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House Dust Mite Extract Activates Apical Cl^- Channels Through Protease-Activated Receptor 2 in Human Airway Epithelia

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ABSTRACT

Adequate fluid secretion from airway mucosa is essential for maintaining mucociliary clearance, and fluid hypersecretion is a prominent feature of inflammatory airway diseases such as allergic rhinitis. House dust mite extract (HDM) has been reported to activate protease-activated receptors (PARs), which play various roles in airway epithelia. However, the role of HDM in regulating ion transporters and fluid secretion has not been investigated. We examined the effect of HDM on ion transport in human primary nasal epithelial cells. The Ca^{2+}-sensitive dye Fura2-AM was used to determine intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by means of spectrofluorometry in human normal nasal epithelial cells (NHNE). Short-circuit current (*I*sc) was measured using Ussing chambers. Fluid secretion from porcine airway mucosa was observed by optical measurement. HDM extract (10 μg/Ml) effectively cleaved the PAR-2 peptide and induced an increase of $[Ca^{2+}]_i$ that was abolished by desensitization with trypsin, but not with thrombin. Apical application of HDM-induced *Isc* sensitive to both a cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor and a Ca^{2+}-activated Cl^- channel (CaCC) inhibitor. HDM extract also stimulated fluid secretion from porcine airway mucosa. HDM extract activated PAR-2 and apical Cl^- secretion via CaCC and CFTR, and HDM-induced fluid secretion in porcine airway mucosa. Our results suggest a role for PAR-2 in mucociliary clearance and fluid hypersecretion of airway mucosa in response to air-borne allergens such as HDM. J. Cell. Biochem. 109: 1254–1263, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: HOUSE DUST MITES; SECRETION; ION CHANNEL; PROTEASE-ACTIVATED RECEPTORS; NASAL EPITHELIUM

Adequate fluid secretion from airway mucosa is essential for maintaining mucociliary clearance, and fluid hypersecretion is a prominent feature of inflammatory airway diseases such as allergic rhinitis. Fluid hypersecretion with mucus hypersecretion also leads to impairment of mcuociliary clearance in several airway disease, such as chronic obstructive pulmonary disease (COPD) and

Abbreviations used: HDM, house dust mite extract; NHNE cells, normal human nasal epithelial cells; PAR, protease-activated receptor; PAR-2AP, PAR-2 agonist peptide; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; PKC, protein kinase C; CaCC, Ca^{2+}-activated Cl^- channel; CFTR, cystic fibrosis transmembrane conductance regulator; NKCC, Na^+-K^+-$2Cl^-$ cotransporter.

Hyung-Ju Cho, Jae Young Choi and Yu-Mi Yang contributed equally to this study.

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asthma [Widdicombe, 2002]. Fluid secretion in the airway is mediated mainly by transepithelial Cl^- movement through apical anion channels, such as the cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+}-activated Cl^- channel (CaCC) [Boucher, 1994]. Altered expression and function of anion channels represents one possible pathogenic mechanism in inflammatory airway disease [Toda et al., 2002], but the exact mechanism behind fluid hypersecretion remains unclear.

Protease-activated receptor (PAR)s, a subfamily of G protein-coupled receptors, are activated by proteolytic cleavage of the N-terminal extracellular domain, leading to stimulation of Gq protein-mediated signal transduction [Nystedt et al., 1994; Ishihara et al., 1997]. Activated Gq protein stimulates phospholipase C (PLC), which hydrolyzes inositol phosphates to generate inositol 1,4,5-trisphosphate (IP3) and the release of Ca^{2+} from the endoplasmic reticulum. Ca^{2+} mobilization plays a variety of physiological roles, influencing differentiation, proliferation, and apoptosis [Berridge et al., 1998]. PARs are widely distributed throughout the apical surface of airway epithelia [Cocks et al., 1999; Ossovskaya and Bunnett, 2004] and there is increasing evidence that PARs play an important role in the inflammation of allergic rhinitis [Dinh et al., 2006] and asthma [Reed and Kita, 2004]. Activation of PARs stimulates IgE production [Gough et al., 1999], mast cell degranulation [Stenton et al., 2002], and cytokine production [Miike et al., 2001]. Interestingly, activation of PAR2 alters ion transport in mouse and human airway epithelial cell cultures. PAR2 activates CaCC in human bronchial epithelial cell lines and mouse tracheas [Kunzelmann et al., 2005]. PAR2 also induces a transepithelial current through CFTR in Calu-3 cells [Sato et al., 2005]. These findings suggest that PARs can regulate anion and fluid secretion in airway epithelia.

House dust mites (HDM) such as *Dermatophagoides pteronyssinus* are a major contributor to the increasing incidence of allergic airway diseases [Avdogan et al., 2007; Shin et al., 2009]. HDM possess endogenous proteases such as cysteine (Der p1) and serine proteases (Der p3, 6, and 9), and can activate PARs, leading to allergic inflammation through epithelial cell detachment and IgE production [Sun et al., 2001; Asokananthan et al., 2002; Adam et al., 2006]. Recently, Asokananthan et al. [2002] reported that a serine protease derived from HDM induces cytokine production and subsequent non-allergic inflammation. However, there is no evidence that HDM can regulate ion transporters in airway epithelia.

We speculated that activation of PARs by HDM extracts could regulate anion channels and subsequent fluid secretion in airway epithelial cells. Accordingly, the purpose of the present study was to examine the effect of HDM extract on ion transport in human primary nasal epithelial cells. We found that HDM-induced apical Cl^- secretion through CFTR and CaCC by activation of PAR-2.

MATERIALS AND METHODS

PREPARATION OF MITE EXTRACT

HDM (*D. pteronyssinus*) extract containing Der p1 and Der p2 was a kind gift from professor C.W. Park (Yonsei University, Korea) [Haselden et al., 1999], created as a lyophilized powder according to a previously reported protocol [Hong et al., 2004]. Preparation and dilution of the HDM extract was performed with phosphate-buffered saline solution (pH 7.4) and diluted to a stock concentration of 1 mg/ml. The amount of total protein in the extract was measured by a Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL). Endotoxin was removed from the HDM extract with Detoxi-Gel™ Endotoxin Removing Gel, and the extract was found to be free of detectable LPS (<0.1 EU/ml) by the Limulus amoebocyte lyase assay (Lonza Walkersville, Inc., Walkersville, MD).

NORMAL HUMAN NASAL EPITHELIAL (NHNE) CELL AND A549 CELL CULTURE

Normal human nasal epithelial (NHNE) cells were cultured as previously reported [Yoon et al., 1997]. Inferior turbinates were harvested from patients without allergy or infection during septoplasty. Informed consent was provided by all patients, and this study was approved by the Institutional Review Board of Yonsei University College of Medicine. Passage 2, primary human nasal epithelial cells (1×10^5 cells/culture) were seeded in 0.5 ml of culture medium onto 24.5 mm, 0.45 μm pore size Transwell clear (Costar Co., Cambridge, MA) culture inserts. Cultured cells were grown submerged, and culture medium was changed on the 1st day and every other day thereafter. After cultures reached confluence, media on the luminal side was removed to create an air–liquid interface (ALI) to encourage differentiation into ciliated columnar epithelial cells with polarity. Epidermal growth factor (EGF) was eliminated from the medium after confluence to avoid multilayer formation, as in a previous report [Kondo et al., 2006]. Cells from the A549 cell line were purchased from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modified Eagle medium and Ham's F-12 medium (1:1) containing 10% FBS and antibiotics.

WESTERN BLOT ANALYSIS OF PAR-2

Nasal epithelial cells were obtained from five patients with infective rhinitis (accompanying sinusitis without allergy) and five patients with allergic rhinitis during sinus surgery. Three normal control tissues were obtained during septoplasty in subjects without inflammation or allergy. The diagnosis of allergic rhinitis was made on the basis of clinical criteria of nasal symptoms (congestion, itching sensation, rhinorrhea, and sneezing) and a positive radio-allergosorbent test (RAST, total IgE level >200 kU IgE/L and specific IgE level ≥2 class) to common aeroallergens. Patients had not taken medicines such as steroids or antihistamines in the 4 weeks before the study. Diagnosis of infective rhinitis without allergy was made based on physical examination of a hypertrophied turbinate accompanying sinusitis with mucopurulent discharge and the absence of allergy proved by the above diagnostic methods. Epithelial cells were harvested using the same methods as for the primary cell culture. Total protein (50 μg) was extracted and separated by 10% SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% skimmed milk in TBST at 4°C overnight, and probed with anti-PAR-2 antibody (1:1,000 in 5% skimmed milk in TBST) and anti-α-tubulin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:1,000 in 5% skimmed milk in TBST) for 4 h at room temperature. After washing, the blots were incubated with anti-mouse secondary antibody (Cell Signaling Technology,

Beverly, MA), diluted 1:2,000 in TTBS for 1 h at room temperature and washed several times.

HYDROLYSIS OF PAR-1 AND PAR-2 SYNTHETIC PEPTIDES

The fluorescence-quenched synthetic substrates PAR-1 {[5-FAM]-Leu-Asp-Pro-Arg-Ser-Phe-Leu-Leu-Lys(Dabcyl)-Asp} and PAR-2 {[5-FAM]-Ser-Lys-Gly-Arg-Ser-Leu-Ile-Gly-Lys(Dabcyl)-Asp} were synthesized as described previously [Hong et al., 2008]. All experiments using HDM extract were performed in physiologic salt solution (PSS; 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose) at 37°C. The PSS (1 ml) was equilibrated at 37°C for 30 min before adding synthetic substrate and the enzyme solution. The cells were illuminated at 490 nm and proteolytic enzyme activities were continuously measuring emitted fluorescence at 520 nm using a PMT chamber system (Photon Technology International Inc., Lawrenceville).

MEASUREMENT OF $[Ca^{2+}]_i$

Primary nasal epithelial cells were isolated from nasal inferior turbinates, seeded onto cover glass slips (22 mm × 22 mm) in 35 mm dishes, and cultured for 2 days. Cells were incubated for 1 h in PSS containing 5 μM Fura2-AM (Teflabs, Austin, TX) in the presence of Pluronic F-127 (Invitrogen) to enhance dye loading. Fura-2-loaded cells were mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan) for imaging. The cells were illuminated at 34 and 380 nm, and the emitted fluorescent images at 510 nm were collected with a CCD camera and analyzed using the MetaFluor system (Universal Imaging Co., Downingtown, PA). The fluorescence ratio (340/380) was taken as measure of $[Ca^{2+}]_i$, and fluorescence images were obtained at 3 s intervals.

USSING CHAMBER EXPERIMENTS

The cultured NHNE cells were mounted in modified Ussing chambers (World Precision Instruments, Sarasota, FL). The epithelium was bathed on both sides with 5 ml of warmed (37°C) regular bicarbonate solution (pH 7.4, 310 mOsm) circulated by gas lifts with 95% O_2 to 5% CO_2. Indomethacin (10 μM) was added in the bath throughout the experiment to minimize endogenously generated prostaglandins. The mounted cells were clamped with an automatic voltage clamp, and the short-circuit current (*I*sc) was measured. Data were analyzed with Acquired and Analysis (version 1.2) software. Cultured NHNE cells were applied in the Ussing chambers, and a 15 min equilibration was achieved to stabilize the transepithelial current. After 2–5 min of current stabilization, HDM extract (10 μg/ml) was added to the apical or basolateral bath and Cl^- channel activity was measured.

PATCH CLAMP MEASUREMENT OF Cl^- CHANNEL ACTIVITIES

Cl^- currents were recorded using the whole-cell configuration of the patch clamp technique. Cl^- currents were isolated by using Cl^- as the only permeant ion in the pipette and bath solutions. All recordings were made at room temperature. Currents were recorded using an Axopatch-200B patch clamp amplifier (Axon Instruments). Cells were stimulated with HDM extract (10 μg/ml) and the currents were measured at a −40 mV holding potential. The I–V relationships were obtained with a ramp pulse from −120 to +120 mV applied at peak current. Bath solution used for patch clamp experiments contained 140 mM *N*-methyl-D-glucamine chloride; 10 mM Hepes (pH 7.2 with Tris); 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose. The pipette solution contained 140 mM *N*-methyl-D-glucamine chloride, 10 mM Hepes (pH 7.2 with Tris), 0.5 mM or 5 mM EGTA, 1 mM Tris–ATP, and 1 mM $MgCl_2$.

OPTICAL MEASUREMENT OF FLUID SECRETION FROM THE AIRWAY MUCOSA

The evaluation of fluid secretion from porcine tracheal mucosa was performed by a previously reported method [Joo et al., 2001]. Porcine tracheal tissue was harvested from an adult Yorkshire female pig that had been killed with an injection of pentobarbital. The mucosa (about 2 cm^2) was dissected free and mounted mucosal-side up at the gas/liquid interface of a 35 mm, Sylgard-lined plastic Petri dish containing 2 ml of KRB buffer. The tissue surface was dried with a gentle stream of 95% O_2 to 5% CO_2 gas and then partly covered with water-saturated mineral oil. The tissue was warmed to 37°C at a rate of 1.5°C/min (TC-102; Medical Systems Corp., Greenvale, NY) and superfused with warmed, humidified 95% O_2 to 5% CO_2. Tissues covered with water-saturated oil were obliquely illuminated to visualize the spherical droplets of secreted mucus within the oil.

REAGENTS

Fura-2 acetoxymethyl ester (fura-2-AM) was purchased from Teflabs. PAR-2 agonist peptides SLIGRL-NH_2 were purchased from the Korea Basic Science Institute (Seoul, Korea). Peptide structure was confirmed by liquid chromatography/mass spectroscopy (HP 1100 series HPLC System). Monoclonal antibodies to PAR-2 were purchased from Santa Cruz Biotechnology. Trypsin, amiloride, indomethacin, niflumic acid, CFTR (inh)-172, soybean trypsin inhibitor (SBTI), clotrimazole, bumetanide, and BAPTA-AM were purchased from Sigma. Thrombin and PKC inhibitor (Ro-31-8220) were purchased from Calbiochem.

RESULTS

HYDROLYSIS OF PAR-1 AND PAR-2 SYNTHETIC PEPTIDES BY HDM EXTRACT

We examined whether HDM extract could catalyze the cleavage of PAR-1 and PAR-2 using fluorescence-quenched PAR-1 and PAR-2 synthetic peptide substrates (10 μM). Because the FAM group only emits light when the quenching group Lys (Dabcyl) is released upon proteolytic cleavage, the fluorescence intensity closely correlates with the amount of cleaved peptide. When we added various doses (10–300 μg/ml) of HDM extract, the fluorescence intensity increased and reached a peak level within 10 s in a dose-dependent manner. HDM cleaved the PAR-2 peptide more effectively than PAR-1 peptide, indicating that PAR-2 is indeed a substrate of HDM extract (Fig. 1).

EXPRESSION OF PARs IN CULTURED NHNE CELLS

We also confirmed the expression of PAR-2 in NHNE cells by RT-PCR. As shown in Figure 2, mRNAs for PAR-1 (366 bp), PAR-2 (369 bp) and PAR-3 (382 bp) were all expressed in the NHNE cells

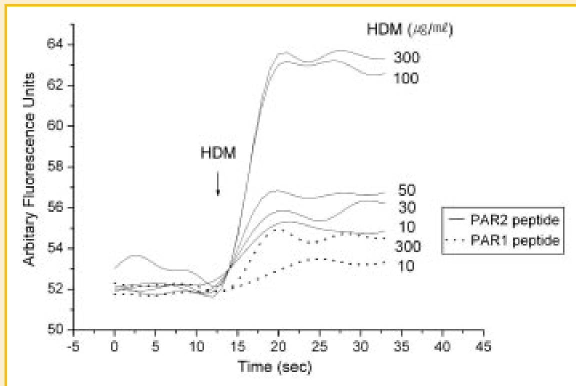

Fig. 1. Hydrolysis of synthetic peptides by HDM extract at different concentrations. HDM extract cleaved PAR-1 and PAR-2 synthetic peptides (10 μM) in a dose-dependent manner.

(Fig. 2). However, the mRNAs of PAR-4 was not expressed. Negative controls for RT-PCR were performed by omitting RT from the reaction, which yielded no product (data not shown).

HDM-INDUCED $[Ca^{2+}]_i$ MOBILIZATION IN CULTURED NHNE CELLS

Next, we examined changes in $[Ca^{2+}]_i$ after HDM application in NHNE. $[Ca^{2+}]_i$ was mobilized immediately after the addition of HDM. A rapid transient biphasic increase of $[Ca^{2+}]_i$ was evoked by HDM (10 μg/ml) within 100 s, followed by a sustained plateau. Although the response was not uniform among the cells, we observed similar trends (Fig. 3A,B). We did not observe the baseline type of $[Ca^{2+}]_i$ oscillation described in a previous report where the airway epithelial cells were stimulated with cockroach extract [Hong et al., 2004]. We also checked the effect of HDM on $[Ca^{2+}]_i$ in A549 cells of human lung cancer cell line. HDM (100 μg/ml) produced spiking response without oscillation in A549 cell (Fig. 3C).

EFFECTS OF PARs DESENSITIZATION ON $[Ca^{2+}]_i$ MOBILIZATION

To clarify whether HDM-induced $[Ca^{2+}]_i$ mobilization is mediated through the PAR-2 signaling pathway in primary nasal epithelial cells, we used a desensitization protocol [Hong et al., 2004]. The activation of PARs by proteolytic cleavage is irreversible, and the cleaved receptors are resistant to further proteolytic activation. Desensitization of all PAR subtypes, including PAR-2 was accomplished by sequential exposure to trypsin at a time interval of 2–3 min. The amplitude of $[Ca^{2+}]_i$ mobilization gradually decreased with repeated application of trypsin, and finally disappeared in spite of HDM extract stimulation (Fig. 4A). In contrast, sequential application of thrombin, which activates PAR-1, -3, and -4, did not affect HDM-induced $[Ca^{2+}]_i$ mobilization (Fig. 4B). Pretreatment with the protease inhibitor SBTI inhibited HDM-induced $[Ca^{2+}]_i$ mobilization, and HDM-induced $[Ca^{2+}]_i$ mobilization was recovered after washout of the SBTI (Fig. 4C).

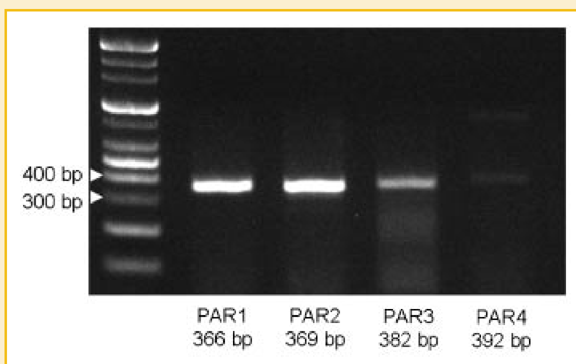

Fig. 2. Protease-activated receptor (PAR)s mRNA expression with RT-PCR. The mRNAs for PAR1, -2, and -3 were expressed in normal human nasal epithelial cells.

HDM-INDUCED TRANSEPITHELIAL CURRENT CHANGES

We further tested whether $[Ca^{2+}]_i$ mobilization by HDM could induce ion transport by measuring *Isc* in cultured NHNE cells. Application of HDM extract (10 μg/Ml) to the apical side of cultured NHNE cells evoked a sustained increase of current. The mean amplitude of the HDM-sensitive *Isc* was 4.82 ± 0.41 μA/cm^2 (n = 40). In contrast, basolateral application of HDM extract yielded minimal current changes (0.23 ± 0.21 μA/cm^2, n = 8). When the cells were pretreated with SBTI for about 30 min, HDM-induced *Isc* was significantly suppressed (0.21 ± 0.18 μA/cm^2; Fig. 5A-1, A-2). These results indicate that a serine protease derived from HDM can induce *Isc* changes.

ION TRANSPORTERS INVOLVED IN HDM-INDUCED *Isc*

We further evaluated the ion transporters involved in HDM-induced *Isc* changes. HDM-induced *Isc* was suppressed by both CFTR inhibitor (CFTRinh172, 20 μM, 3.08 ± 0.57 μA/cm^2, n = 4) and CaCC inhibitors (Niflumic acid, 100 μM, 2.25 ± 0.68 μA/cm^2, n = 4). Combined treatment of CFTRinh-172 and niflumic acid almost suppressed HDM-induced *Isc* (Fig. 5B-1, B-2). Basolateral application of 20 μM clotrimazole, an inhibitor of Ca^{2+}-activated K^+ channels, also decreased HDM-induced *Isc* to the level of 1.63 ± 0.36 μA/cm^2 (n = 5). When we blocked the Cl^- source from basolateral NKCC1 with bumetanide (100 μM), HDM-induced *Isc* dropped to 1.85 ± 0.48 μA/cm^2 (n = 5; Fig. 5C). Pretreatment with 50 μM of a Ca^{2+} chelating agent (BAPTA-AM) or 10 μM PKC inhibitor (Ro-31-8220) also inhibited *Isc* induced by HDM (1.97 ± 0.42 μA/cm^2 with BAPTA-AM, 2.27 ± 0.39 μA/cm^2 with Ro-31-8220 (Fig. 5D). Collectively, these results demonstrate that HDM may induce apical Cl^- secretion through CaCC and CFTR, with the driving force provided by basolateral Ca^{2+}-activated K^+ channels. $[Ca^{2+}]_i$ mobilization and PKC activation are involved in this process.

PATCH CLAMP MEASUREMENT OF Cl^- CHANNEL ACTIVITIES

We further confirmed the role of CFTR and CaCC in a HDM-induced current with whole-cell patch clamp tests. NHNE cells were patch-clamped in a whole-cell configuration, and the Cl^- current was isolated by removing all cations from the pipette and bath solutions so that the only permeable ion under the experimental conditions was Cl^-. The Ca^{2+}-dependent Cl^- current was recorded by including 0.5 mM EGTA and 1 mM ATP in the pipette solution

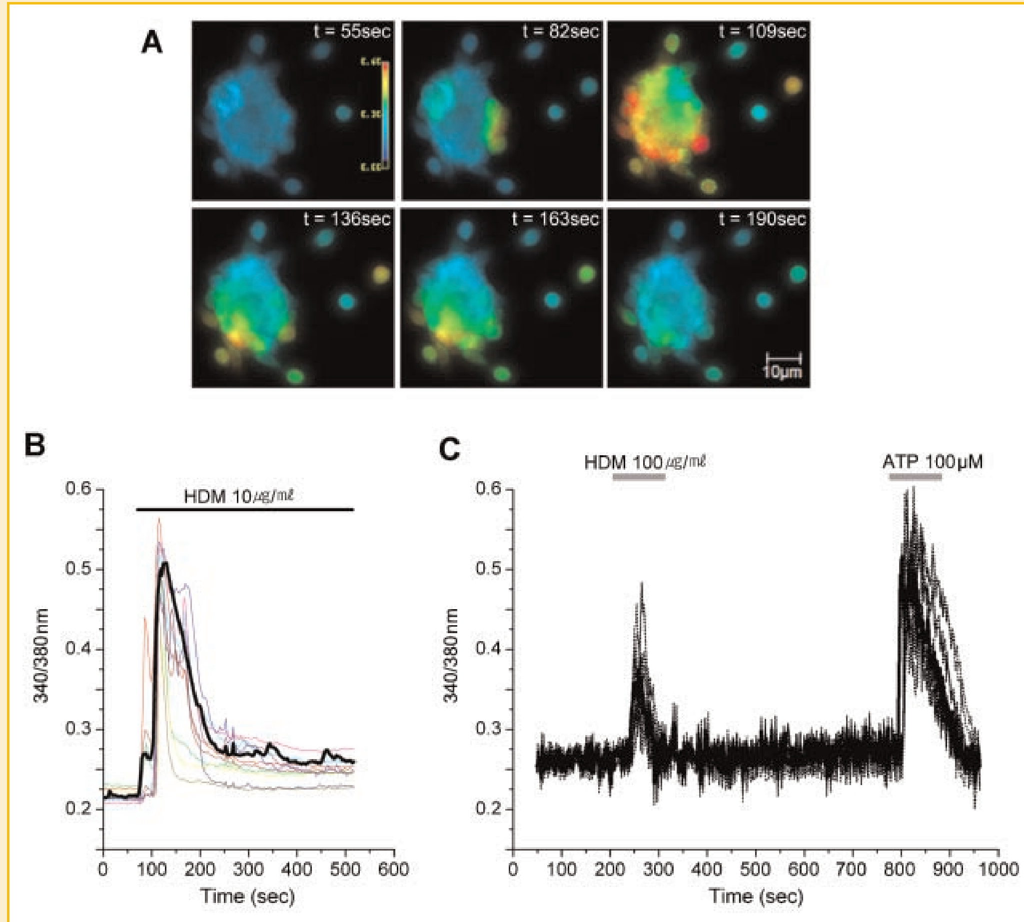

Fig. 3. $[Ca^{2+}]_i$ mobilization induced by HDM in human nasal epithelial cells and A549 cells. A: Fluorescence changes in response to HDM (10 μg/Ml) in normal human nasal epithelial cells. B: $[Ca^{2+}]_i$ versus time for 10 cells from images in A. The response was evoked immediately after application of HDM and followed by a sustained plateau. Colored lines show the trace of $[Ca^{2+}]_i$ mobilization in individual cells, and the bold line indicates the mean value of 10 individual cells. C: HDM (100 μg/Ml) also evoked spiking elevation of $[Ca^{2+}]_i$ in A549 cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 6A). The current was activated by incubating the cells for 2–3 min with HDM extract (10 μg/Ml). This current showed outward rectification, time-dependent activation, and substantial tail currents. The CFTR-like Cl^- currents were recorded by including 5 mM EGTA and 5 mM ATP in the pipette solution, which completely chelated free Ca^{2+} (Fig. 6B). The current was activated by incubating the cells for 5–10 min with HDM extract, and showed kinetic properties resembling those for CFTR [Zeng et al., 1997]. That is, the

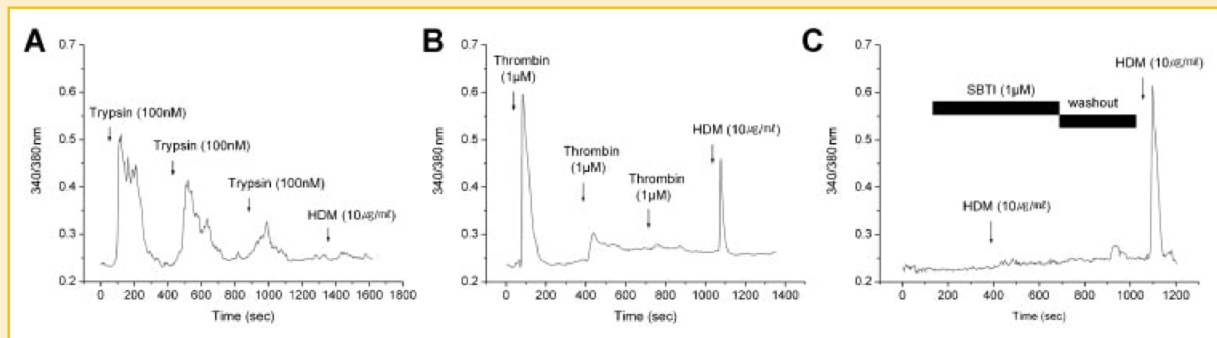

Fig. 4. The involvement of PAR-2 in HDM-induced $[Ca^{2+}]_i$ mobilization in human nasal epithelial cells. A: Desensitization of all PAR subtypes by repeated stimulation with trypsin (100 nM) abolished HDM-induced $[Ca^{2+}]_i$ mobilization (10 μg/Ml). B: Desensitization of PAR-1, -3, and -4 by repeated stimulation with thrombin (1 μM) did not affect HDM-induced $[Ca^{2+}]_i$. C: Serine protease inhibitor (SBTI, 1 μM) suppressed HDM-induced $[Ca^{2+}]_i$, which recovered after washout of the SBTI.

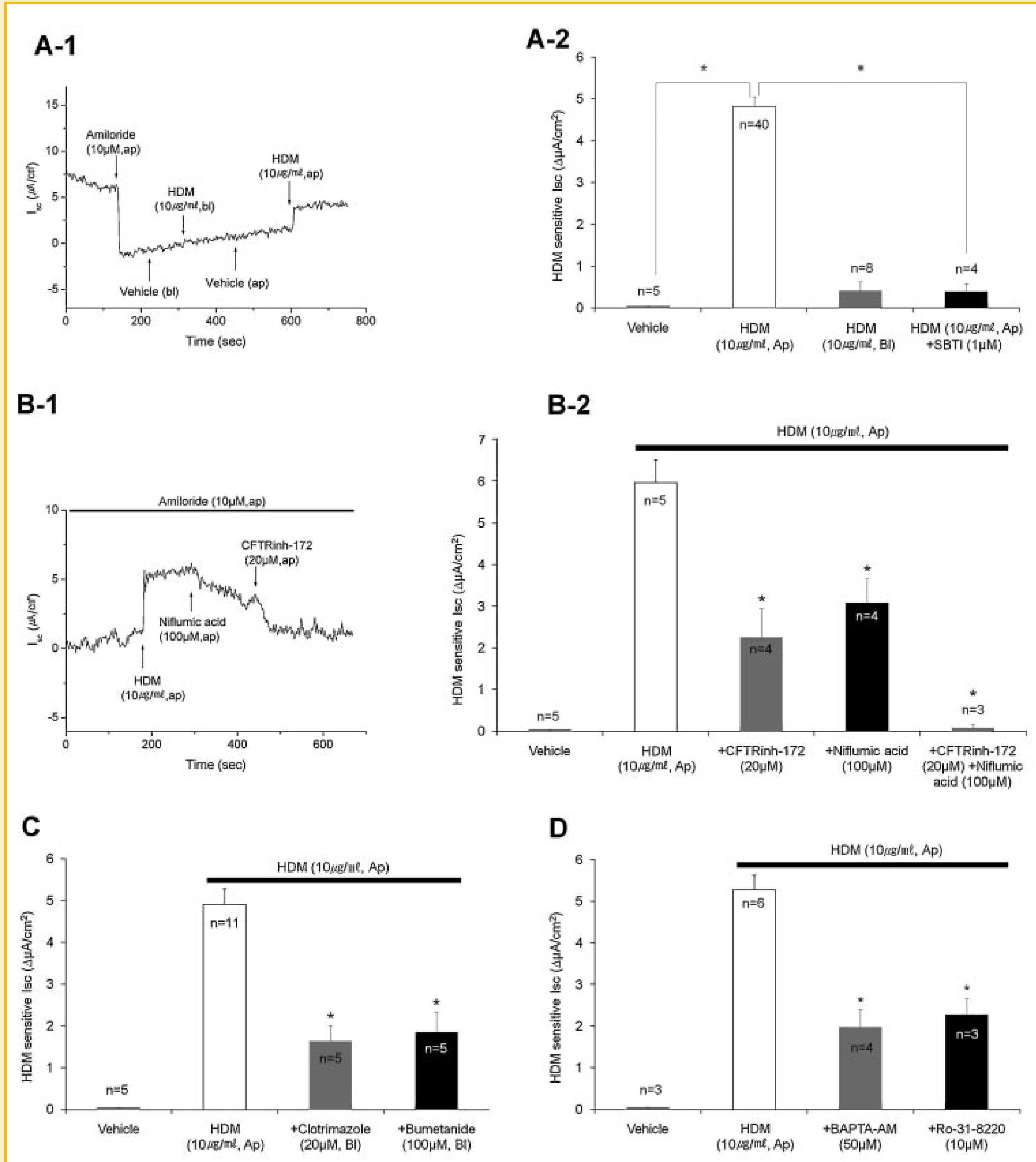

Fig. 5. HDM-induced transepithelial current changes in cultured human nasal epithelial cells. A-1: Typical short circuit current (*I*sc) recording following apical (Ap) or basolateral (Bl) application of HDM (10 µg/Ml). Indomethacin (10 µM) was added in the bath throughout the experiment to minimize endogenously generated prostaglandins. To block the outward Na^+ current amiloride (10 µM) was applied on the apical cell surface before HDM application. A-2: Summary of the effects of application of HDM on *I*sc. B-1: Typical short circuit current (*I*sc) recording showing the effects of niflumic acid (100 µM) and CFTR(inh)-172 (20 µM) on HDM-induced *I*sc. B-2: Summary of the effects of niflumic acid and CFTR(inh)-172 on HDM-induced *I*sc. C: Summary of the effects of basolateral application of 20 µM clotrimazole and 100 µM bumetinide on HDM-induced *I*sc. D: Comparison summary of the effects of HDM extract in the absence or presence of 50 µM BAPTA-AM or 10 µM PKC inhibitor (Ro-31-8220) on *I*sc. $^*P < 0.05$ compared with apical application of HDM.

current was voltage- and time-dependent without tail current. In addition, the I–V relationship was linear, between −120 and +120 mV. These results provide further evidence that HDM induces apical Cl^- secretion via CaCC and CFTR in NHNE cells.

PAR-2 EXPRESSION IN INFECTIOUS RHINITIS AND ALLERGIC RHINITIS

We compared the protein expression of PAR-2 in nasal epithelial cells from the patients with infective rhinitis or allergic rhinitis with

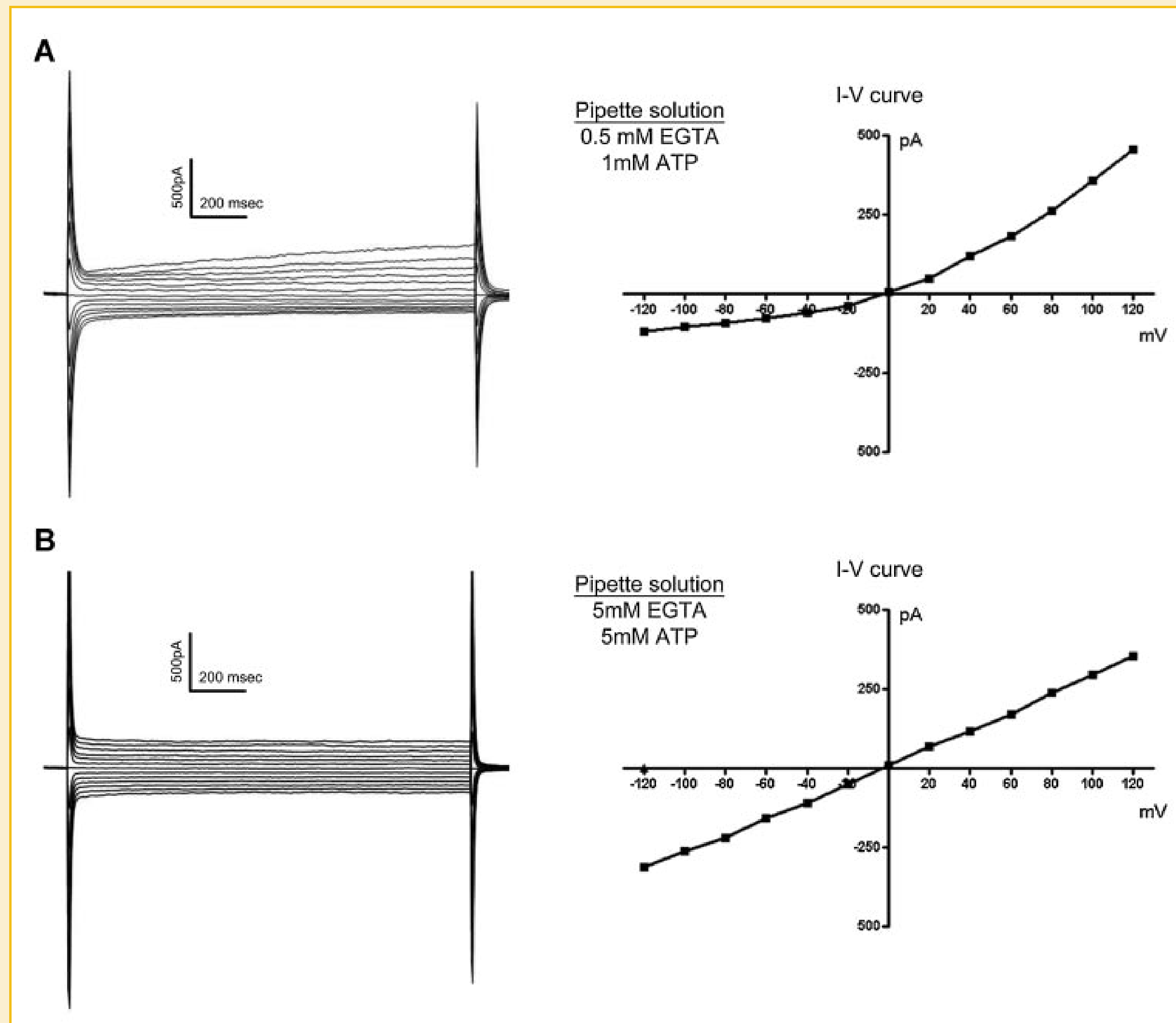

Fig. 6. Activation of Ca^{2+}-activated Cl^- channel and CFTR by HDM in single human nasal epithelial cell. A: Ca^{2+}-dependent Cl^- current were recorded by including 0.5 mM EGTA and 1 mM ATP in pipette solution. The current was activated by incubating the cells for 2–3 min with 10 μg/Ml HDM. B: CFTR-like Cl^- current were recorded by including 5 mM EGTA and 5 mM ATP in pipette solution. The potential was held at −40 mV for 1.2 s between pulse potential of between −120 and +120 mV in increment of 20 mV.

those from the nasal epithelial cells from normal control. When we compare the relative protein abundances, PAR-2 expression is significantly increased in infective rhinitis (1.98 ± 0.43) and allergic rhinitis (5.04 ± 0.72) than in normal controls (0.65 ± 0.03; Fig. 7). In this respect, we supposed that HDM-induced Cl^- secretion, which is mediated by PAR-2, was enhanced under infective or allergic rhinitis.

FLUID SECRETION FROM PORCINE TRACHEAL MUCOSA BY HDM

Finally, we investigated whether HDM extract would stimulate fluid secretion in the airway mucosa using the optical measurement of bubbles in airway mucosa. Since at least 1 cm^2 of flat airway mucosa was needed, we used pig tracheal tissue instead of human nasal mucosa. Basal secretion for 20 min before HDM application was minimal (Fig. 8A-1, B-1). However, 5 min after the addition of HDM extract (100 μg/Ml) or PAR-2 activating peptide (PAR-2 AP, 10 μM) to the bath solution on the basolateral, tiny droplets started to develop, until many tiny bubbles were present 30 min after application (Fig. 8A-2, B-2).

DISCUSSION

Air-borne allergens such as cockroach, fungi, and pollen contain various proteases that can activate PAR-2 leading in turn to elevate $[Ca^{2+}]_i$ levels [Reed and Kita, 2004]. We showed that PARs are expressed in primary nasal epithelial cells and that HDM hydrolyzes mainly PAR-2, mobilizing $[Ca^{2+}]_i$ in primary nasal epithelial cells. These findings confirmed that $[Ca^{2+}]_i$ mobilization was mainly mediated by PAR-2 signaling in human nasal epithelial cells. The dominant role of PAR-2 with HDM is consistent with previous reports from airway epithelial cell lines. Der p1 induces proinflammatory cytokine from HeLa cells by activating PAR-2, and not PAR-1 [Asokananthan et al., 2002]. Der p3 and Der p9 induce

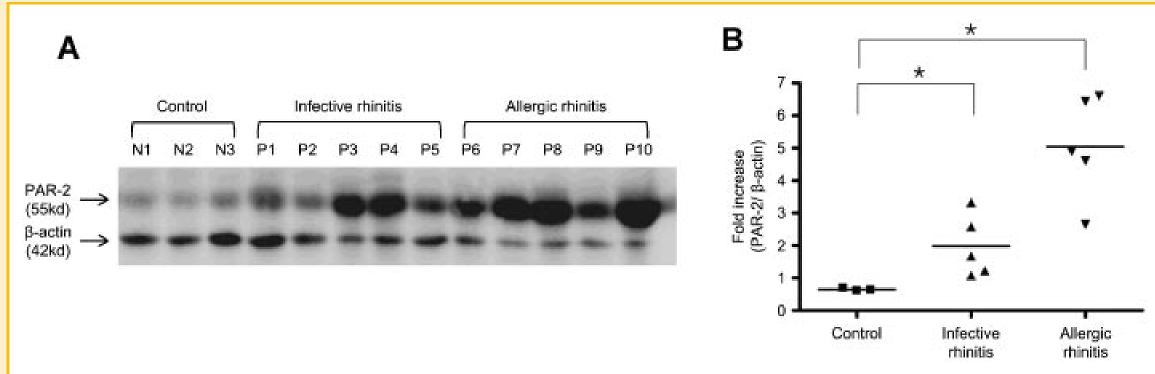

Fig. 7. Comparison of the PAR-2 expression levels in control, infective and allergic rhinitis. A: Western blot analysis shows that PAR-2 expression in nasal epithelial cells from the patients with infective and allergic rhinitis is greater than those from control tissues. B: Summary of relative protein expression in 3 control and 5 infective rhinitis and 5 allergic rhinitis. $^{*}P < 0.05$ compared with apical application of HDM.

PAR-2-dependent in the A549 cell line [Sun et al., 2001]. The role of PAR-1 in nasal epithelial cells is still unclear. PAR-1 mRNA was expressed in NHNE cell and high-dose HDM extract-induced hydrolysis of PAR-1, and thrombin, a PAR-1, -3, and -4 activator, also increased $[Ca^{2+}]_i$ in our experiments. The mobilization pattern in response to HDM was somewhat different from other allergens that activate PAR-2 in airway epithelia. In contrast to our data, cockroach extract induces a baseline type of $[Ca^{2+}]_i$ oscillation from

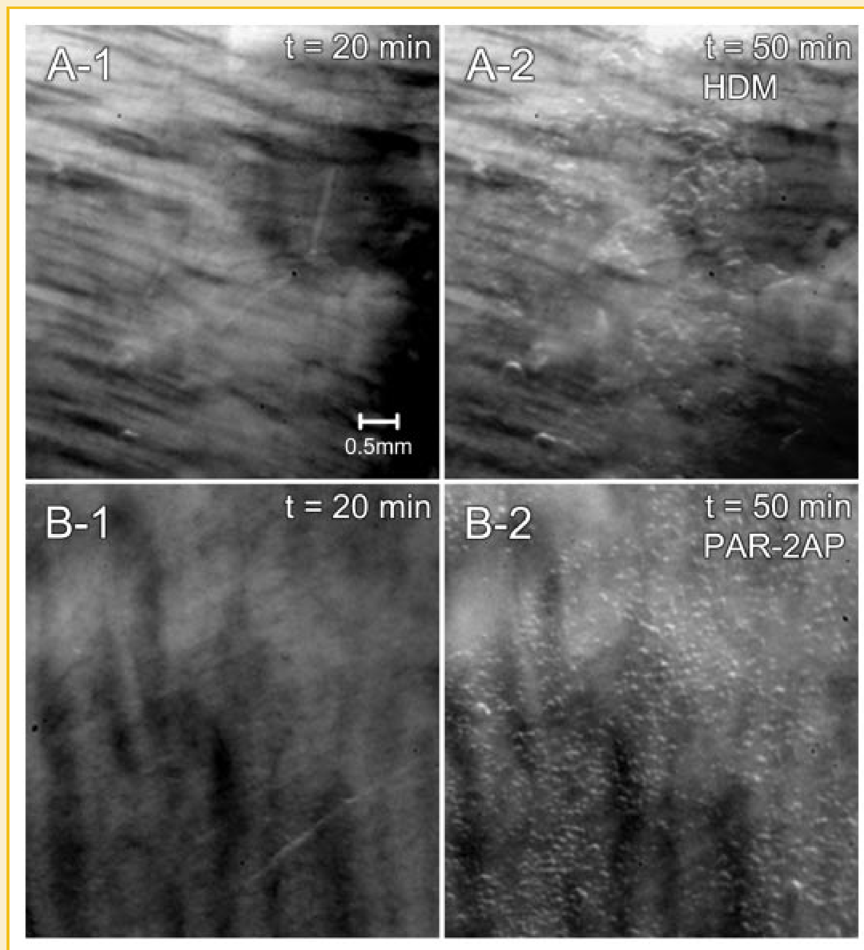

Fig. 8. Optical measurement of fluid secretion from porcine airway mucosa after stimulation with HDM extract (A) or PAR-2AP (B). There was no basal secretion for 20 min without stimulation (A-1, B-1). Thirty minutes after application of HDM extract (A-2) or PAR2-AP (B-2), many tiny bubbles emerged from the mucosa. HDM; house dust mite; PAR-2AP, PAR2 activating peptide.

the thapsigargin-sensitive Ca^{2+} store through the activation of PAR-2 in A549 cells [Hong et al., 2004]. The pattern of $[Ca^{2+}]_i$ mobilization in response to cockroach extract is dose-dependent, and a high dose of cockroach extract induces a typical biphasic $[Ca^{2+}]_i$ increase similar to our observations with HDM. We found similar spiking response in response to HDM in A549 cell. The pathway for HDM-induced $[Ca^{2+}]_i$ mobilization in human nasal epithelial cells needs further dissection.

The most notable finding of this study was that apical application of HDM extract activates Cl^- channels in primary human airway epithelial cells. Furthermore, we observed that HDM and PAR2-AP-induced fluid secretion in porcine airway mucosa. HDM-induced *Isc* was dependent on both CFTR and CaCC in cultured NHNE cells. We further confirmed the presence of both CFTR- and CaCC-dependent currents with patch clamp experiments. Involvement of both CFTR and CaCC in PAR2-induced *Isc* is a somewhat different finding from previous studies. PAR-2-induced Cl^- secretion is totally dependent on CFTR in the airway serous cell model (Calu-3 cells) [Palmer et al., 2006], but PAR-2-evoked *Isc* is mainly dependent on CaCC in the mouse trachea [Kunzelmann et al., 2005]. This inconsistency could originate from the different expression patterns of apical Cl^- channels of various cell types and species. CFTR is the only apical Cl^- channel in Calu-3 cells, and it mediates the Ca^{2+} activated Cl^- current [Haws et al., 1994]. In contrast, CaCC is more dominant than CFTR in the mouse trachea [Kunzelmann et al., 2005]. Because both CFTR and CaCC are expressed in human airway epithelia [Choi et al., 2007], HDM-induced *Isc* can be dependent on both Cl^- channels. The mechanism of CFTR activation by HDM still needs to be elucidated. PAR stimulates CFTR-dependent Cl^- secretion by prostaglandin E_2 synthesis in Calu-3 cells [Palmer et al., 2006], but inhibition of PGE_2 synthesis by indomethacin did not block HDM-induced Cl^- secretion in our experiment. Interestingly, HDM extract-induced Cl^- secretion was partially inhibited by a PKC inhibitor (Ro-31-8220). We assume that $[Ca^{2+}]_i$ elevated by HDM activates PKC and, subsequently, CFTR. PKC activation by $[Ca^{2+}]$-elevating agents can open the CFTR in various cell types [Chappe et al., 2003; Chen et al., 2004]. We also showed that HDM-induced fluid secretion in porcine airway mucosa. The apical surface of the mucosa was covered by oil in our experimental setting, and so we added high-dose HDM extract or PAR2-AP to the bath solution on the basolateral side. Based on the experiment with Ussing chamber the *Isc* in NHNE cell the response to PAR-2 AP is much clearer on the apical side than that on the basolateral side. If we were able to apply HDM or PAR2-AP on the apical side, we expect faster and big response. Because both CFTR and CaCC are expressed, porcine airway is a good model for human airway disease [Choi et al. 2007]. We expect that HDM also induces fluid secretion in human airway mucosa. The physiological role of HDM-induced Cl^- secretion remains unclear. Fluid secretion subsequent to HDM-induced Cl^- transport might provide the airway surface liquid essential for maintaining adequate mucociliary clearance, facilitating the elimination of the pathogen from the airway. On the other hand, HDM-induced transepithelial fluid secretion could induce watery rhinorrhea which is chief manifestation of allergic airway disease.

PARs are present on virtually all of the cells involved in inflammatory airway disease. Interestingly, PAR-2 expression is greater in patients with asthma [Reed and Kita, 2004], allergic rhinitis [Lee et al., 2007] on airway smooth muscle, submucosal glands, and epithelial cells. We also found that PAR-2 expression was significantly higher in nasal epithelial cells from patients with allergic or infective rhinitis than normal controls. Overexpression of PAR-2 is thought to be one of the underlying mechanisms behind airway hyperreactivity in both allergic airway diseases. Schmidlin et al. [2002] reported the effect of PAR-2 on an ovalbumin challenge of immunized mice. They found that eosinophil infiltration and airway hyperreactivity to inhaled methacholine was inhibited in mice lacking PAR-2 and increased in mice overexpressing PAR-2 compared to wild-type animals. In addition to enhancing allergic response, increased expression of PAR-2 in allergic and infectious conditions can lead to fluid oversecretion when the airway mucosa is exposed to HDM, representing one possible mechanism of fluid hypersecretion. As a follow-up, we are currently investigating whether the response of PAR-2 and the resulting fluid secretion in patients with allergic airway disease differs from that in healthy subjects.

In summary, HDM extract activated PAR-2 and apical Cl^- secretion via CaCC, CFTR in NHNE cells and HDM also induced fluid secretion in porcine airway mucosa. Our results suggest a role for PAR-2 in host defense and hyperreactivity of airway mucosa in response to air-borne allergens such as HDM.

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