5,000 for COX-2 (sc-2033, donkey anti-goat IgG) and 1:10,000 for COX-1 (sc-2317, donkey anti-rabbit IgG), respectively. Membranes were washed three times for 5 minutes each with TBST and once for 5 minutes with TBS. The protein bands and molecular weight markers were detected using Hyperfilm® and ECL plus reagent (Amersham Bioscience, Piscataway, NJ). The protein levels and molecular weights were determined using a personal densitometer and the ImageQuant program (Molecular Dynamics, Sunnyvale, CA). Band densities were normalized to the signal from AM not exposed to zymosan and drugs.

For identification of endoprostanoïd receptor proteins, polyclonal antibodies against these receptors (EP₁, EP₂, EP₃ and EP₄) were purchased from Cayman Chemical (Ann Arbor, MI). SGC were collected using discontinuous Percoll gradient (1.5 × 10⁶ ~ 2.5 × 10⁶ cells/sample) and protein was extracted and measured as described above. Whole cell lysates were precleared by adding 0.25 µg/ml normal rabbit or goat IgG together with 20 µl of Protein A/G-PLUS-Agarose (Santa Cruz Biotechnology) and incubating for 30 min at 4 °C. Supernatant was collected after centrifugation at 2500 rpm for 5 min. To 1 ml supernatants primary antibodies to EP₁, EP₂, EP₃ or EP₄ (2 µg each) were added and incubated for 1 hour at 4 °C. Protein A/G PLUS-Agarose (20 µl) was then added to each sample and after 1 hour
incubation, immunoprecipitates were collected by centrifugation at 3,000 rpm for 5 min (Mamoon, et al., 2004). After washing the pellets 4 times with PBS, 40 µl of electrophoresis sample buffer was added to each sample and boiled for 90 seconds. Proteins were separated, transferred to nitrocellulose membranes and detected as described above.

**Materials.** Protein purification reagents were purchased from Santa Cruz Biotechnology Inc. Fetal bovine serum was purchased from Hyclone laboratories Inc. PC-1 medium containing 2 mM Glutamax and serum substitutes, was purchased from Cambrex Bio Science. AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid) and SC19220 (8-chlorodibenzo[b,f][1,4]oxazepine-10(11H)-carboxylic acid, 2-acetylhydrazide) were purchased from Cayman chemicals. Chromanol 293B was purchased from Tocris Cookson Inc. Amiloride, ChTX, collagenase I, collagen type IV, dexamethasone, DIDS, DMEM, DMSO, DNAse, DPC, glibenclamide, indomethacin, PGD₂, PGE₂, PGF₂α, Percoll, protease, Zymosan A, and other reagents, were purchased from Sigma. DMSO was used to dissolve water-insoluble reagents used in Ussing chamber studies and stored at -20 °C. These stocks were further diluted in Krebs-Ringers buffer solution immediately before use. The final dilution of DMSO in the macrophage culture or Ussing chamber solution was equal to or less than 0.1% (v/v). Charybdotoxin was dissolved in water as a 100 µM stock solution. DPC (Sigma) was dissolved in with 0.1 N NaOH as a 1 M stock solution.

**Data Analysis.** All data are expressed as mean ± SEM, and n values reported are the number of animals used for each experiment. To examine the relative sensitivities of SGC to agonists under different experiment conditions, EC50 values were calculated from individual cumulative concentration-response data using sigmoid fit functions of Origin 7.0 (Originlab) and then averaged. Data from multiple treatment groups were analyzed by one-way ANOVA or one-way repeated measures ANOVA followed by the Student-Newman-Keuls pair-wise
test for multiple comparisons, whenever appropriate (Sigma Stat software, SPSS Inc., Chicago, IL). In some cases, ANOVA on ranks or repeated measures ANOVA on ranks were used instead when the variances of the data were not equal among all treatment groups. Student's $t$-test was used to compare the data from 2 groups with either paired or unpaired tests as appropriate. A value of $p < 0.05$ was regarded as significant.
Results

The resting transepithelial potentials, currents, and resistances for confluent SGC monolayers were 5.1 ± 0.5 mV, 5.0 ± 0.7 µA/cm², and 1.8 ± 0.2 KΩ/cm² respectively, at 3~5 days after primary culture (n=11). At the beginning of all Ussing chamber experiments 10 µM amiloride was added apically to block sodium absorption. This caused an average 1.0 ± 0.1 µA/cm² decrease in ∆I_{SC} (n=11). Supernatant taken from 24-hour zymosan-exposed AM had average osmolarities of 320 mOsm/L and pH values of 7.2, close to values of fresh DMEM (320 and 7.4, respectively). The addition of DMEM to the Ussing chambers at up to 10% (v/v) had little effect on the total osmolarity or pH value of the Krebs-Rings buffer solution (310 mOsm/L and pH 7.4, respectively), and did not change I_{SC} of SGC monolayers.

Effect of supernatant from zymosan-activated AM in inducing ∆I_{SC}. To examine the direct effect of substances released by activated-AM in increasing I_{SC}, supernatant from 24-hour zymosan-exposed AM (Mϕ + Zymosan Supernatant) was applied cumulatively to the serosal side of the SGC monolayers. The supernatant-induced ∆I_{SC} is illustrated in Fig 1A. A small increase in I_{SC} was induced at 0.3% dilution (ΔI_{SC} = 1.1 ± 0.3 µA/cm²). I_{SC} increased with increasing volume added. The ΔI_{SC} induced by 1% and 10% dilution were 3.1 ± 0.7 µA/cm² and 9.0 ± 0.8 µA/cm², respectively (n=5). The increase in I_{SC} was persistent reaching a plateau after about 5 minutes exposure to Mϕ + Zymosan Supernatant. The transmembrane resistance also decreased as shown by the increase in amplitude of the currents induced by voltage pulses (2 mV every 30 seconds) applied to the epithelium. The I_{SC} increased by Mϕ + Zymosan Supernatant was abolished by 1 mM DPC but not by 100 µM DIDS applied apically (n=3). The concentration-response relationship of Mϕ + Zymosan Supernatant-induced ΔI_{SC} is shown in Fig 1D (filled squares, n=5). A maximal response was not reached at the dilutions used (up to 10%) in these experiments. Apical addition of Mϕ + Zymosan Supernatant (up to
10%) did not induce significant increases in $I_{SC}$ (n=3). Supernatant applied serosally from 1, 2, 3, 6, 12 hours zymosan-treated AM did not cause significant increases in $I_{SC}$ (data not shown).

Supernatant removed from AM incubated for 24 hours without zymosan exposure ($M_\Phi$-only Supernatant) applied to the serosal side of the SGC epithelium induced $\Delta I_{SC}$ of $1.4 \pm 0.8 \mu A/cm^2$ at 10% dilution (Fig 4A, middle trace marked with $M_\Phi$-Only, n=3), similar in magnitude to that caused by 0.3% $M_\Phi + Zymosan$ Supernatant ($\Delta I_{SC} = 1.1 \pm 0.3 \mu A/cm^2$, n= 5, p>0.05).

**Effects of indomethacin and dexamethasone in inhibiting the production of active substance by zymosan-activated AM.** There are numerous substances released from macrophages on activation, the mix changing with the stimulus used and the length of time after activation (Lohmann-Matthes, et al., 1994). The active substances produced that elicited a change in $\Delta I_{SC}$ were not observed in supernatant until more than 12 hours after zymosan exposure, suggesting that release of pre-formed mediators was not involved. In addition, the substances produced must be fairly stable to accumulate in culture medium for 24 hours at 37 °C without being totally degraded. Therefore, since it has been shown that zymosan can induce the expression of cyclooxygenase (Vicente, et al., 2001) and that some prostanoids, such as PGE$_2$ and PGF$_{2\alpha}$ are quite stable in culture medium, we examined the possibility that the active products were prostaglandins.

As shown in Fig 1B, indomethacin (5 μM), a nonselective COX blocker, was added to the AM culture during 24-hour zymosan exposure. 1% dilution of supernatant from AM exposed to both indomethacin and zymosan did not increase $I_{SC}$ (trace 3: $M_\Phi + Indo + Zymosan$ Supernatant), $\Delta I_{SC} = 0 \pm 0 \mu A/cm^2$, while 1% $M_\Phi + Zymosan$ Supernatant induced an increase in $I_{SC}$ (trace 1: $M_\Phi + Zymosan$ Supernatant), $\Delta I_{SC} = 2.7 \pm 0.3 \mu A/cm^2$ (n = 5 each, p<0.05). Supernatant from AM exposed to indomethacin alone had no effect on $\Delta I_{SC}$ (trace 2: $M_\Phi +$...
Indo Supernatant: 0 ± 0 µA/cm², n= 5). Residual indomethacin in the supernatant had no direct effect on the SGC, since application of equivalent dilution of indomethacin (50 nM, n=5) directly to the Ussing chamber did not change the response of SGC to Mφ + Zymosan Supernatant (data not shown).

We also treated AM culture with 1 µM dexamethasone, a known suppressor of macrophage function (Becker and Grasso, 1985). Supernatant from AM treated with dexamethasone (1%) during 24-hour zymosan exposure induced significantly less ∆I_{SC} compared with ∆I_{SC} induced by 1% Mφ + Zymosan Supernatant. Dexamethasone treatment caused a 70.5 ± 2.4% inhibition of ∆I_{SC} compared with Mφ + Zymosan Supernatant (n = 3, p<0.05). There was no direct action of the residual dexamethasone in the supernatant on the SGC, since application of equivalent dilution of dexamethasone (10 nM, n=3) directly to the Ussing chamber had no effect on the SGC response to Mφ + Zymosan Supernatant (data not shown). DMSO vehicle (0.1% dilution) in macrophage culture did not influence the supernatant-induced ∆I_{SC}.

The comparison of prostaglandins and supernatant in inducing-∆I_{SC}. The ability of prostaglandins to increase I_{SC} of SGC was examined In Fig 1C. PGE 2 or PGF 2α applied cumulatively to the serosal side of SGC monolayers increased I_{SC}. I_{SC} rises to a stable plateau within 5 min after the agonist application, similar to the response to Mφ + Zymosan Supernatant. PGE 2-induced ∆I_{SC} reach a maximum at approximately 10^{-6} M in the case of PGE 2 (Fig 1D, open squares, maximal ∆I_{SC} = 15.7 ± 1.4 µA/cm², n = 4). The EC_{50} for serosal PGE 2-induced ∆I_{SC} is 10.0 ± 1.8 nM (n = 4). Serosal PGF 2α was less potent than PGE 2 in inducing ∆I_{SC}, with an estimated EC_{50} of 1.1 ± 0.1 µM (Fig 1D, ∆I_{SC} = 6.4 ± 0.7 µA/cm² at 10^{-5} M, open circles, n = 3). We also applied another prostaglandin, PGD 2, to the serosal side of SGC monolayer, which induced ∆I_{SC} with an EC_{50} of ~19 nM (one experiment, data not
shown). As also shown in the Fig 1D, 100% Mφ + Zymosan Supernatant is estimated to induce $\Delta I_{\text{SC}}$ comparable to that induced by $10^{-7}$ M PGE$_2$.

**PGE$_2$ concentration in the supernatant of zymosan-activated AM.** PGE$_2$ levels in Mφ-only Supernatant measured using radioimmunoassay was $10 \pm 1$ ng/ml ($\sim 28 \pm 3$ nM, n = 3). The PGE$_2$ concentration reached $195 \pm 28$ ng/ml ($\sim 550 \pm 79$ nM, n = 3) in Mφ + Zymosan Supernatant, an approximate 20-fold increase in PGE$_2$ release into the culture medium compared with AM not exposed to zymosan (p < 0.05). PGE$_2$ was not detectable in fresh culture media.

**Cyclooxygenase expression and zymosan exposure of AM.** The delay in production of active substance in inducing $\Delta I_{\text{SC}}$ is consistent with increased expression of a protein. We examined the effects of zymosan exposure on cyclooxygenase (COX-1 and COX-2) expression levels in AM. As shown in Fig 2, Western blots for both COX-1 and COX-2 yielded single bands with estimated molecular weights of approximately 79-90 Kd. COX-1 levels, as estimated using western blot (Fig 2A and inset), were not affected by zymosan exposure for 24 hours (98 ± 13% of basal level, n = 3). Indomethacin (5 $\mu$M) or dexamethasone (1 $\mu$M) present during zymosan exposure did not alter COX-1 expression levels (Fig 2A, bar graph, 153 ± 17% and 122 ± 29% of basal level, respectively, n = 3 each). COX-1 expression levels were not significantly different among all treatment groups (p > 0.05).

COX-2 expression levels were increased as early as 6 hours but markedly increased by 24 hours zymosan-exposure (data not shown). As shown in the western blot and bar graph in Fig 2B and inset, zymosan exposure (24-hour) significantly increased COX-2 expression levels in non-drug, 5 $\mu$M indomethacin-, and 1 $\mu$M dexamethasone-treated groups (filled bars for zymosan-treated AM versus empty bars for non zymosan-treated AM, n = 3, p < 0.05). Zymosan exposure increased COX-2 expression level to $540 \pm 100\%$ of basal level in non-
drug and non-zymosan AM, to 850 ± 200% in indomethacin- and zymosan-treated AM (versus 220 ± 50% in AM treated with indomethacin alone), and to 120 ± 20% in dexamethasone- and zymosan-treated AM (versus 20 ± 10% in macrophage treated with dexamethasone alone). The presence of dexamethasone during zymosan exposure significantly suppressed the expression of COX-2 (120 ± 20% of basal level versus 540 ± 100% of basal level in zymosan-treated AM with and without dexamethasone, respectively, p<0.05). Indomethacin (5 µM) treatment alone significantly increased COX-2 expression to 225 ± 52% of basal level (p < 0.05).

**Serosal and apical PGE$_2$ application in inducing $\Delta I_{SC}$.** Since AM are resident on the luminal surface of the airway, would substances, specifically PGE$_2$, produced luminally cause an effect? As shown in Fig 3A trace 2, PGE$_2$ applied to the apical side of the SGC monolayers increased $I_{SC}$ with similar characteristics to those observed upon serosal addition of PGE$_2$ or supernatant. The EC$_{50}$ for apical PGE$_2$ was 3.6 ± 1.8 µM (n=3, Fig 3B circles), significantly higher than that for serosal PGE$_2$ application in parallel experiments (EC$_{50}$ = 15.5 ± 1.3 nM, n=4, squares, p < 0.05 using t-test).

PGE$_2$ is less potent in inducing $\Delta I_{SC}$ in collagenase-dissociated SGC than protease-dissociated SGC (167 ± 34 nM, n = 4 versus 10.0 ± 1.8 nM n = 4 in Fig 1D, or 15.5 ± 1.3 nM in Fig 3B, n = 4. p < 0.05). PGE$_2$ had similar EC$_{50}$ values in two separate protease-dissociated SGC groups shown in Fig 1D and Fig 3B (p>0.05).

**Receptors responsible for PGE$_2$-induced $\Delta I_{SC}$.** We examined the types of EP receptor involved in the PGE$_2$ actions. As shown in Fig 3C, Control trace, PGE$_2$ increased $I_{SC}$ with an estimated EC$_{50}$ of 167 ± 34 nM (Fig 3D, circles, n = 4). When 10 µM SC19220, an EP$_1$ antagonist, or 30 µM AH6809, an EP$_1$ and EP$_2$ antagonist was applied to the serosal side of SGC monolayers, AH6809 caused right-shift of the concentration-response relationship of PGE$_2$-induced $\Delta I_{SC}$ (Fig 3D, upward triangles, EC$_{50}$ = 666 ± 162 nM, n=3), but SC19220 did
not (Fig 3D, squares, EC$_{50}$ = 103 ± 33 nM, n=3). EC$_{50}$ for AH6809-treated group was significantly different from Control group and SC19220-treated group (p < 0.05).

Fig 3E shows that, all four EP receptor subtypes (EP$_{1-4}$) were detected using immunoprecipitation and Western blot methods in SGC isolated with protease or collagenase-dissociated fresh SGC. The estimated molecular weights were approximately 42, 52, 53, and 65Kd, respectively (n= 3-5).

**Effect of supernatant, PGE$_2$, and forskolin on ACh-induced $\Delta$I$_{SC}$.** We tested the ability of supernatant to modulate ACh-induced $\Delta$I$_{SC}$. To estimate the concentration-response relationship for ACh-induced $\Delta$I$_{SC}$, we applied ACh cumulatively to the serosal side of SGC monolayers. As shown in Fig 4A, ACh transiently increased I$_{SC}$ followed by a slow decay. Changes in I$_{SC}$ were observed from 10$^{-7}$ M to a maximum at 10$^{-5}$ M ACh (trace marked by “Control”). The estimated EC$_{50}$ for ACh-induced $\Delta$I$_{SC}$ in “Control” SGC was 438 ± 64 nM (Fig 4B, circles, n= 6,). In parallel inserts, prior to ACh treatment, SGC monolayers were treated serosally with $M\phi$-only Supernatant or $M\phi$ + Zymosan Supernatant as described previously (traces marked by “$M\phi$-only” and “$M\phi$ + Zymosan” respectively). As mentioned before, $M\phi$-only Supernatant at up to 10% (v/v) only induced a small increase in $\Delta$I$_{SC}$, while $M\phi$ + Zymosan Supernatant induced a stable increase in I$_{SC}$ that is similar to that shown in Fig 1A. Subsequent cumulative application of ACh to the same SGC monolayers induced increases in I$_{SC}$ similar in characteristics to those in the “Control” trace. ACh-induced $\Delta$I$_{SC}$ had an EC$_{50}$ of 335 ± 67 nM for SGC pretreated with $M\phi$-only Supernatant (Fig 4B, squares, n=3), a value not significantly different from the EC$_{50}$ in “Control” SGC (p > 0.05). However, the EC$_{50}$ for ACh-induced $\Delta$I$_{SC}$ in SGC treated with $M\phi$ + Zymosan Supernatant was significantly reduced to 149 ± 40 nM (Fig 4B, upward triangles, n=3, p < 0.05 compared with EC$_{50}$ values in “Control” SGC or $M\phi$-only Supernatant-treated SGC).
In addition, the pretreatments with $\text{Mφ} + \text{Zymosan Supernatant}$ and $\text{Mφ-only Supernatant}$ significantly increased the maximal increase in ACh-induced $\Delta I_{\text{SC}}$ in SGC, with average values being $140 \pm 6\%$ and $122 \pm 4\%$ of the ACh-induced maximal $\Delta I_{\text{SC}}$ in “Control” SGC run in parallel ($p<0.05$).

We further examined the effect of PGE$_2$ or forskolin pretreatment on ACh-induced $\Delta I_{\text{SC}}$. SGC monolayers were pretreated with PGE$_2$ or forskolin ($5 \times 10^{-6}$ M, to elevating cytosolic cAMP level) serosally for 5 minutes before ACh application. Both PGE$_2$ and forskolin induced persistent increases in $I_{\text{SC}}$ in 3 to 5 minutes (Fig 4C, middle and upper traces, respectively). Pretreatment of SGC monolayers with $10^{-7}$ M PGE$_2$ or $5 \times 10^{-6}$ M forskolin sensitized SGC to ACh, ACh-induced increases in $I_{\text{SC}}$ were now observed from $10^{-8}$ M to the maximum at $10^{-6}$ M. This is compared with range of $10^{-7}$ M to $10^{-5}$ M ACh concentrations in SGC not pretreated with PGE$_2$ or forskolin (Control trace, Fig 4C). $10^{-7}$ M PGE$_2$ and $5 \times 10^{-6}$ M forskolin pretreatment shifted the EC$_{50}$ for ACh response to $121 \pm 20$ nM ($n = 10$) and $84 \pm 19$ nM ($n=4$) (Fig. 4D, upper triangles and squares, respectively), while the EC$_{50}$ for ACh-induced $\Delta I_{\text{SC}}$ in controls was $418 \pm 28$ nM (Fig 4D, circles, n=12). The shift in the sensitivity to ACh depended on the concentration of PGE$_2$. An increase in sensitivity to ACh could be detected at $10^{-8}$ M PGE$_2$ and reached maximum at $10^{-6}$ M PGE$_2$. PGE$_2$ pretreatment shifted the EC$_{50}$ for ACh-induced $\Delta I_{\text{SC}}$ to $292 \pm 37$ nM ($n = 3$), $188 \pm 35$ nM ($n = 3$), and $103 \pm 35$ nM ($n = 3$) at $10^{-9}$ M, $10^{-8}$ M, and $10^{-6}$ M PGE$_2$ concentrations, respectively. The EC$_{50}$ values for PGE$_2$- (from $10^{-8}$ M and up) and forskolin-pretreated groups were significantly different from control ($P < 0.01$). Pretreatment with $10^{-7}$, $10^{-6}$ M PGE$_2$ or $5 \times 10^{-6}$ M forskolin had similar effects in sensitizing ACh-induced $\Delta I_{\text{SC}}$, and the EC$_{50}$ values for these three groups were not significantly different from each other ($p > 0.05$).
PGF$_{2\alpha}$ ($10^{-5}$ M) was also applied serosally before ACh application. The EC$_{50}$ was shifted for the ACh-induced $\Delta I_{SC}$ to $87 \pm 8$ nM ($n = 3$), significantly different from that in Controls ($418 \pm 28$ nM, $n=12$, $p<0.05$).

The ACh-induced maximal $\Delta I_{SC}$ in groups treated with $10^{-8}$ M, $10^{-7}$ M, and $10^{-6}$ M PGE$_2$ were significantly greater than control monolayers tested in parallel, the average increases being $143 \pm 6\%$, $135 \pm 5\%$, and $131 \pm 7\%$ of ACh-induced maximal $\Delta I_{SC}$ in control ($p<0.05$), respectively. ACh-induced maximal $\Delta I_{SC}$ in SGC treated with $10^{-9}$ M PGE$_2$ or $5 \times 10^{-6}$ M forskolin were not significantly different from those of control, the average values being $104 \pm 9\%$ and $107 \pm 8\%$ of control, respectively ($p > 0.05$).

**K$^+$ and Cl$^-$ channels involved in PGE$_2$- or ACh-induced $\Delta I_{SC}$**. ChTX, a $K_{Ca}$ blocker, and chromanol 293B, a blocker of $K_v$LQT1(KCNQ1) / KCNE3 $K^+$ channels (Lohrmann, et al., 1995), were used to examine the involvement of these $K^+$ channels in the PGE$_2$- and ACh-induced increases in $I_{SC}$. Previous reports showed that the $K_v$LQT1 (KCNQ1) are present in human bronchial epithelial cells and the Calu-3 serous cell line. KvLQT1 are involved in cAMP-mediated Cl$^-$ secretion (Mall, et al., 2000; Cowley and Linsdell, 2002). Chromanol 293B, at 200 µM (supramaximal concentration), was applied serosally to SGC monolayers after $10^{-7}$ M PGE$_2$ but before ACh applications, which caused an average $63 \pm 3\%$ decrease in PGE$_2$-induced $\Delta I_{SC}$ (Fig 5A trace 3, $n=4$). However, Chromanol 293B did not change the amplitude or potency of ACh-induced increase in $I_{SC}$. ACh-induced maximal $\Delta I_{SC}$ in PGE$_2$ + 293B treated group was $108 \pm 13\%$ of PGE$_2$-treatment group (Fig 5B, $p> 0.05$). Chromanol 293B treatment did not alter the PGE$_2$–induced reduction in EC$_{50}$ of ACh-induced $\Delta I_{SC}$ induce (Fig 5B circles, $123 \pm 36$ nM for PGE$_2$-treatment group versus $191 \pm 82$ nM for PGE$_2$ + 293B treatment group, upper triangles, $n=4$ each, $p > 0.05$).
ChTX (100 nM) was added serosally after $10^{-7}$ PGE$_2$ application and blocked 24 ± 4% (n=4) of $10^{-7}$ M PGE$_2$-induced $\Delta I_{SC}$. ChTX also blocked 66 ± 1% of ACh-induced maximal $\Delta I_{SC}$ compared with that in SGC not treated with ChTX (Fig. 5A trace 2 and Fig 5B squares n=4). EC$_{50}$ values for ACh-induced $\Delta I_{SC}$ were 171 ± 37nM and 109 ± 26 nM for SGC monolayers treated with and without 100 nM ChTX, respectively (n=4 each, p>0.05). All these SGC monolayers were pretreated with $10^{-7}$ M PGE$_2$. The effect of K$^+$ channel inhibition on the ACh concentration response relationships for PGE$_2$-pretreated SGC is shown in Fig 5B. In SGC monolayers not treated with PGE$_2$, ChTX (100nM) added before ACh actions also blocked 60 ± 7% of ACh-induced maximal $\Delta I_{SC}$ (n = 4).

To test the possibility that PGE$_2$ activates CFTR channels and facilitates the ACh response, 2 mM (maximum concentration) glibenclamide was applied apically to the SGC monolayers to block CFTR channel activity. Glibenclamide, a relatively non-specific CFTR channel blocker, has been used to study the role of CFTR in the epithelial cells (Krouse, et al., 2004). Glibenclamide abolished $10^{-7}$ M PGE$_2$-induced increase in $I_{SC}$ to below baseline levels (Fig 5C, n = 3). After PGE$_2$ and glibenclamide treatment, ACh-induced increases in $I_{SC}$ were also inhibited (Fig 5D, a partial enlarged-view of 5C, averaged maximal $\Delta I_{SC}$ was ~ 1 $\mu$A/cm$^2$, n = 3). The EC$_{50}$ of ACh-induced increases in $I_{SC}$ after PGE$_2$ and glibenclamide treatment was 182 ± 28 nM (n=3), which was not significantly different from control (123 ± 36 nM, n=4, p>0.05). These data were consistent with results that supernatant and PGE$_2$-induced increases in $I_{SC}$ were abolished by apically applied 1 mM DPC, but less effected by 100 $\mu$M DIDS (Fig 1A).

**Effect of PGE$_2$ on the ACh-induced serosal K$^+$ current.** To study the modulation of ACh-induced increases in serosal K$^+$ current by PGE$_2$, we used 180 $\mu$g/ml nystatin to permeabilize the apical membrane of the SGC monolayers to small monovalent ions,
replaced Cl\(^-\) in the solution with gluconate ion, and established a high apical to serosal K\(^+\) ion concentration gradient as described in the methods. About 10 min after addition of nystatin, basal \(I_{SC}\) increased and then stabilized an indication of successful permeablization of the apical membrane (Fig 6A). ACh addition increased serosal K\(^+\) current with an EC\(_{50}\) of 720 ± 111 nM (Fig 6B, circles, \(n=3\)). Pretreating permeablized SGC monolayers with 10\(^{-7}\) M PGE\(_2\) prior to ACh application increased \(\Delta I_{SC}\) (3.6 ± 1.1 \(\mu\)A/cm\(^2\), \(n = 3\)) and significantly sensitized ACh-induced increases in K\(^+\) current (EC\(_{50}\) = 185 ± 52 nM. Fig 6B, upward triangles, \(p<0.05\), \(n=3\)). No enhancement of ACh-induced maximal K\(^+\) current by PGE\(_2\) pretreatment occurred, ACh-induced maximal K\(^+\) current was 82 ± 5% of Control (\(p > 0.05\), \(n = 3\)).
Discussion

The effects of AM-released substances in stimulating and enhancing increases in $I_{sc}$ in the SGC monolayers were examined. Zymosan activated AM by inducing the expression of COX-2 and the production of PGE$_2$. Supernatant from activated-AM or prostanoids increased $I_{sc}$ in SGC monolayers. Pretreatment of SGC monolayers with supernatant from activated-AM, PGE$_2$ or forskolin enhanced ACh-induced $\Delta I_{sc}$. Our findings suggest that prostanoids produced by AM enhance SGC secretory functions.

Unopsonized zymosan activates naïve macrophages via the mannose receptor and Toll-like receptors to initiate innate immune responses (Takeuchi and Akira, 2001), leading to phagocytosis, arachidonic acid release, and COX-catalyzed prostanoid productions (Girotti, et al., 2004), as well as cytokine production (Lohmann-Matthes, et al., 1994). In this study, it took more than 12 hours for sufficient amount of active substances to increase $\Delta I_{sc}$ to appear in the supernatant, suggesting that protein synthesis was required. COX-2 expression was increased at 6-24 hours after zymosan exposure, an effect blunted by glucocorticoid exposure. The ability of zymosan-activated AM supernatant to increase $I_{sc}$ was blocked by indomethacin and reduced by dexamethasone, suggesting that the active components of supernatant were COX-related prostanoids. PGE$_2$ accumulated in the supernatant to a concentration of ~5×10$^{-7}$ M after 1.6×10$^6$/ ml AM were exposed to 0.1 mg/ml zymosan for 24 hours. At this concentration, PGE$_2$ induces significant $\Delta I_{sc}$ when applied serosally (Fig 1C and 1D). About 2×10$^8$ AM were recovered by a single lavage of the lungs, we estimated that if this number of AM were to be activated in vivo to the same degree as in vitro, 5×10$^{-7}$ M of PGE$_2$ could be reached in >100 ml of airway fluid.

Products released by AM in the lumen are secreted onto the apical surface of the epithelium. In Calu-3 cells, a cell line with both serous and mucus gland cell properties (Shen,
et al., 1994), isoprostane 8-iso-prostaglandin E₂ induced ∆I_{SC} with apical EC_{50} higher than serosal EC_{50} (Cowley, 2003). In this study, PGE₂ applied apically significantly induced ∆I_{SC} at 10^{-7} M, but its EC_{50} was significantly higher than that for serosal application (3.6 µM versus 15.5 nM, Fig 3A and 3B). Supernatant applied apically (10% dilution) did not induce significant ∆I_{SC}, but applying undiluted supernatant would induce significant ∆I_{SC}, comparable to 10^{-7} M PGE₂ (Fig 1D). Although PGE₂ is poorly metabolized in cell culture, it has a half-life of less than 30 seconds in the circulation (Camus and Jeannin, 1984), thus PGE₂ is an autocrine or paracrine hormone acting locally to stimulate airway secretion when released into the surface airway liquid. In vivo, products secreted by activated-AM cross the epithelium to induce both local and systemic actions. Lung instillation of products from co-cultures of epithelial cells and macrophages exposed to PM10 causes bone marrow stimulation in rabbits (Fujii, et al., 2002). Inhaling particulates induces airway hyperreactivity (Walters, et al., 2001) and has systemic inflammatory effects (van Eeden and Hogg, 2002). Kitano et al. (1992) showed that intact airway constricted and secreted mucus after intraluminal application of methacholine, although the EC_{50} was significantly higher than that for serosal application. Relatively lipophilic compounds in supernatant from activated-AM, such as PGE₂, should cross the epithelium to affect underlying tissues by activating serosal receptors, although we cannot rule out specific receptors in the apical cell surface.

PGE₂ binds to four EP receptor types (EP₁,₄). All are present in SGC (Fig 3E). EP₁,₃ receptors are linked to phospholipase C and EP₂,₄ receptors are linked to adenylyl cyclase (Breyer, et al., 2001). In SGC, supernatant, PGE₂, or forskolin induced stable ∆I_{SC} similar to cAMP-elevating agents-induced ∆I_{SC} in Calu-3 cells (Cowley, 2003) by activation of CFTR, since both supernatant and PGE₂-induced ∆I_{SC} were blocked by apically applied DPC or glibenclamide, but less by DIDS. In addition, a cAMP-activated K⁺ channel (KvLQT1) is also
involved since PGE$_2$-induced $\Delta I_{SC}$ were reduced by serosally applied chromanol 293B. The preferential inhibition by the EP$_{1+2}$ receptor antagonist AH6809, but not by the EP$_1$ receptor antagonist SC19220 suggests that the EP$_2$ receptor induces $\Delta I_{SC}$ to PGE$_2$.

Unlike PGE$_2$, ACh induced transient $\Delta I_{SC}$ followed by plateaus. ACh belongs to Ca$^{2+}$-elevating neurotransmitters released by parasympathetic nerves (Coulson and Fryer, 2003). ACh activates M$_3$ receptors to increase $I_{SC}$ (Liu and Farley, 2005) and to initiate fluid secretion via SGC (Yang, et al., 1988; Ishihara, et al., 1992). The increase in $I_{SC}$ is mostly likely brought about through production of inositol 1, 4, 5-trisphosphate and Ca$^{2+}$ release from internal stores. Ca$^{2+}$ not only activates $K_{Ca}$, which induces membrane hyperpolarization and net flux of Cl$^-$/$HCO_3^-$ into the lumen (Ballard and Inglis, 2004), but also stimulates mucus secretion (Ishihara, et al., 1992). ACh-induced $\Delta I_{SC}$ were significantly blunted by ChTX, suggesting the ACh response depended on the $K_{Ca}$. An intermediate conductance $K_{Ca}$ has been found in Calu-3 cells (Cowley and Linsdell, 2002). The cAMP-activated $K_VLQT1$ $K^+$ channels are not likely to be involved in the ACh-induced $\Delta I_{SC}$ since the latter was not affected by chromanol 293B.

In SGC there are two cell types with characteristics consistent with serous and mucus cells. Serous cells and the Calu-3 cells respond to cAMP-elevating agents with increases in both CFTR and $K_VLQT1$ $K^+$ currents, the net effect being $HCO_3^-$/Cl$^-$ exit through CFTR. In Calu-3 cells, muscarinic activation also induces membrane hyperpolarization and net flux of Cl$^-$/$HCO_3^-$ (Ballard and Inglis, 2004). Mucus gland cells have fewer CFTR, and cAMP-elevating agents do not induce significant ionic current (Tamada, et al., 2000). Our previous data showed that SGC isolated using discontinuous Percoll® gradients (Yang, et al., 1991; Chan, et al., 1996) consist of about 70% mucus cells and 30% serous cells using periodic acid-Schiff and alcian blue staining methods. Thus, $\Delta I_{SC}$ in Ussing chamber
measurements are combination of ionic currents mediated by serous and mucus gland cells. Farley, et al. (1991) reported that, the magnitude of ACh-induced $\Delta I_{SC}$ was independent of isoproterenol-induced $\Delta I_{SC}$ in isolated tracheal epithelium preparations at supramaximal drug concentrations ($10^{-5}$ M each). They concluded that isoproterenol and ACh responses occurred in different cell types, presumably serous and mucus cells. In this experiment, supernatant from zymosan-activated AM, PGE$_2$, or forskolin shifted the concentration-response relationships for the ACh-induced $\Delta I_{SC}$ to the left relative to untreated controls resulting in a greater than 3-fold increase in apparent sensitivity to ACh. ACh-induced maximal $\Delta I_{SC}$ were increased by supernatant or PGE$_2$ pretreatment. Forskolin pretreatment did not increase ACh-induced maximal $\Delta I_{SC}$, similar to the effect of isoproterenol on ACh response in isolated tracheal epithelium as reported by Farley et al. (1991). Therefore, PGE$_2$ or supernatant from zymosan-activated AM increases cytosolic cAMP concentration to enhance the apparent sensitivity to ACh, but may activate another pathway resulting in an increased maximal response to ACh. The increase in maximal response to ACh may not involve increased maximal activation of serosal K$^+$ channels since the amplitude of ACh-induced serosal K$^+$ current in nystatin permeabilized SGC was not influenced by PGE$_2$ (Fig 6). PGE$_2$ is known to activate tyrosine kinase / PI$_3$Kinase via the EP$_4$ receptor, in addition to activation of PKA (Regan, 2003), but whether this pathway is activated in SGC is not known. EP$_4$ receptors are present in isolated SGC (Fig 3E).

The apparent increase in sensitivity to ACh occurred rapidly after exposure to PGE$_2$ and is therefore probably not due to an increased expression of receptors. Also, PGE$_2$ sensitized SGC to histamine-induced $\Delta I_{SC}$ (unpublished observation) that are mediated via H$_1$ receptors (Liu and Farley, 2005). It seems likely that steps common to both ACh and histamine transduction pathways are enhanced by PGE$_2$. One common event may be the sensitization of ion channels by PKA-mediated phosphorylation. PKA reportedly sensitizes large
conductance Ca\(^{2+}\)-activated K\(^{+}\) channels to Ca\(^{2+}\) (Tian, et al., 2001). PGE\(_{2}\) pretreatment apparently sensitized ACh-induced serosal K\(^{+}\) current without enhancing its maximal response (Fig 6A, 6B). ChTX reduced the ACh-induced maximal \(\Delta I_{SC}\) without changing the apparent sensitization of ACh response by PGE\(_{2}\) treatment (Fig 5B). Thus PGE\(_{2}\)-induced direct sensitization of ChTX-sensitive \(K_{Ca}\) is not solely responsibly for the apparent sensitization of SGC to ACh. Chromanol 293B-sensitive cAMP-activated K\(^{+}\) channels are not involved in such sensitization either, because 293B had no effect on the ACh-induced \(\Delta I_{SC}\) in both sensitivity and magnitude, even though it inhibited the PGE\(_{2}\)-induced \(\Delta I_{SC}\) (Fig 5B).

CFTR channels are reported to be the exclusive Cl\(^{-}\) conductance in Calu-3 cells for cholinergically mediated gland secretions (Moon, et al., 1997). Our data show that glibenclamide applied apically reduced both PGE\(_{2}\) and ACh-induced \(\Delta I_{SC}\). However Joo et al. (2002) demonstrated that cholinergic-stimulated fluid secretion occurred in tracheal/bronchial epithelium from cystic fibrosis patients although cAMP-induced secretion did not. It is possible that CFTR loss in serous cells is compensated for partially by Ca\(^{2+}\)-activated Cl\(^{-}\) channels in mucus cells (Ballard and Inglis, 2004). Glibenclamide may also have nonspecific effects, blocking important ion transporters essential for ACh-induced \(\Delta I_{SC}\) (Ballard and Inglis, 2004). Although glibenclamide almost completely blocked PGE\(_{2}\) and ACh-induced \(\Delta I_{SC}\), the sensitization of the EC\(_{50}\) for ACh-induced \(\Delta I_{SC}\) was not affected by glibenclamide (Fig 5D). These data suggest that the apparent sensitization of SGC to ACh by PGE\(_{2}\) is not due to the direct sensitization of ion channels such as CFTR and \(K_{Ca}\), but rather through effects on the signal transduction pathway activating ion channels, presumably by enhancing the elevation of intracellular Ca\(^{2+}\).

Overall implications of this study are that exposure to particulates induce the release of PGE\(_{2}\) from AM, which have significant effects on the mucosa of the airway. The products
released increase ion flux (and therefore secretion of fluid) into the airway, and sensitize the SGC to respond to secretagogues. Inhibition of CFTR and $K_{Ca}$ changed the magnitude of ACh-induced response without affecting PGE$_2$-induced sensitization to ACh. If PGE$_2$-induced sensitization of SGC to ACh was due to enhanced Ca$^{2+}$ mobilization, we suggest that events activated by Ca$^{2+}$, such as ACh-induced mucus release, would also be enhanced by PGE$_2$ even if CFTR function was lost. PGE$_2$ generally has been consider anti-inflammatory in the lung (Vancheri, et al., 2004). Our study suggests that PGE$_2$-induced enhancement of SGC secretory response to ACh may constitute a protective mechanism during acute exposure of airway to particulates by aiding in particulate clearance; however, it also may lead to excessive fluid/mucus secretion and therefore exacerbate pathological conditions found in asthma, cystic fibrosis or chronic obstructive pulmonary disease.
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Fig. 1. Supernatant from zymosan-activated AM increased I_{SC} across the confluent SGC monolayers in the Ussing chamber. A: I_{SC} was recorded under voltage-clamp at 0 mV potential difference across SGC monolayers. 2 mV pulses (every 30 seconds) were used to monitor conductance changes. Supernatant from zymosan-activated macrophage or control macrophage (shown in Fig 4A trace “M_Ф-only”) was added cumulatively to the serosal side of monolayers to give final dilutions marked by arrows (from 0.1% to 10%). The difference of I_{SC} from plateau to baseline at each dilution was measured as the response to supernatant normalized to the area of the SGC monolayers (0.6 cm^2) as ΔI_{SC} (µA/cm^2). DIDS or DPC was added to the apical side to inhibit Cl^- channels marked at arrows. B: Indomethacin (5 µM) was added to macrophage cultures during 24-hour zymosan exposure. Indomethacin treatment abolished the increase in I_{SC} induced by supernatant (trace 3: M_Ф + Indo + Zymosan Supernatant, n=5). Residual indomethacin (~50 nM) in the Ussing chamber originating from supernatant did not affecting I_{SC} (trace 2: M_Ф + Indo. Supernatant, n=5). C: PGE_2 or PGF_2α was applied serosally to the SGC monolayers from 3 x 10^{-11} M to 10^{-5} M concentrations (cumulative), which induced persistent increases in I_{SC}. D: Summary of concentration response data for M_Ф + Zymosan Supernatant (filled squares, n=5), PGE_2 (open squares, n=4), and PGF_2α (open circles, n=3) in inducing ΔI_{SC} from A and C. Data shown as Mean ± SEM, n is the number of animals used. The dotted line shows that 100% M_Ф + Zymosan supernatant has an estimated equivalent potency comparable to 10^{-7} M PGE_2.

Fig. 2. Western blot analysis of COX-1 and COX-2 expression in AM exposed to zymosan, dexamethasone, and indomethacin. A: The effect of Dex. (dexamethasone 1 µM) or Indo. (indomethacin 5 µM) applied during exposure of AM to zymosan treatment on COX-1
expression levels. The expression levels were normalized to the basal COX-1 level in AM not treated with zymosan or drugs as shown in lane 1 (n=3 for each treatment). B: The effect of Dex. (1 µM) or Indo. (5 µM) applied with or without zymosan-treatment on COX-2 expression levels in AM. The COX-2 expression levels were normalized to the basal COX-2 level in AM not treated with zymosan or drugs as shown in lane 1 (n=3 for each treatment. *significantly different from paired-macrophage groups not treated with zymosan (empty bars, p<0.05). †significantly different from zymosan-treated AM (first filled bar, p<0.05). Data shown as Mean ± SEM, and n is the number of animals used.

Fig. 3. The difference in the serosal and apical PGE2 sensitivities and the role of EP1 and EP2 receptors in PGE2-induced ∆I_{sc} in SGC monolayers. A: PGE2 was applied cumulatively from 3 x 10^{-11} M to 10^{-5} M to the serosal side (Trace 1) or apical side (Trace 2) of SGC monolayers in the Ussing chamber. B: Concentration-response relationships (normalized to maximal serosal response) for PGE2-induced ∆I_{sc} are shown. The estimated EC50 for serosal PGE2 was 15.5 ± 1.3 nM (squares, n=4) and 3.6 ± 1.8 µM (circles, n=3) for apical PGE2. C: The inhibition of PGE2-induced ∆I_{sc} by EP receptor antagonists. PGE2 applied cumulatively from 3 x 10^{-9} M to 10^{-5} M concentrations to the serosal side of SGC monolayers induced similar response (control trace) to those shown in Fig. 1C and Fig. 3A. In parallel inserts, SC19220 (10 µM) and AH6809 (30 µM) were applied serosally prior to PGE2 application. D. PGE2-induced ∆I_{sc} and its inhibition by SC19220 and AH6809. Control SGC has an EC50 of 167 ± 34nM (circles, n=4) for PGE2-induced ∆I_{sc}, SC19220-treated SGC had an EC50 of 103 ± 33nM (squares, n=3), and AH6809-treated SGC had an EC50 of 666 ± 162 nM (upward-triangles, n=3). E. Immunoprecipitation and Western blot indicated the presence of all four subtypes of endoprosthanoid receptors in SGC dissociated with either protease or collagenase
(EP1-4, 3~5 animals were used in each group), with estimated molecular weights being 42, 52, 53, and 65 Kd for EP1, EP2, EP3, and EP4 subtypes, respectively. Data points are shown as Mean ± SEM, and n is the number of animals used in the experiment.

**Fig. 4.** Pretreatment of SGC monolayers with supernatant from zymosan-activated AM, PGE2, and forskolin sensitized ACh-induced ΔIsc. A: 0.1% to 10% dilutions of supernatant from AM without zymosan exposure (trace: Mϕ−only) or from AM with zymosan exposure (trace: Mϕ + Zymosan) were applied cumulatively to the serosal side of SGC followed by cumulative application of ACh (marked at arrows). ACh-induced increases in ISc in SGC not treated with supernatants (trace: Control) were used as control. B: Concentration-response relationships for ACh-induced ΔIsc in SGC pretreated with Mϕ + Zymosan Supernatant (upward triangles, n=3) and Mϕ−only Supernatant (squares, n=3), and in Control SGC (circles, n=6). C: In the bottom trace (Control), DMSO vehicle applied before ACh treatment had no effect on the responsiveness to ACh. The top two current recordings show that, five minutes prior to ACh addition, 10−7 M PGE2 or 5 × 10−6 M forskolin caused stable increases in ISc. Notice that PGE2 or forskolin pretreatment sensitized SGC monolayers to low ACh concentrations (10−8 M ~ 3 × 10−7 M) to increase ISc. D: The effect of forskolin (5 × 10−5 M, squares, n=4) or PGE2 (10−7 M, upward triangles, n=10) pretreatment on the concentration responses for ACh-induced ΔIsc (normalized to the maximal ACh-induced ΔIsc response in Control SGC monolayers, circles, n=6). Data are expressed as Mean ± SEM and the number of animals used in each group is indicated in the figure.

**Fig. 5.** PGE2 and ACh-induced ΔIsc were blocked by K+ and Cl− channel blockers. A, Trace 1: control is a similar current recording to Fig 4C, PGE2 (10−7 M) was applied before ACh
treatment. Also, in parallel inserts, ChTX (100 nM, trace 2, n=3) or chromanol 293B (200 nM, trace 3, n=4) was applied serosally after PGE₂ treatment, but before ACh treatment. B. Concentration-response relationships for ACh-induced ΔI_{sc} in SGC pretreated with PGE₂ alone (circles, n=6), PGE₂ + ChTX (squares, n=4), or PGE₂ + 293B (upward triangles, n=4). C: similarly, 2 mM glibenclamide was applied apically after PGE₂-treatment, but before ACh treatment (n=3). D is an enlarged-view (greater than 5 ×) of partial current recording in C, which shows ACh-induced increase in I_{sc} after PGE₂ and glibenclamide treatment. The straight line represents the estimated baseline. n represents the numbers of animals used in each group.

Fig. 6. PGE₂ sensitized ACh-induced serosal K⁺ current in nystatin-permeablized SGC monolayers. A. Recording of K⁺ current in response to PGE₂ and ACh. The apical membrane of SGC monolayers was permeabized with 180 µg/ml nystatin (at first arrow), causing a stable increase in current. NaCl was replaced with potassium gluconate and sodium gluconate in the apical and serosal solutions, respectively. 10⁻⁷ M PGE₂ was added serosally 5 min before ACh treatment (gray trace). The black trace is a control recording without PGE₂ pretreatment. Cumulative concentration response relationships for ACh-induced K⁺ current were then generated. B. The average concentration-response relationships for ACh-induced K⁺ current (calculated as % of ACh-induced maximal response in control). Data are expressed as mean ± SEM; and the number of animals used in each group is indicated in the figure.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6