JPET Fast Forward. Published on July 29, 2005 as DOI: 10.1124/jpet.105.088542
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Prostanoids secreted by alveolar macrophages enhance ionic currents in swine tracheal submucosal gland cells

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Running title: Macrophage-secreted PGE_2 enhances airway epithelial currents

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Number of text pages: 25

Number of tables: 0

Number of figures: 6

Number of references: 40

Number of word in abstract: 249

Introduction: 366

Discussion: 1695

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; ΔI_{SC}: changes in short circuit current; I_{SC}: short circuit current; K_{Ca}: Ca^{2+}-activated potassium channels; Mϕ: macrophages; PGE_2: prostaglandin E_2; $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$; PKA: cAMP dependent protein kinase A; SC19220: 8-chloro-dibenzo[b,f][1,4]oxazepine-10(11*H*)-carboxylic acid, 2-acetylhydrazide; SGC: submucosal gland cells.

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

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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 (ΔI_{SC}) in SGC monolayers in Ussing chambers. Dexamethasone (1 μM) or indomethacin (5 μM) during zymosan exposure of AM reduced or abolished the supernatant-induced ΔI_{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 ΔI_{SC} with an EC_{50} of 15.5 ± 1.3 nM (n=4), and 3.6 ± 1.8 μM (n=3) when applied apically. Four types of endoprostanoid receptors ($EP_{1\sim4}$) were detected in SGC using Western blot. PGE_2-induced ΔI_{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 ΔI_{SC}. PGE_2-induced ΔI_{SC} were blocked by charybdotoxin (ChTX), chromanol 293B or glibenclamide, ACh-induced ΔI_{SC} were only blocked by ChTX or glibenclamide. None of these blockers altered PGE_2 pretreatment-induced sensitization of ACh-induced ΔI_{SC}. These results demonstrate that prostanoids released from activated-AM directly increase CFTR and K^+ channel activity. ACh-induced ΔI_{SC} are also enhanced due to enhanced activation of Ca^{2+}-activated K^+ channels (K_{Ca}).

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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 ΔI_{SC} across SGC monolayers; 2) the substance(s) secreted modulate ionic current changes induced by other secretagogues and neurotransmitters such as ACh. The latter

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effect may contribute to hypersecretory changes during airway inflammation. We examined the effect of substances released by zymosan-activated AM and PGE_2 on SGC, measured as ΔI_{SC} across confluent SGC monolayers in Ussing chambers. Our results demonstrate that zymosan induces COX-2 expression and PGE_2 release by AM. Supernatant from zymosan activated-AM or PGE_2 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.

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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_2HPO_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 ^oC). The cells were recovered from the lavage solution by centrifugation, washed in the same lavage solution (100 g $\times$ 10 min at 4 ^oC) 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 x 10^5 cells / cm^2 ml, or 1.6 x 10^6 cells / ml media. Usually at least 1~2 x 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

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was quickly removed and transported to the lab in physiological saline solution containing (mM): NaCl, 140; KCl, 5.5; $CaCl_2$, 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, 10^6 SGC were plated onto each Millicell®–HA insert (12 mm diameter; Millipore, Billerica, MA) pre-coated with human placental collagen type IV (20 μg/cm^2). Inserts were maintained in PC-1 medium in the cell incubator (37 °C, 5% CO_2) 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 ^oC, 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.

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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_2PO_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 ^oC. 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). ΔI_{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, ΔI_{SC} reflected primarily K^+ channel activity in the serosal membrane. Using chamber solution

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components for the apical side were (mM): potassium gluconate ($C_6H_{11}O_7K$) 120, KH_2PO_4 25, K_2HPO_4 0.8, $MgSO_4$ 1.2, $CaCl_2$ 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 ($C_6H_{11}NaO_7$). Solutions were bicarbonate-free and bubbled continuously with 100% 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_3VO_4) at 0 ^oC. 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

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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 endoprostanoid receptor proteins, polyclonal antibodies against these receptors (EP_1, EP_2, EP_3 and EP_4) were purchased from Cayman Chemical (Ann Arbor, MI). SGC were collected using discontinuous Percoll gradient ($1.5 \times 10^6 \sim 2.5 \times 10^6$ 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 EP_1, EP_2, EP_3 or EP_4 (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

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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-chloro-dibenzo[b,f][1,4]oxazepine-10(11*H*)-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, PGD_2, PGE_2, $PGF_{2\alpha}$, 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 ^oC. These stocks were further diluted in Krebs-Ringers buffer solution immediately before us. 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, EC_{50} 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

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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.

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Results

The resting transepithelial potentials, currents, and resistances for confluent SGC monolayers were 5.1 ± 0.5 mV, 5.0 ± 0.7 μA/cm^2, and 1.8 ± 0.2 KΩ/cm^2 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/cm^2 decrease in ΔI_{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 ΔI_{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 ΔI_{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/cm^2). I_{SC} increased with increasing volume added. The ΔI_{SC} induced by 1% and 10% dilution were 3.1 ± 0.7 μA/cm^2 and 9.0 ± 0.8 μA/cm^2, 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

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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 (*MΦ-only Supernatant*) applied to the serosal side of the SGC epithelium induced ΔI_{SC} of 1.4 ± 0.8 μA/cm^2 at 10% dilution (Fig 4A, middle trace marked with *MΦ-Only*, n=3), similar in magnitude to that caused by 0.3% *MΦ + Zymosan Supernatant* (ΔI_{SC} = 1.1 ± 0.3 μA/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 ΔI_{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 ^{o}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: *Mϕ + Indo + Zymosan Supernatant*), ΔI_{SC} = 0 ± 0 μA/cm^2, while 1% *MΦ + Zymosan Supernatant* induced an increase in I_{SC} (trace 1: *Mϕ + Zymosan Supernatant*), ΔI_{SC} = 2.7 ± 0.3 μA/cm^2 (n = 5 each, p<0.05). Supernatant from AM exposed to indomethacin alone had no effect on ΔI_{SC} (trace 2: *Mϕ +*

Indo Supernatant: 0 ± 0 $\mu A/cm^2$, 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 ΔI_{SC} compared with ΔI_{SC} induced by 1% *Mϕ + Zymosan Supernatant*. Dexamethasone treatment caused a 70.5 ± 2.4% inhibition of ΔI_{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 ΔI_{SC}.

The comparison of prostaglandins and supernatant in inducing-ΔI_{SC}. The ability of prostaglandins to increase I_{SC} of SGC was examined In Fig 1C. PGE_2 or $PGF_{2\alpha}$ 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 ΔI_{SC} reach a maximum at approximately 10^{-6} M in the case of PGE_2 (Fig 1D, open squares, maximal ΔI_{SC} = 15.7 ± 1.4 $\mu A/cm^2$, n = 4). The EC_{50} for serosal PGE_2-induced ΔI_{SC} is 10.0 ± 1.8 nM (n = 4). Serosal $PGF_{2\alpha}$ was less potent than PGE_2 in inducing ΔI_{SC}, with an estimated EC_{50} of 1.1 ± 0.1 μM (Fig 1D, ΔI_{SC} = 6.4 ± 0.7 $\mu A/cm^2$ at 10^{-5} M, open circles, n = 3). We also applied another prostaglandin, PGD_2, to the serosal side of SGC monolayer, which induced ΔI_{SC} with an EC_{50} of ~19 nM (one experiment, data not

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shown). As also shown in the Fig 1D, 100% *Mϕ + Zymosan Supernatant* is estimated to induce ΔI_{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 ± 1 ng/ml (~28 ± 3 nM, n = 3). The PGE_2 concentration reached 195 ± 28 ng/ml (~550 ± 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 ΔI_{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 μM) or dexamethasone (1 μM) present during zymosan exposure did not altered 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 μM indomethacin-, and 1 μM 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 ±100% of basal level in non-

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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 ΔI_{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 ΔI_{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 ΔI_{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 ΔI_{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 ($EP_{1\sim4}$) 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 ΔI_{SC}. We tested the ability of supernatant to modulate ACh-induced ΔI_{SC}. To estimate the concentration-response relationship for ACh-induced ΔI_{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 ΔI_{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 *Mϕ-only Supernatant* or *Mϕ + Zymosan Supernatant* as described previously (traces marked by "Mϕ–only" and "Mϕ + Zymosan" respectively). As mentioned before, *Mϕ–only Supernatant at* up to 10% (v/v) only induced a small increase in ΔI_{SC}, while *Mϕ + 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 ΔI_{SC} had an EC_{50} of 335 ± 67 nM for SGC pretreated with *Mϕ–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 ΔI_{SC} in SGC treated with *Mϕ + 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 *Mϕ–only Supernatant*-treated SGC).

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In addition, the pretreatments with *Mϕ + Zymosan Supernatant* and *Mϕ–only Supernatant* significantly increased the maximal increase in ACh-induced ΔI_{SC} in SGC, with average values being 140 ± 6% and 122 ± 4% of the ACh-induced maximal ΔI_{SC} in "Control" SGC run in parallel ($p<0.05$).

We further examined the effect of PGE_2 or forskolin pretreatment on ACh-induced ΔI_{SC}. SGC monolayers were pretreated with PGE_2 or forskolin (5 x 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_{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 x 10^{-6} M forskolin sensitized SGC to ACh, ACh-induced increases in I_{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 x 10^{-6} M forskolin pretreatment shifted the EC_{50} for ACh response to 121 ± 20 nM (n = 10) and 84 ± 19 nM (n=4) (Fig. 4D, upper triangles and squares, respectively), while the EC_{50} for ACh-induced ΔI_{SC} in controls was 418 ± 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 ΔI_{SC} to 292 ± 37 nM (n = 3), 188 ± 35 nM (n = 3), and 103 ± 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 x 10^{-6} M forskolin had similar effects in sensitizing ACh-induced ΔI_{SC}, and the EC_{50} values for these three groups were not significantly different from each other ($p > 0.05$).

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$PGF_{2\alpha}$ (10^{-5} M) was also applied serosally before ACh application. The EC_{50} was shifted for the ACh-induced ΔI_{SC} to 87 ± 8 nM (n = 3), significantly different from that in Controls (418 ± 28 nM, n=12, p<0.05).

The ACh-induced maximal ΔI_{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 ± 6%, 135 ± 5%, and 131 ± 7% of ACh-induced maximal ΔI_{SC} in control (p<0.05), respectively. ACh-induced maximal ΔI_{SC} in SGC treated with 10^{-9} M PGE_2 or 5 x 10^{-6} M forskolin were not significantly different from those of control, the average values being 104 ± 9% and 107 ± 8% of control, respectively (p > 0.05).

K^+ and Cl^- channels involved in PGE_2- or ACh-induced ΔI_{SC}. ChTX, a K_{Ca} blocker, and chromanol 293B, a blocker of K_VLQT1(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_VLQT1 (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 ± 3% decrease in PGE_2-induced ΔI_{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 ΔI_{SC} in PGE_2 + 293B treated group was 108 ± 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 ΔI_{SC} induce (Fig 5B circles, 123 ± 36 nM for PGE_2-treatment group versus 191 ± 82 nM for PGE_2 + 293B treatment group, upper triangles, n=4 each, p > 0.05).

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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 ΔI_{SC}. ChTX also blocked 66 ± 1% of ACh-induced maximal ΔI_{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 ΔI_{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 ΔI_{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 ΔI_{SC} was ~ 1 μA/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 μM 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 μg/ml nystatin to permeablize the apical membrane of the SGC monolayers to small monovalent ions,

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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 ΔI_{SC} (3.6 ± 1.1 μA/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).

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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 ΔI_{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 ΔI_{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 ΔI_{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,

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et al., 1994), isoprostane 8-iso-prostaglandin E_2 induced ΔI_{SC} with apical EC_{50} higher than serosal EC_{50} (Cowley, 2003). In this study, PGE_2 applied apically significantly induced ΔI_{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 ΔI_{SC}, but applying undiluted supernatant would induce significant ΔI_{SC}, comparable to 10^{-7} M PGE_2 (Fig 1D). Although PGE_2 is poorly metabolized in cell culture, it has a half-life of less than 30 seconds in the circulation (Camus and Jeannin, 1984), thus PGE_2 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 PGE_2, should cross the epithelium to affect underlying tissues by activating serosal receptors, although we cannot rule out specific receptors in the apical cell surface.

PGE_2 binds to four EP receptor types (EP_{1-4}). All are present in SGC (Fig 3E). $EP_{1,3}$ receptors are linked to phospholipase C and $EP_{2,4}$ receptors are linked to adenylyl cyclase (Breyer, et al., 2001). In SGC, supernatant, PGE_2, or forskolin induced stable ΔI_{SC} similar to cAMP-elevating agents-induced ΔI_{SC} in Calu-3 cells (Cowley, 2003) by activation of CFTR, since both supernatant and PGE_2-induced ΔI_{SC} were blocked by apically applied DPC or glibenclamide, but less by DIDS. In addition, a cAMP-activated K^+ channel (KvLQT1) is also

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involved since PGE_2-induced ΔI_{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 ΔI_{SC} to PGE_2.

Unlike PGE_2, ACh induced transient ΔI_{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 ΔI_{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 ΔI_{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, ΔI_{SC} in Ussing chamber

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measurements are combination of ionic currents mediated by serous and mucus gland cells. Farley, et al. (1991) reported that, the magnitude of ACh-induced ΔI_{SC} was independent of isoproterenol-induced ΔI_{SC} in isolated tracheal epithelium preparations at supramaximal drug concentrations (10^{-5} M each). They concluded that isoproteronol 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 ΔI_{SC} to the left relative to untreated controls resulting in a greater than 3-fold increase in apparent sensitivity to ACh. ACh-induced maximal ΔI_{SC} were increased by supernatant or PGE_2 pretreatment. Forskolin pretreatment did not increase ACh-induced maximal ΔI_{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 permeablized SGC was not influenced by PGE_2 (Fig 6). PGE_2 is known to activate tyrosine kinase / PI_3Kinase 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 ΔI_{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

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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 ΔI_{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 ΔI_{SC} in both sensitivity and magnitude, even though it inhibited the PGE_2-induced ΔI_{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 ΔI_{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 ΔI_{SC} (Ballard and Inglis, 2004). Although glibenclamide almost completely blocked PGE_2 and ACh-induced ΔI_{SC}, the sensitization of the EC_{50} for ACh-induced ΔI_{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

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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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Legends for Figures

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_{Φ}-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} ($\mu A/cm^2$). 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_{Φ} + Indo + Zymosan Supernatant, n=5). Residual indomethacin (~50 nM) in the Ussing chamber originating from supernatant did not affecting I_{SC} (trace 2: M_{Φ} + Indo. Supernatant, n=5). C: PGE_2 or $PGF_{2\alpha}$ 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_{Φ} + Zymosan Supernatant (filled squares, n=5), PGE_2 (open squares, n=4), and $PGF_{2\alpha}$ (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_{Φ} + 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

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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 PGE_2 sensitivities and the role of EP_1 and EP_2 receptors in PGE_2-induced ΔI_{sc} in SGC monolayers. A: PGE_2 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 PGE_2-induced ΔI_{sc} are shown. The estimated EC_{50} for serosal PGE_2 was 15.5 ± 1.3 nM (squares, n=4) and 3.6 ± 1.8 µM (circles, n=3) for apical PGE_2. C: The inhibition of PGE_2-induced ΔI_{sc} by EP receptor antagonists. PGE_2 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 PGE_2 application. D. PGE_2-induced ΔI_{sc} and its inhibition by SC19220 and AH6809. Control SGC has an EC_{50} of 167 ± 34nM (circles, n=4) for PGE2-induced ΔI_{sc}, SC19220-treated SGC had an EC_{50} 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 endoprostanoid receptors in SGC dissociated with either protease or collagenase

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($EP_{1\sim4,}$ 3~5 animals were used in each group), with estimated molecular weights being 42, 52, 53, and 65 Kd for EP_1, EP_2, EP_3, and EP_4 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, PGE_2, and forskolin sensitized ACh-induced $\Delta I_{sc.}$ 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 I_{SC} in SGC not treated with supernatants (trace: Control) were used as control. B: Concentration-response relationships for ACh-induced ΔI_{sc} 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 PGE_2 or 5×10^{-6} M forskolin caused stable increases in I_{sc}. Notice that PGE_2 or forskolin pretreatment sensitized SGC monolayers to low ACh concentrations (10^{-8} M ~ 3 x 10^{-7} M) to increase I_{SC}. D: The effect of forskolin (5 x 10^{-5} M, squares, n=4) or PGE_2 (10^{-7} M, upward triangles, n=10) pretreatment on the concentration responses for ACh-induced ΔI_{SC} (normalized to the maximal ACh-induced ΔI_{SC} 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. PGE_2 and ACh-induced ΔI_{SC} were blocked by K^+ and Cl^- channel blockers. A, Trace 1: control is a similar current recording to Fig 4C, PGE_2 (10^{-7} M) was applied before ACh

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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 PGE_2 treatment, but before ACh treatment. B. Concentration-response relationships for ACh-induced ΔI_{sc} in SGC pretreated with PGE_2 alone (circles, n=6), PGE_2 + ChTX (squares, n=4), or PGE_2 + 293B (upward triangles, n=4). C: similarly, 2 mM glibenclamide was applied apically after PGE_2-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 PGE_2 and glibenclamide treatment. The straight line represents the estimated baseline. n represents the numbers of animals used in each group.

Fig. 6. PGE_2 sensitized ACh-induced serosal K^+ current in nystatin-permeablized SGC monolayers. A. Recording of K^+ current in response to PGE_2 and ACh. The apical membrane of SGC monolayers was permeablized 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 PGE_2 was added serosally 5 min before ACh treatment (gray trace). The black trace is a control recording without PGE_2 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.

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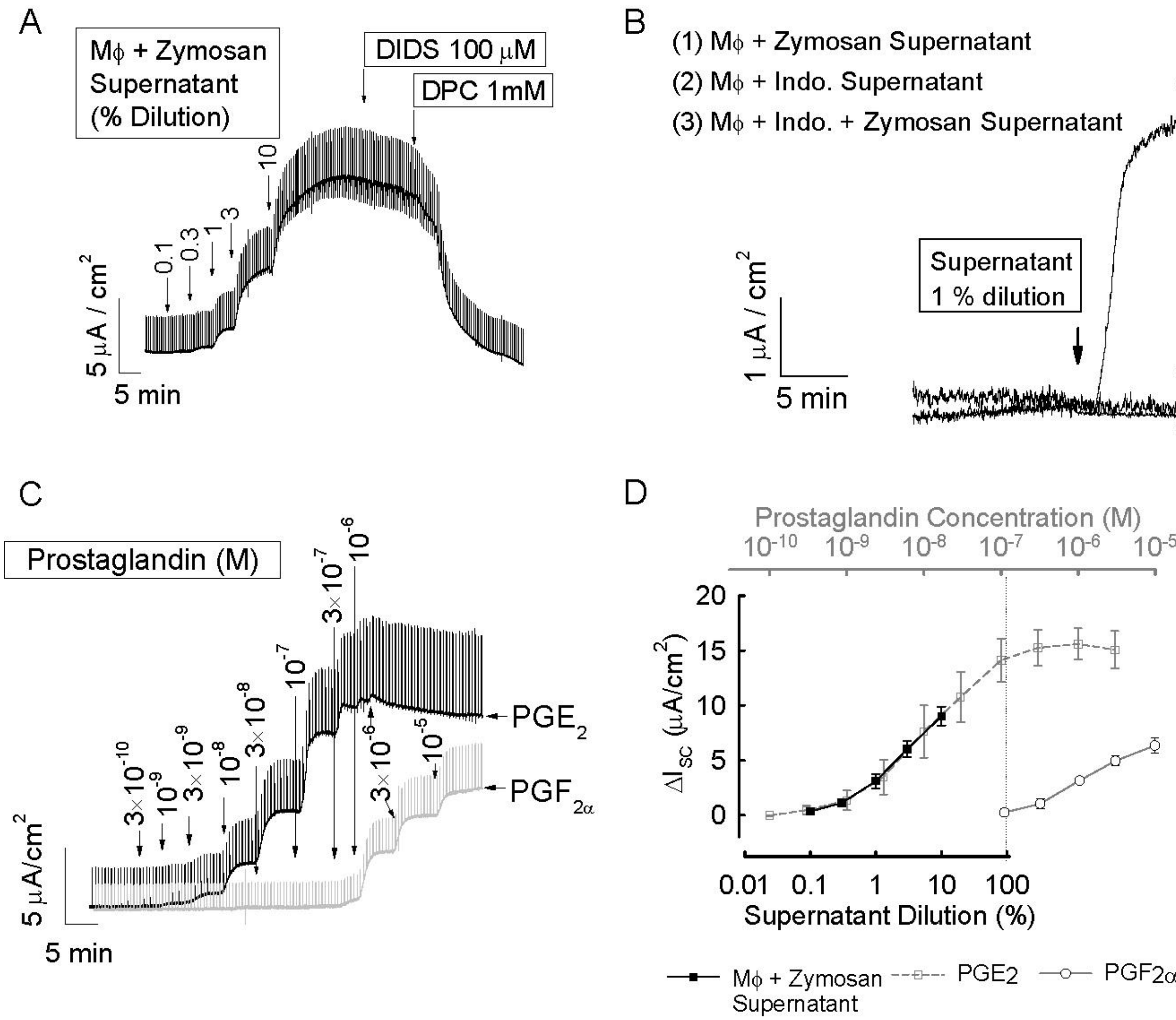

Figure 1

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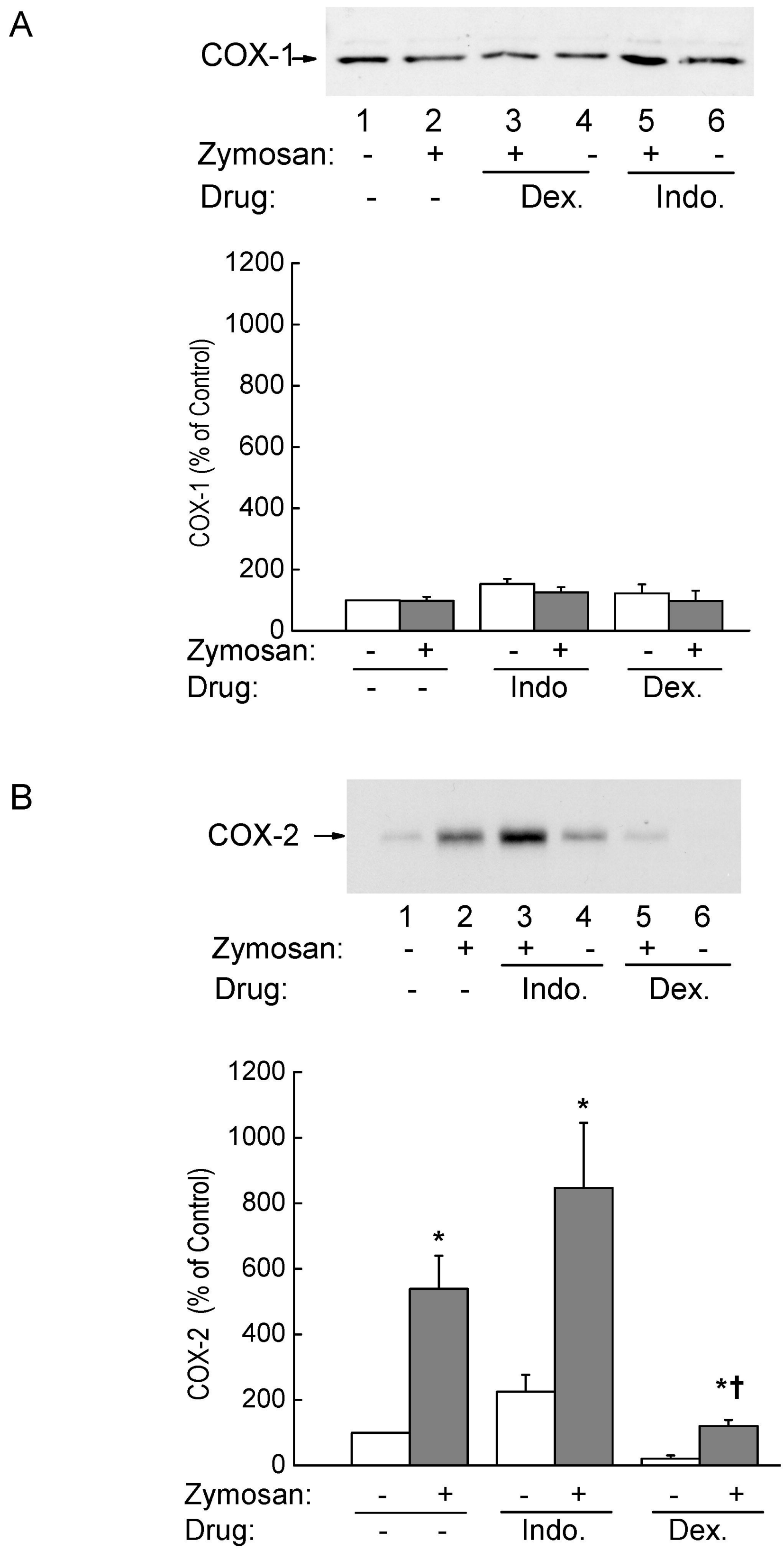

Figure 2

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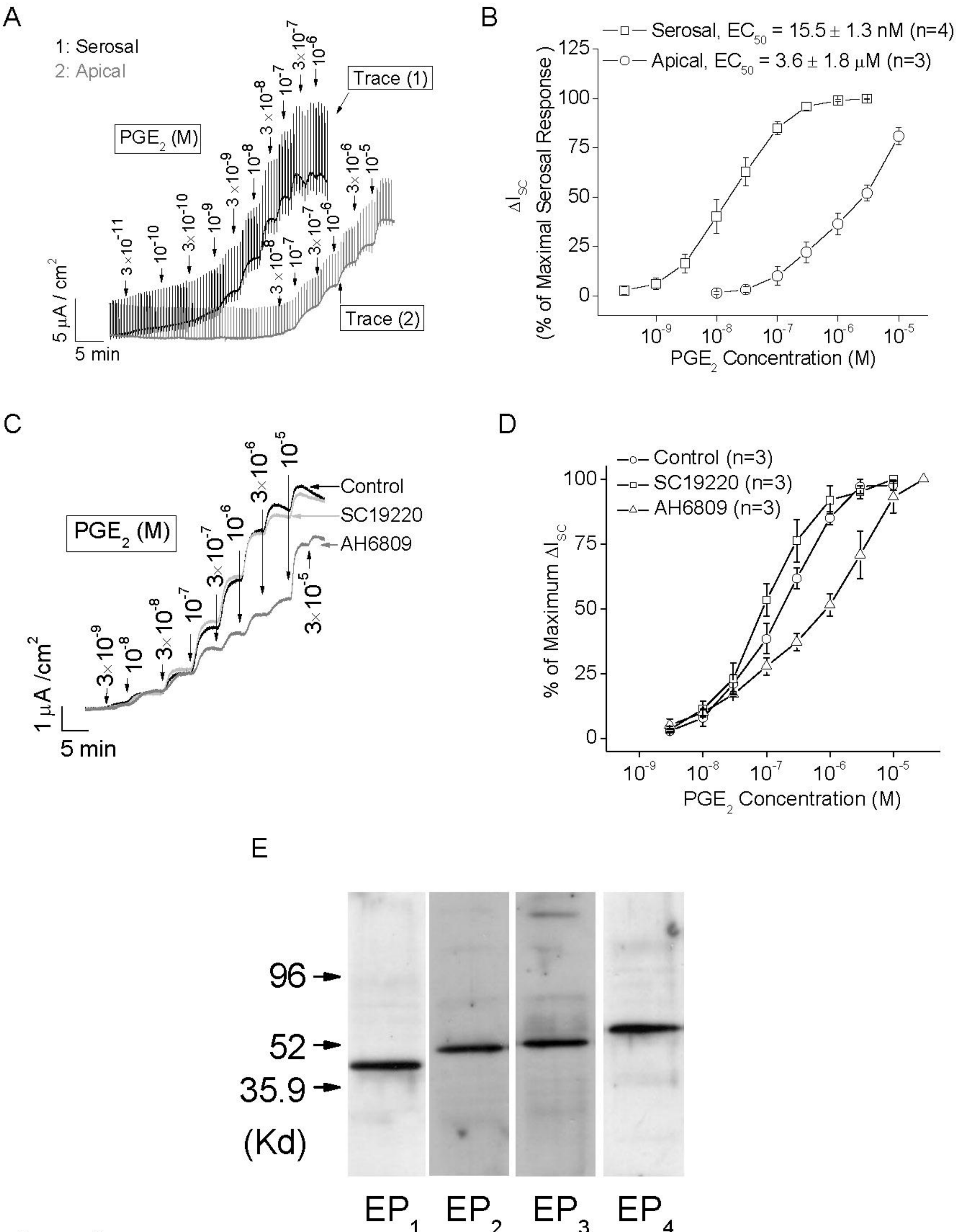

Figure 3

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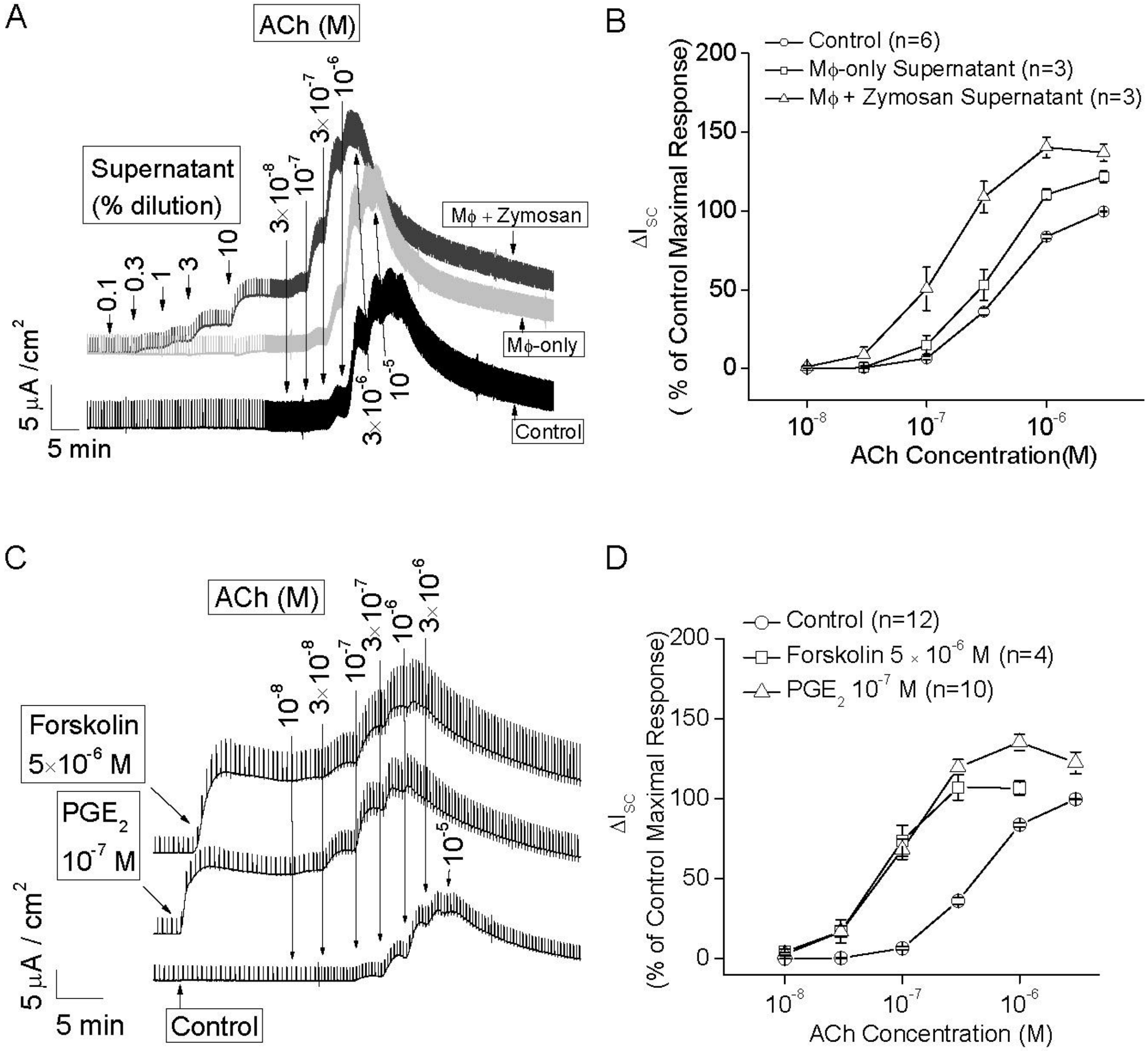

Figure 4

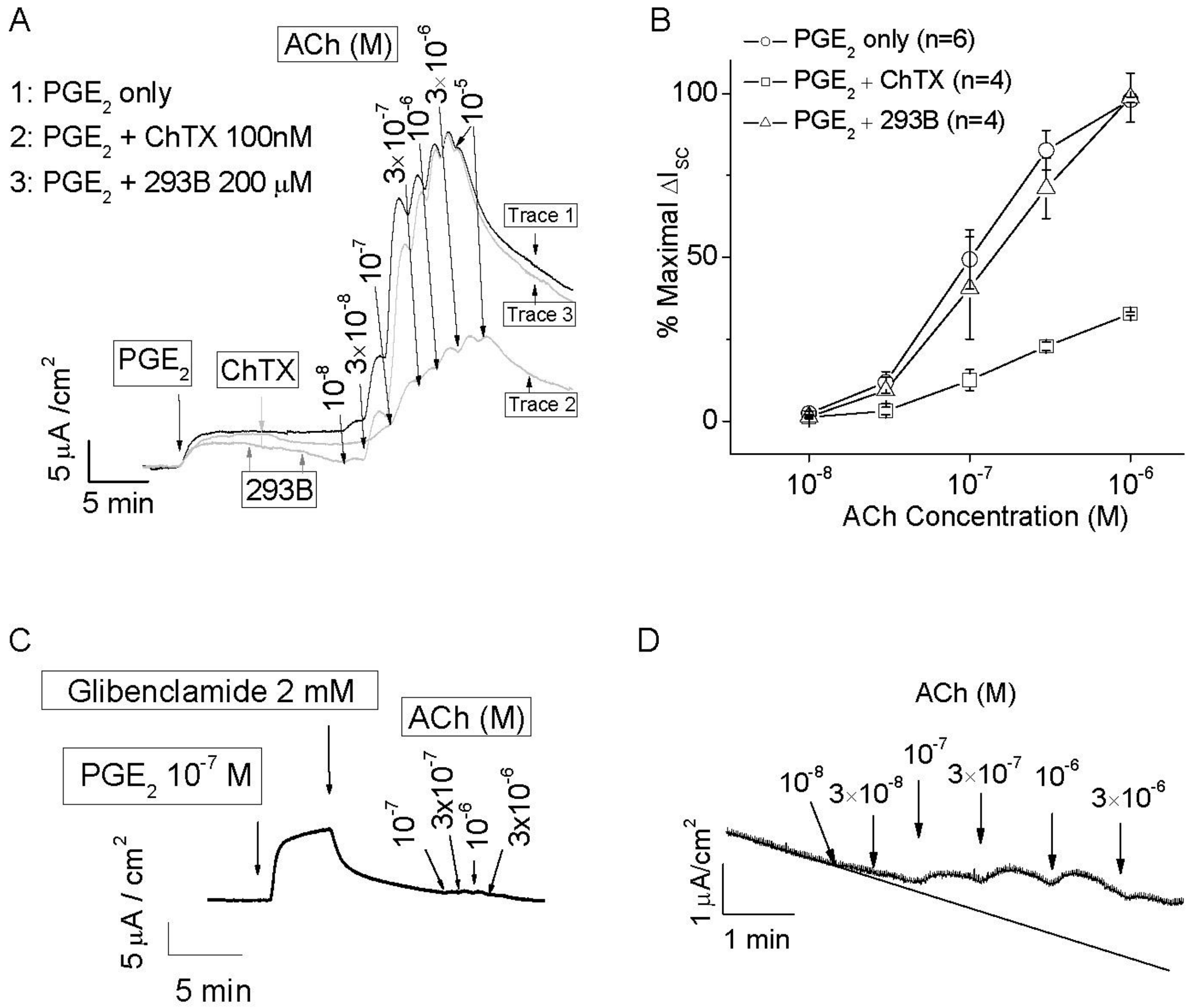
A
ACh (M)
1: PGE2 only
2: PGE2 + ChTX 100nM
3: PGE2 + 293B 200 μM
Trace 1
Trace 3
Trace 2
PGE2
ChTX
293B
5 μA /cm2
5 min
B
PGE2 only (n=6)
PGE2 + ChTX (n=4)
PGE2 + 293B (n=4)
% Maximal ΔIsc
ACh Concentration (M)
C
Glibenclamide 2 mM
ACh (M)
PGE2 10-7 M
5 μA / cm2
5 min
D
ACh (M)
1 μA/cm2
1 min

Figure 5

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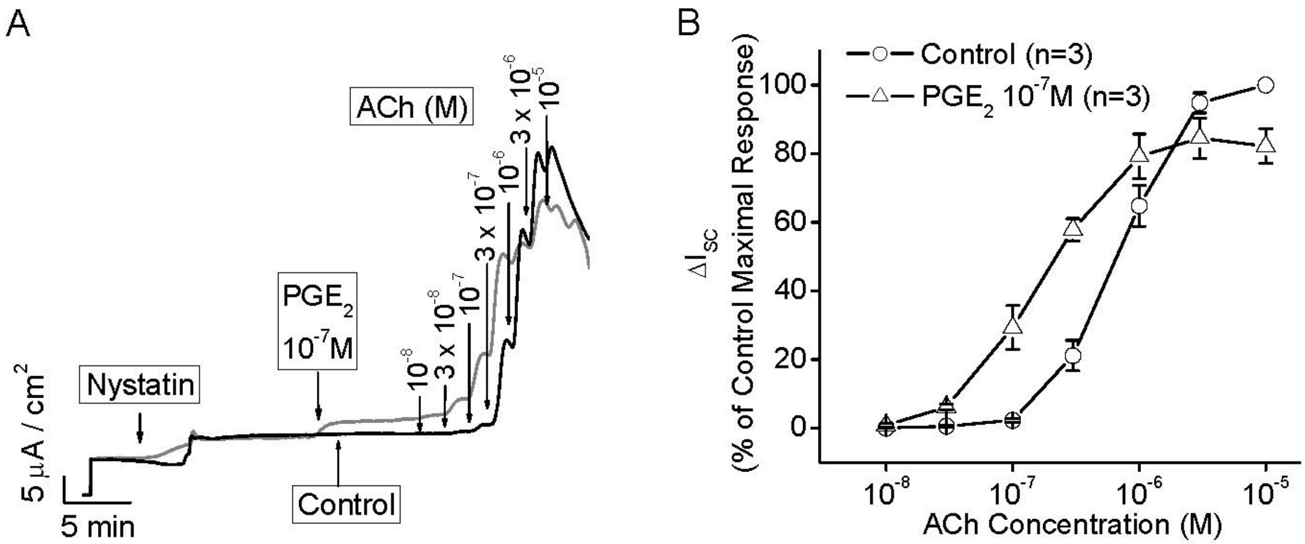

Figure 6