DA-6034–Induced Mucin Secretion Via Ca^{2+}-Dependent Pathways Through P2Y Receptor Stimulation

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Purpose. We evaluated whether DA-6034 is involved in mucin secretion via P2Y receptor activation and/or intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) change. Also, we investigated the effect of P2Y receptor inhibitors or Ca^{2+} chelators on the DA-6034–induced mucin secretion and $[Ca^{2+}]_i$ increases.

Methods. Effects of DA-6034 on mucin expression in primary, cultured, conjunctival epithelial cells was studied using RT-PCR, Western blot analysis, and periodic acid-schiff (PAS) staining. To evaluate thin film layer thickness generated by mucin and fluid secretion, cells were incubated in DA-6034 with/without P2Y antagonists or extracellular/intracellular Ca^{2+} chelators, and were imaged with confocal microscope using Texas Red-dextran dye. In addition, DA-6034–induced Ca^{2+}-dependent Cl^- channels opening was evaluated using perforated patch clamp. Fluo-4/AM was used to measure changes in $[Ca^{2+}]_i$ induced by DA-6034 in Ca^{2+}-free or Ca^{2+}-containing buffered condition, as well as P2Y antagonists.

Results. DA-6034 induced the expression of mucin genes, production of mucin protein, and increase of number of mucin-secreting cells. P2Y antagonists inhibited DA-6034–induced mucin and fluid secretion, which was also affected by extracellular/intracellular Ca^{2+} chelators. DA-6034 stimulated Cl^- channel opening and $[Ca^{2+}]_i$ elevation. Further, $[Ca^{2+}]_i$ increases induced by DA-6034 were lacking in either P2Y antagonists or Ca^{2+}-free buffered condition, and diminished when endoplasmic reticulum Ca^{2+} was depleted by cyclopiazonic acid in Ca^{2+}-free buffered condition.

Conclusions. This study demonstrated that DA-6034 has a potential to induce mucin secretion via Ca^{2+}-dependent pathways through P2Y receptors in multilayer, cultured, human conjunctival epithelial cells.

Keywords: cultured conjunctival epithelial cells, mucins, purinergic receptors, calcium signaling, DA-6034

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) are tightly controlled, and local and temporal increases therein dominate cellular processes, including tear and mucin secretion.[1,2] In exocrine gland cells, Ca^{2+} signaling is initiated by Ca^{2+} release from intracellular stores such as the endoplasmic reticulum (ER) and mitochondria.[3] After rapid global Ca^{2+} signals, the clearance of cytoplasmic Ca^{2+} is accomplished by activities of Ca^{2+}-adenosine triphosphatase (ATPases) localized to the ER and plasma membrane.[3] Mechanisms that control influx and extrusion of Ca^{2+} contribute to Ca^{2+} homeostasis in the cell.[2] Thus, defective Ca^{2+} homeostasis causes lacrimal gland diseases or corneal epithelial wound healing delay.[4,5]

Mucins are distributed along the epithelial surface in mucosal tissues, including the airway tract, oral cavity, and ocular surface.[6] Both secreted and membrane-tethered mucins (MUCs) of the corneal and conjunctival epithelia are necessary for protecting the ocular surface and conserving the major refractive surface of the eye.[7,8] Goblet cells intercalated within the stratified conjunctival epithelia express and secrete gel-forming mucins, which are responsible for epithelium protection, maintenance of optical clarity, and refractive power.[9] MUC5AC, the most abundant gel-forming mucin, moves over the glycocalyx, collecting and eliminating debris and pathogens.[10]

The apical cells of the stratified epithelium of cornea and conjunctiva express membrane-tethered mucins such as MUC1, 4, and 16.[9,11] MUC4 acts as an antiadhesive molecule, lubricating the ocular surface and preventing infection from bacteria.[11] MUC1 and MUC16 interact with galectin-3 in the glycocalyx and provide a mucosal barrier against infectious agents and foreign particles.[12,13]

Alteration of secreted and membrane-tethered mucins is related to ocular surface disease.[10] Argueso et al.[14] reported that MUC5AC protein and corresponding mRNA levels were reduced in both the tear fluid and conjunctival epithelium of patients with Sjögren's syndrome. The distribution of the carbohydrate epitope of MUC16 is altered in patients with dry eye symptoms.[15] Accompanied by worse clinical signs and symptoms, mRNA levels of MUC5AC and MUC16 were shown

to be lower in aqueous-deficient dry eye.[16] Other studies have shown that MUC1 and MUC16 levels are increased in patients with Sjögren's syndrome or in postmenopausal women with non-Sjögren dry eye, which may represent compensatory mechanisms to maintain a healthy ocular surface.[17–19]

The P2Y family can be subdivided into two groups based on their coupling to specific G proteins. The P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors couple to Gq, to activate phospholipase C (PLC), and the P2Y12, P2Y13, and P2Y14 receptors couple to Gi, to inhibit adenylyl cyclase.[20] The P2Y2 receptor has been believed to be the major coordinator of mucociliary clearance in the lung.[21] Activation of this receptor increases mucin and water secretion.[22–24] In addition, activation of P2Y2 receptors leads to stimulation of PLC and inositol 1,4,5-trisphosphate (IP_3) release, which ultimately leads to release of Ca^{2+} from the ER Ca^{2+} store.[25,26] P2Y receptors, most likely P2Y2 receptor, coordinate regulation of epithelial Na^+ channels and Ca^{2+}-activated Cl^- channels (CaCCs) in many secretory epithelium including conjunctival epithelium.[27–30] Recently, P2Y2 receptor gene has been found in conjunctival epithelium, corneal epithelium, meibomian gland, and ductal cells on the ocular surface.[31]

Several authors have suggested that P2Y2 receptor agonists can regulate tear film secretion.[32–34] Diquafosol promoted tear and mucin secretion via elevated $[Ca^{2+}]_i$.[35] Moreover, Diquafosol elicited increases in net Cl^- transport through P2Y2 receptor stimulation in rabbit conjunctiva.[29]

The DA-6034 (7-carboxymethyloxy-3′, 4′, 5-trimethoxyflavone monohydrate), has been known to treat gastric ulcers by stimulating the synthesis of endogenous prostaglandin E2 to increase gastric mucin secretions and by promoting gastric epithelial cell migration and wound healing through the mammalian target of rapamycin (mTOR) pathway.[36,37] Recently, several studies reported the therapeutic possibility for effects of DA-6034 in the dry eye model.[38–40] DA-6034 increases MUC5AC production in conjunctival goblet cells and improves ocular surface integrity in dry eye animal model.[38] In vivo study using a mouse dry eye model, DA-6034 increases tear fluid production and conjunctival goblet cell number.[39] Another study reported that DA-6034 shows therapeutic efficacy in restoring tear function and inhibiting inflammatory processes by downregulating the mitogen-activated protein kinase signaling in an experimental dry eye model.[40] Considering the effect of DA-6034 on tear and mucin secretion, it could be postulated that DA-6034 may involve the mechanisms of the Ca^{2+}-activated mucin secretion. However, there is no evidence that DA-6034 regulates Ca^{2+} signaling during mucin secretion in multilayer, cultured, human conjunctival epithelial cells.

In the present study, we hypothesized that activation of P2Y receptors by DA-6034 would regulate anion channels and subsequent mucin secretion in multilayer, cultured, human, conjunctival epithelial cells. Therefore, we sought to determine whether DA-6034 is involved in the expression of mucin genes, production of mucin protein, and mucin secretion. We also investigated whether DA-6034 alters expression of P2Y receptors and induces changes in Cl^- channel activities and $[Ca^{2+}]_i$. The effect of intracellular/extracellular Ca^{2+} or P2Y receptors antagonists on DA-6034–induced mucin secretion and $[Ca^{2+}]_i$ were assessed to investigate the physiologic role of DA-6034.

Methods

Isolation and Culture of Primary Conjunctival Epithelial Cells

During preparation for cornea buttons, human conjunctival tissues were saved and donated by the YONSEI eye bank (Severance EYE & ENT hospital, Seoul, Korea) with informed and written consent from the family of the donor for use of research purposes. Eight different conjunctivas were used. Regarding donor demographics, subjects had no ocular disease and their ages ranged from 29 to 49 years. The entire conjunctiva was dissected approximately 2-mm lateral to the limbus of the cornea. Conjunctival cells were isolated by first incubating conjunctival specimens in PBS (Millipore, Billerica, MA, USA) with 1.4 U dispase (Sigma-Aldrich Corp., St Louis, MO, USA) for 1 hour in a 37°C incubator. The loosened cells were then removed with a cell scraper, washed two times, seeded on 60-mm culture dishes, and incubated at 37°C in a humidified 5% CO_2 atmosphere. The culture medium used was KBM-Gold basal medium (Lonza, Walkersville, MD, USA) with KGM-Gold SingleQuots (Lonza). The culture medium was changed 1 day after seeding and every other day thereafter until the cells reached 60% to 70% confluence (~5–6 days), at which time they were dissociated with 0.25% trypsin EDTA (Lonza). Subcultures were seeded on 100-mm culture dishes at 2×10^3 cells/cm^2. After reaching 60% to 70% confluence (~5–6 days), passage 2 cells (1×10^5 cells) were seeded onto 24-well plates containing transwell polyester membrane inserts (Corning, Union City, CA, USA). After cells covered the bottom of transwell plates (~3 days), apical media was removed and culture was fed from the basal compartment with differentiation media (1:1, KGM-Gold + BSA [HyClone, Logan, UT, USA] + retinoic acid [Alfa Aesar, Ward Hill, MA, USA]: low glucose Dulbecco's Minimal Essential Medium). The medium was changed every day (~10 days) during air-lifting, and cultured until three to approximately four layers of conjunctival cells were identified.

Periodic Acid-Schiff Staining

After treatment with DA-6034 for 24 hours, primary multilayer, cultured, human conjunctival epithelial cells were fixed in 2% formaldehyde and embedded in paraffin. Central vertical sections (3-μm thick) were cut and stained with Periodic Acid-Schiff (PAS) reagent using a standard protocol. Images of conjunctival epithelial cells were obtained using a model E800 microscope (Nikon, Melville, NY, USA). The number of PAS-positive cells was counted in three consecutive light microscopic fields per culture set using three different sections and averaged.

Cell Proliferation Assay

Cells were incubated with various concentrations of DA-6034 (1, 10, and 100 μM) for 24 hours, after which cell viability was measured using the (dimethylthiazol-diphenyltetrazolium bromide) MTS and Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay. Cells were cultured in a 96-well plate (BD Falcon, Franklin Lakes, NJ, USA) at 1×10^4 cells per well overnight and treated with DA-6034. DA-6034 was synthesized by Dong-A Pharmaceutical Company (Kyunggi-do, Korea) and diluted with 0.2% dimethyl sulfoxide (DMSO).

For the MTS assay, cell proliferation was determined using the CellTiter 96 AQueous One Solution Reagent Cell Kit (Promega, Madison, WI, USA). Briefly, 20 μL of Cell Titer 96 AQueous One Solution Reagent containing MTS was added to each well of the 96-well plate containing the samples in 100 μL of culture medium. The cultures were incubated at 37°C for 1 to 4 hours in a humidified, 5% CO_2 atmosphere. The reaction was stopped by adding 25 μL of 10% SDS to each well. Optical density was measured using a plate reader with a 490-nm filter. For the CCK-8 assay, 10 μL CCK-8 solution was added to each well of the plate containing the samples in 100 μL of culture

medium. Optical density was measured using a plate reader with a 450-nm filter. Experiments were carried out in triplicate.

Reverse Transcription-Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction

Total RNA was isolated from cultured conjunctival cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). For RT-PCR, cDNA was synthesized from 1 μg of total RNA using cDNA EcoDry Premix (Clontech, Mountain View, CA, USA). One microgram of cDNA was subsequently used in RT-PCR. The housekeeping gene β-Actin was used as an internal control. For real-time PCR, we used the SYBR green Premix Ex Taq (Clontech) with a Step One Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). After initial denaturation at 95°C, targets were amplified using individually optimized thermocycling conditions. For MUC: 35 cycles of 30 seconds at 95°C (denaturation), 30 seconds at 57°C (annealing), and 30 seconds at 72°C (extension); for P2Y receptor: 35 cycles of 30 seconds at 95°C, 30 seconds at 55°C to 62°C, depending on the primers and 30 seconds at 72°C, terminating with 7 minutes at 72°C. The real-time PCR protocol consisted of an initial denaturation for 10 seconds at 95°C, followed by 40 cycles of denaturation for 15 seconds at 95°C and annealing for 60 seconds at 60°C. β-Actin amplification was used as an endogenous reference for determination of mRNA integrity. Amplification products were separated by electrophoresis on 1.5% agarose DNA gels and visualized by ethidium bromide staining. Results are representative of at least three independent experiments. Primer sequences are listed in the Table.

Western Blot Analysis

Cells were lysed with radioimmunoprecipitation (RIPA) buffer (Biosesang, Seoul, Korea) containing 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μg/mL pepstatin, and 10 μg/mL leupeptin for 20 minutes at 4°C, scraped with a cell scraper, and centrifuged at 15,000*g*, for 15 minutes, at 4°C. Cell lysates were boiled in Laemmli's sample buffer (Bio-Rad, Hercules, CA, USA) for 5 minutes. Proteins were separated by SDS-PAGE (Bio-Rad) on 6.5% gels for mucin and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked overnight at 4°C in 3% BSA or 5% nonfat dry milk in buffer containing 10 mM Tris-HCl (pH 8.0; Sigma-Aldrich), 150 mM NaCl (Sigma-Aldrich), and 0.05% Tween-20 (Sigma-Aldrich), and then incubated overnight with primary antibody for MUC1 (sc-7313, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), MUC4 (ab60720, 1:1000; Abcam, Cambridge, MA, USA), MUC5AC (ab24070, 1:200; Abcam), MUC16 (ab10033, 1:300; Abcam), P2Y1 (1:1000; Abcam), P2Y2 (1:1000; Abcam), P2Y4 (1:1000; Abcam), P2Y6 (1:1000; Abcam), or β-Actin (1:1000; Santa Cruz Biotechnology), followed by incubation with peroxidase-labeled anti-mouse IgG secondary antibody (1:3000; KPL, Gaithersburg, MD, USA) or peroxidase-labeled anti-rabbit IgG secondary antibody (1:3000; KPL).

β-Actin served as a loading control. Cells exposed to 10 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich) for 1 hour served as a positive control. Immunoreactive bands were visualized using an enhanced chemiluminescence Western blotting kit (Amersham Pharmacia, Piscataway, NJ, USA). Results are representative of at least three independent experiments.

Confocal Microscopy Measurement of Thin Films of Mucin and Fluid

Multilayer cell cultures were exposed to media containing DA-6034 (100 μM) and each P2Y antagonist for 12 hours; suramin (P2Y2 antagonist, 50 μM; Tocris, Bristol, UK), pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, P2Y4 antagonist, 100 μM; Tocris), or MRS (P2Y6 antagonist, 5 μM; Tocris). Cell cultures were also exposed to media containing DA-6034 (100 μM) and each different calcium condition for 12 hours to demonstrate the association between increase in $[Ca^{2+}]_i$ and DA-6034 induced mucin secretion; Ca^{2+}-free buffer (normal buffer with EGTA 1-2 mM), Ca^{2+}-free buffer + BAPTA (50 μM; Sigma-Aldrich), or Ca^{2+}-free buffer + BAPTA/thapsigargin (Tg, 1 μM; Sigma-Aldrich). BAPTA is a selective intracellular Ca^{2+} chelator. Thapsigargin, an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase, blocks pumping the calcium into the ER, which causes calcium stores to become depleted.

During 12 hours of the incubation with DA-6034, cultures were incubated at 37°C and 5% CO_2 with Texas Red-dextran (Molecular Probes, Eugene, OR, USA) which was used at concentrations of 2 mM and loaded into upper chamber of transwell insert. Texas Red conjugated to dextran, which has a good quantum yield and is relatively impermeable across the epithelia, was routinely used to label the airway surface layer.[41] All samples were immersed in perfluorocarbon for maintenance of hydration and reduction of artifacts. Z-stack images were acquired by laser scanning confocal microscopy (LSM 700; Carl Zeiss, Jena, Germany) and analyzed using ZEN 2011 (blue edition) imaging software (Carl Zeiss) to measure the thickness of the thin film layer. Five predetermined points on the culture were scanned, and the thickness of five different sections in one image generated from a confocal Z-stack using the Ortho setting was measured and averaged.

Patch Clamp Measurement of Cl^- Channel Activities

Whole-cell voltage-clamp recordings were made using the perforated patch clamp method at room temperature. For Cl^- currents recordings, patch electrodes with resistance of 3 to 5 MΩ were filled with an internal solution containing 140 mM N-methyl-D-glucamine (NMDG)-Cl, 0.5 mM EGTA, 10 mM HEPES, 1 mM $MgCl_2$, and 1 mM Mg-ATP, adjusted to pH 7.2. The composition of external solution was as follows: 140 mM NMDG-Cl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.4.[42] Cells were stimulated with DA-6034 (100 μM). The Cl^- currents were elicited by voltage ramps from −100 to + 100 mV (800-millisecond duration) applied every 10 seconds from a holding potential of −40 mV. Nystatin was prepared as a stock solution (25 mg/mL) in DMSO, diluted to a concentration of 250 μg/mL using the internal solution, and back-filled into the pipette after the tip of the pipette was initially filled with the nystatin-free solution. Currents were recorded using a Multi-Clamp 700B amplifier (Axon, Foster City, CA, USA), subsequently digitized with a sampling rate of 10 kHz, and analyzed using pCLAMP10 software (Axon).

Measurement of $[Ca^{2+}]_i$

Cells stimulated with DA-6034 (100 μM) or ATP were incubated for 30 minutes in physiological salt solution (PSS) containing 2 μM Fluo-4/AM (Invitrogen) in the presence of 0.05% Pluronic F-127 (Invitrogen) at room temperature. For comparison with DA-6034, purinergic agonist ATP, which activates Ca^{2+} dependent pathways was used. The emitted fluorescent images at 490 nm (fluorescence intensity = F/F_0) were collected with a charge-coupled device (CCD) camera (Photometrics, Tucson, AZ, USA) attached to an inverted microscope. Fluorescence images were obtained at 2-second intervals. We also evaluated the effect of P2Y antagonist on DA-6034–stimulated Ca^{2+} mobilization, at which inhibitors were

TABLE. Primer Sequence Used for RT-PCR and Real-Time PCR

Name	Accession Number	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Product Size, bp	Temperature, °C
Actin	NM001101.3	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	233	55
Actin*	NM001101.3	GGCATCCTCACCCTGAAGTA	AGGTGTGGTGCCAGATTTTC	65	60
MUC1	NM001204286.1	TCTCACCTCCTCCAATCAC	GAAATGGCACATCACTCAC	368	55
MUC1*	Argueso et al.,[14] NM002456	GTGCCCCCTAGCAGTACCG	GACGTGCCCCTACAAGTTGG	123	60
MUC4	NM018406.6	TTCTAAGAACCACCAGACTCAGAGC	GAGACACACCTGGAGAGAATGAGC	466	62
MUC4*	Argueso et al.,[14] GenBank AF058803	GCCCAAGCTACAGTGTGACTCA	ATGGTGCCGTTGTAATTTGTTGT	102	60
MUC5AC	Argueso et al.,[14] GenBank Z48314	TCCACCATATACCGCCACAGA	TGGACCGACAGTCACTGTCAAC	103	62
MUC16	NM024690.2	GCCTCTACCTTAACGGTTACAATGAA	GATCAGTTGGCAACCCAAGTAGAAG	293	62
MUC16*	NM024690.2	GCCTCTACCTTAACGGTTACAATGAA	GGTACCCCATGGCTGTTGTG	114	60
P2Y1	NM002563.3	TGTGGTGTACCCCCTCAAGTCCC	ATCCGTAACAGCCCAGAATCAGCA	259	58
P2Y2	NM176072.2	GAGCATCCTGACCTGGAGAG	AGTGCATCAGACACAGCCAG	279	58
P2Y4	NM002565.3	CCACCTGGCATTGTCAGACACC	GAGTGACCAGGCAGGGCACGC	424	64
P2Y6	NM176798.2	CGCTTCCTCTTCTATGCCAACC	CCATCCTGGCGGCACAGGCGGC	365	63

* Real-time PCR.

added 15 minutes before DA-6034. Suramin (P2Y2 antagonist, 50 μM), PPADS (P2Y4 antagonist, 100 μM), or MRS (P2Y6 antagonist, 5 μM) was used. DA-6034 induced changes of $[Ca^{2+}]_i$ after treatment with cyclopiazonic acid (CPA, 25 μM; Tocris) in the absence or presence of extracellular Ca^{2+} were measured. Ionomycin (5 μM; Tocris) was added at the end of every experiment to verify the cell viability from an increase in $[Ca^{2+}]_i$ by direct actions on cell membrane. All data were analyzed using MetaFlour software (Molecular Devices, Downingtown, PA, USA).

Statistical Analysis

Data are presented as means ± SEM. The data were analyzed by one-way ANOVA, and statistical significance was determined using Bonferroni's multiple comparison test. Statistical analyses were performed using GraphPad PRISM (version 4; GraphPad Software, Inc., San Diego, CA, USA). Differences were considered statistically significant for P values less than 0.05.

RESULTS

Effects of DA-6034 on Mucin Gene and Protein Expression

DA-6034 treatment for 4 hours induced MUC5AC and MUC16 expression according to the results of Western blot analysis (Fig. 1). Reverse transcription–PCR showed that mRNA expression of all mucins existed over the course of the DA-6034 treatment time (Fig. 2A). In real-time PCR, peak MUC1, MUC4, and MUC16 were achieved within 1 hour, and peak MUC5AC was achieved within 4 hours (Fig. 2B). In Western blot analysis, MUC4 protein expression increased at 4 hours after treatment, and MUC5AC protein expression increased at

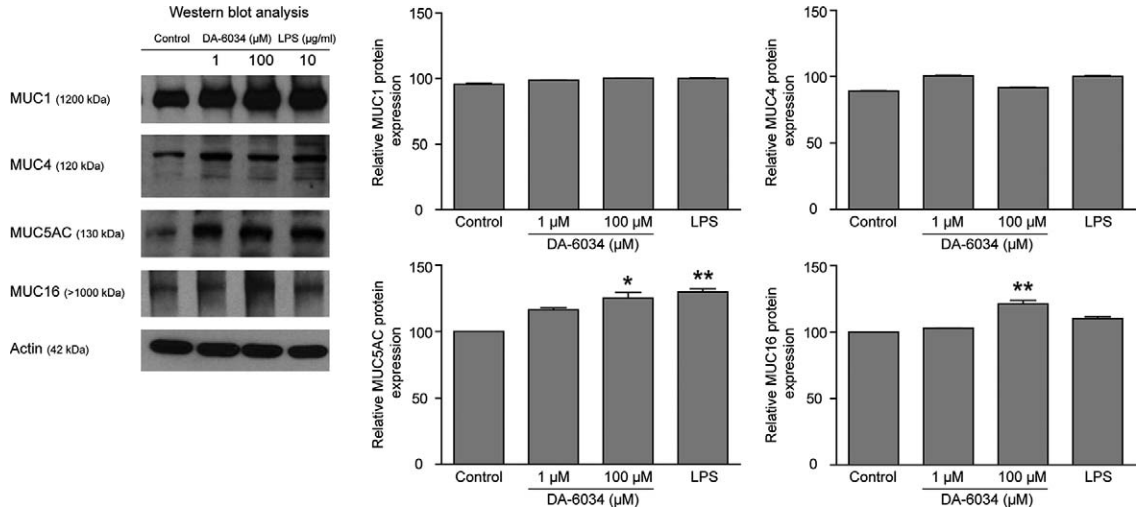

FIGURE 1. Expression of mucin protein after application of DA-6034 (1, 100 μM) for four hours in multilayer-cultured human conjunctival epithelial cells. β-Actin was used as an internal control. Lipopolysaccharide (10 μg/mL) was used as a positive control. *Error bars* represent SEM (**$P < 0.01$, *$P < 0.05$).

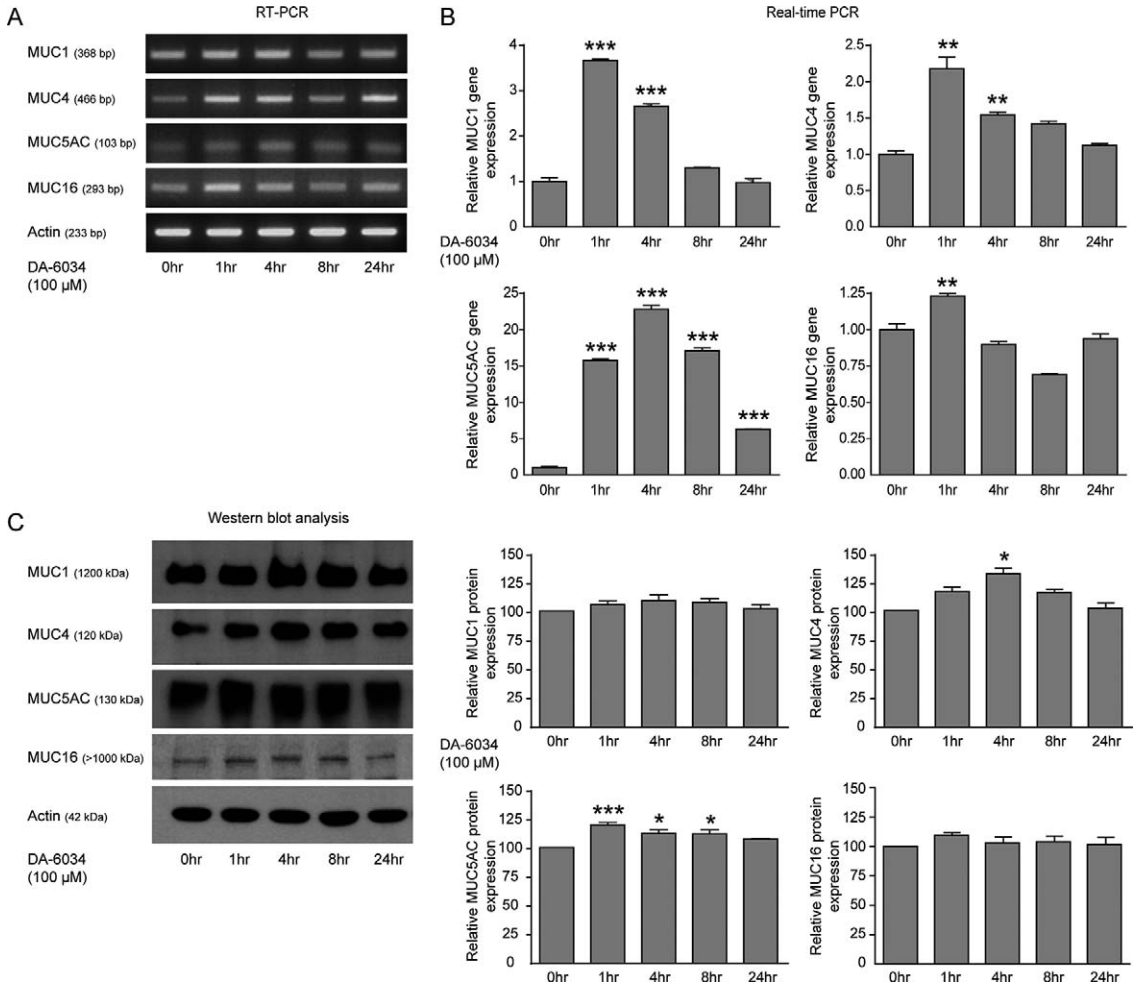

Figure 2. Expression of mucin genes and protein after application of DA-6034 (100 μM) in a time-dependent manner in multilayer, cultured, human conjunctival epithelial cells. (**A**) Reverse transcription–PCR. (**B**) Real-time PCR. (**C**) Western blot analysis. β-Actin was used as an internal control. *Error bars* represent SEM (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

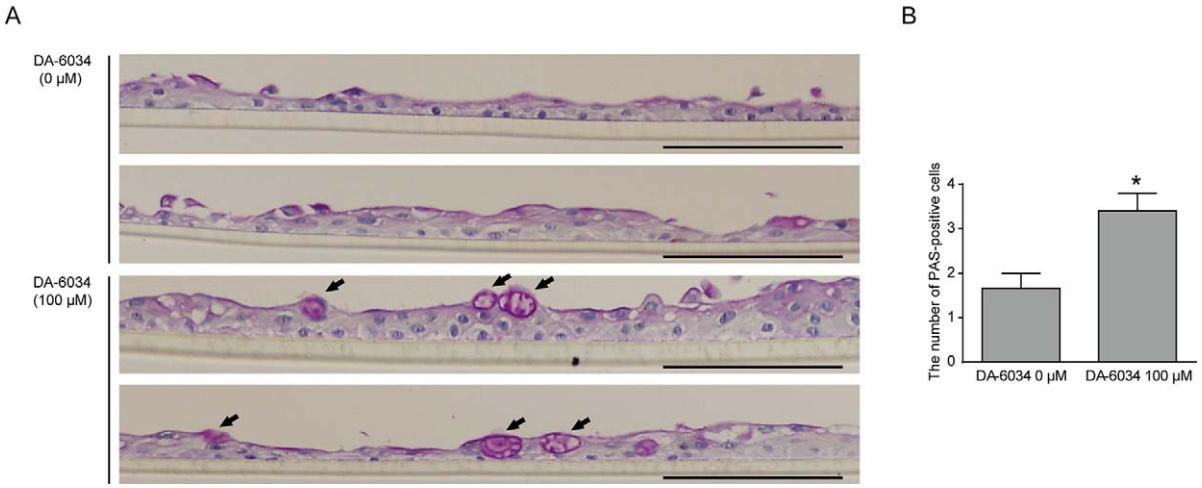

Figure 3. Changes in PAS-positive cell numbers after application of DA-6034 (100 μM) in multilayer, cultured, human conjunctival epithelial cells. (**A**) Periodic Acid-Schiff staining of cells (*black arrows*). (**B**) Comparison of the number of PAS-positive cells. *Error bars* represent SEM (*$P < 0.05$). All micrographs are of the same magnification. *Scale bars*: 200 μM.

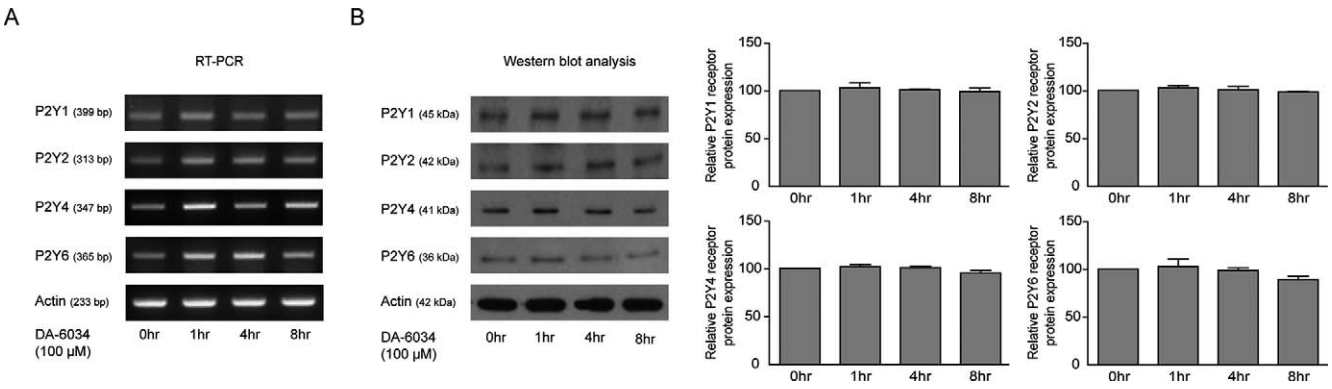

FIGURE 4. Expression of P2Y receptor genes and protein after application of DA-6034 (100 μM) in multilayer, cultured, human conjunctival epithelial cells at different time points. (**A**) Reverse transcription–PCR. (**B**) Western blot analysis. *Error bars* represent SEM.

1 hour and stayed elevated until at least 8 hours after treatment (Fig. 2C).

Effect of DA-6034 on PAS-Positive Cells

DA-6034 (100 μM) induced the expression of PAS-positive cells. The purple color-stained round cells (black arrows) are PAS-positive cells (Fig. 3A). A significant increase in the number of cells was observed in DA-6034–treated cells compared with buffer only treated cells (Fig. 3B).

Effects of DA-6034 on Cell Proliferation

Regarding the dose-dependent effects of DA-6034, there was no significant increase or reduction in the number of cultured human conjunctival epithelial cells (data not shown).

Effects of DA-6034 on P2Y Receptor Gene and Protein Expression

DA-6034 induced the expression of mRNA of P2Y2, P2Y4, and P2Y6 receptor in RT-PCR test (Fig. 4A). However, according to the results of Western blot analysis, there was no significant difference in the P2Y receptor protein expression (Fig. 4B).

Effects of DA-6034 on Mucin and Fluid Secretion in P2Y Antagonist Cotreated Cultured Human Conjunctival Epithelial Cells

DA-6034 (100 μM) induced the mucin and fluid secretion from cultured human conjunctival epithelial cells into extracellular space. When treating the cells with P2Y antagonist (suramin, PPADS, or MRS) and DA-6034 for 24 hours, the thickness of the thin film layer was significantly reduced, compared with DA-6034–treated cells ($P < 0.001$, Fig. 5A).

Effects of DA-6034 on Mucin and Fluid Secretion in Extracellular/Intracellular Ca^{2+} Chelator Cotreated Cultured Human Conjunctival Epithelial Cells

Treatment of cells with DA-6034 in different calcium conditions (Ca^{2+}-free buffer, Ca^{2+}-free buffer + BAPTA, or Ca^{2+}-free buffer + BAPTA/Tg) showed that the thickness of the thin film layer was significantly reduced, compared with DA-6034 alone-treated cells ($P < 0.01$ for Ca^{2+}-free buffer and $P < 0.001$ for the others, Fig. 5B). This finding suggested that both intracellular and extracellular Ca^{2+} may involve in DA-6034–induced mucin and fluid secretion.

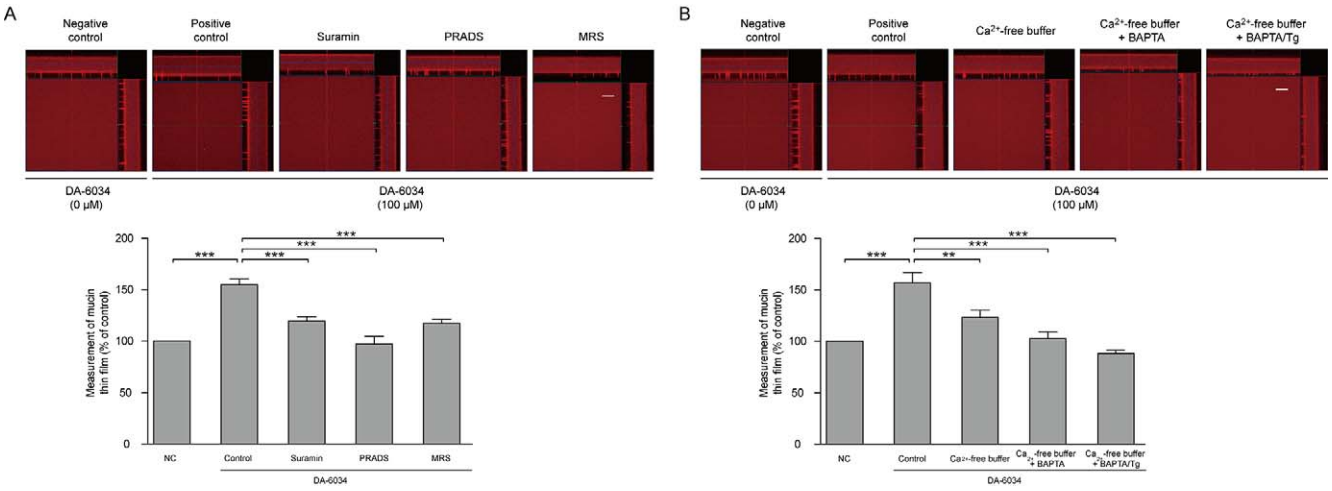

FIGURE 5. Changes in thin film layer after application of DA-6034 (100 μM) for 12 hours with/without (**A**) P2Y antagonists or (**B**) extracellular/intracellular Ca^{2+} chelators in multilayer, cultured, human conjunctival epithelial cells. Z-stack images of the Texas Red–labeled thin film layer were acquired by laser scanning confocal microscopy and analyzed using ZEN 2011 (blue edition) imaging software to measure the thickness of red color-stained thin film layer. *Error bars* represent SEM (***$P < 0.001$, **$P < 0.01$). All micrographs are of the same magnification. *Scale bars*: 20 μM.

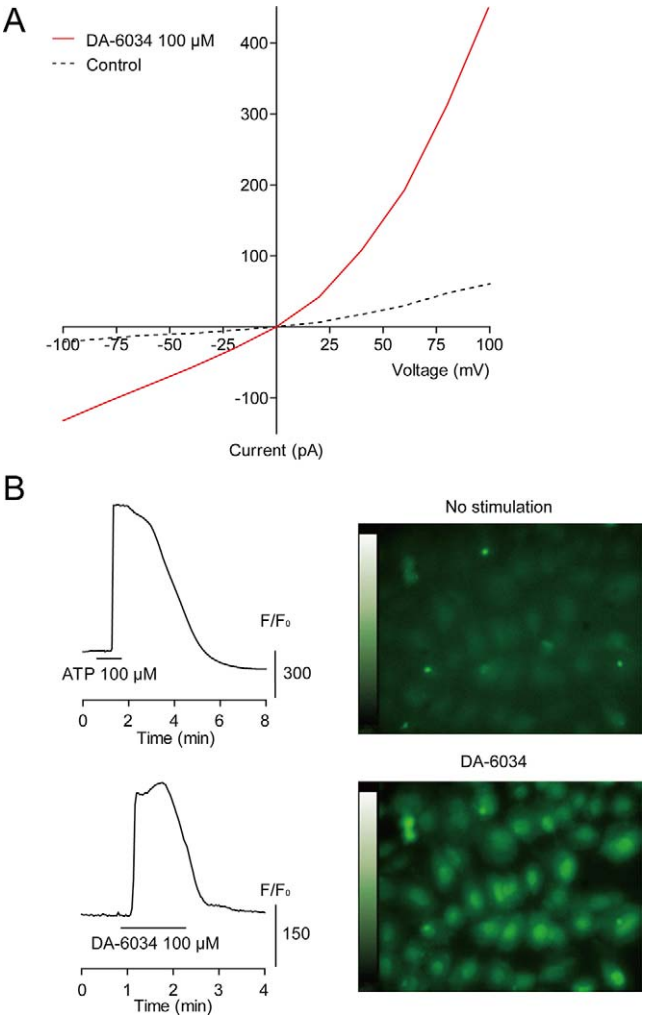

Figure 6. Changes in Cl^- currents and $[Ca^{2+}]_i$ after application of DA-6034 (100 μM) in multilayer, cultured, human conjunctival epithelial cells. Cells were incubated with 2 μM Fluo-4/AM and 0.05% Pluronic F-127 for 30 minutes in physiological salt solution at room temperature. Fluo-4/AM fluorescence was measured at 490 nm (fluorescence intensity = F/F_0). Fluorescence images were obtained at 2-second intervals. (**A**) Cl^- currents activated by DA-6034 (100 μM). (**B**) Adenosine triphosphatase or DA-6034-induced increases in $[Ca^{2+}]_i$ using Fluo-4 fluorescence dye.

Effects of DA-6034 on Cl^- Channel Activities and Ca^{2+} Mobilization

DA-6034 (100 μM) activated Ca^{2+}-dependent Cl^- current across cells, when compared with control. We observed a current-voltage curve after application of DA-6034, indicating that DA-6034 opened the CaCCs (Fig. 6A). We showed that extracellular application of high concentration of ATP evoked transient increases of $[Ca^{2+}]_i$ (Fig. 6B). Rapid transient increase of $[Ca^{2+}]_i$ was also evoked by DA-6034, followed by a sustained plateau. Increased intracellular Ca^{2+} response (as shown by fluorescence changes) was observed after application of DA-6034 (Fig. 6B).

Effects of DA-6034 on Ca^{2+} Mobilization in P2Y Antagonist Pretreated Cultured Human Conjunctival Epithelial Cells

We confirmed that suramin, PPADS, or MRS blocked the $[Ca^{2+}]_i$ increases induced by DA-6034. Adenosine triphosphatase–stimulated Ca^{2+} mobilization under each inhibitor was also reduced but not completely blocked (Figs. 7A, 7B). Ionomycin treatment led to an increased $[Ca^{2+}]_i$ in all cases.

Effects of Extracellular/Intracellular Ca^{2+} in DA-6034–Induced $[Ca^{2+}]_i$ Increase

To determine the sources of increased $[Ca^{2+}]_i$ induced by DA-6034, experiments with Ca^{2+}-free buffered solution and/or CPA were performed. DA-6034–induced $[Ca^{2+}]_i$ increases were completely inhibited by Ca^{2+}-free buffered solution. When cells were re-exposed to Ca^{2+}-containing buffered solution, DA-6034–induced $[Ca^{2+}]_i$ increases persisted, although they were reduced by 40% of the initial reaction (Fig. 8A). To investigate whether DA-6034–induced $[Ca^{2+}]_i$ increases are related to Ca^{2+} release from internal stores, cells were treated with CPA to deplete ER calcium stores, after which DA-6034 was applied. Treatment with CPA in nominally Ca^{2+}-free buffered solution showed inhibitory effects against the increases in $[Ca^{2+}]_i$ induced by DA-6034 (Fig. 8B).

Discussion

In the present study, we demonstrated a role for Ca^{2+} signaling in mucin secretion by DA-6034 in multilayer, cultured, human conjunctival epithelial cells. This signaling pathway may involve P2Y receptor activation, followed by Ca^{2+} entry from extracellular space or Ca^{2+} release from intracellular stores.

DA-6034, a synthetic derivative of eupatilin, plays a role in a variety of activities, such as wound healing, mucus secretion, cell cycle arrest, endogenous prostaglandin synthesis, nuclear factor-κB, and matrix metalloproteinase-9 inhibition.[36,38,40,43] Of these, DA-6034–induced activities, mucin secretion is important to treatment of dry eye syndrome. Intracellular Ca^{2+} plays an important role in Ca^{2+}-regulated ocular physiologic processes, such as tear and mucin secretion.[1,44] These processes are not only controlled by the release of Ca^{2+} from intracellular stores, but also by influx of Ca^{2+} into the cell through Ca^{2+} channels. It has been well established that activation of P2Y2 receptor induces an increase in $[Ca^{2+}]_i$ from release of intracellular Ca^{2+} stores via PLC/IP_3 pathways. In rabbit conjunctival epithelial cells, P2Y2 agonist activated PLC, thereby triggering an increase in IP_3 production via G proteins.[45] However, in primary, cultured, human conjunctival epithelial cells, the effects of DA-6034 on ocular mucin secretion and mechanisms of DA-6034 in stimulating P2Y receptors and ion transport have yet to be fully elucidated. In this study, we hypothesize that DA-6034 induced mucin secretion via activation of P2Y receptors and Ca^{2+} regulation.

We demonstrated that DA-6034 induced the expression of mucin genes, production of mucin protein, and increase of PAS-positive cells. Regarding the expression of P2Y receptors after treatment with DA-6034, we observed the elevation of mRNA level in short period, but there was no significant change of the protein expression level of each receptor.

In the present study, we demonstrated a useful way to quantitatively measure the mucin and fluid secretion from the multilayer, cultured, conjunctival epithelial cells using confocal microscopy. Previously, Texas Red-dextran was used to measure airway surface layers consisting of two separate layers, the mucus layer and the periciliary layer. The mucus layer has been known to comprise gel-forming mucins (muc-5b and muc-5ac) secreted by glands and goblet cells, and the periciliary layer was thought to be a low-viscosity aqueous layer.[46] Recently, Randell[47] suggested that the periciliary layer is also a gel layer composed of cell surface tethered mucins (muc-1 and muc-4) and glycolipids. Thus, we believed that it

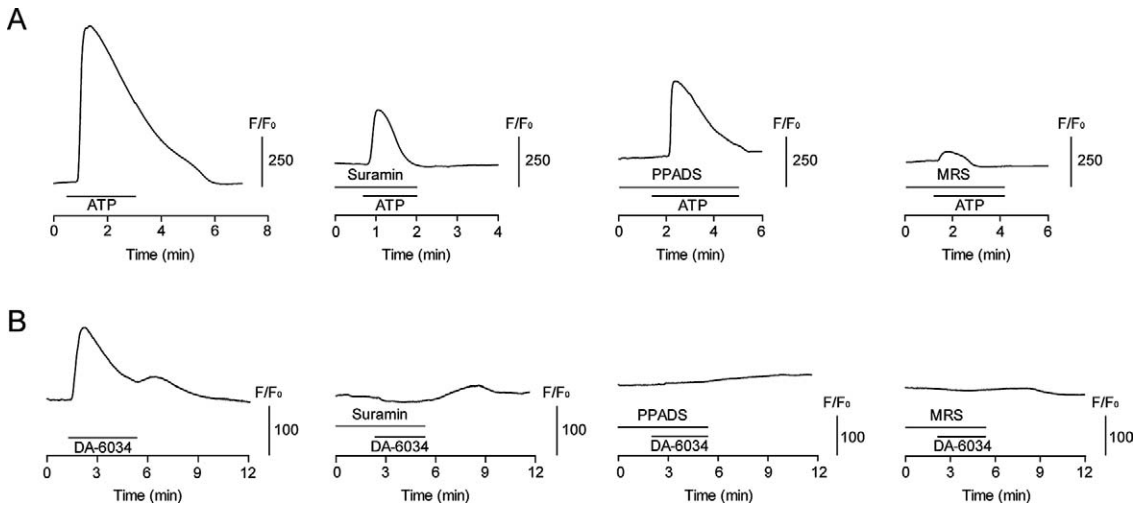

FIGURE 7. Changes in $[Ca^{2+}]_i$ after application of DA-6034 (100 μM) in P2Y antagonist pretreated multilayer, cultured, human conjunctival epithelial cells. Cells were incubated with 2 μM Fluo-4/AM and 0.05% Pluronic F-127 for 30 minutes in physiological salt solution at room temperature. Fluo-4/AM fluorescence was measured at 490 nm (fluorescence intensity = F/F_0). (**A**) Adenosine triphosphatase (**B**) DA-6034 (100 μM).

would be possible to measure the thickness of the thin film layer, consisting of secreted and membrane-tethered mucins, which are secreted by conjunctival epithelial cells. Based on the result of our study, we suggest that DA-6034 induced the formation of thin film layer composed by mucin, glycoprotein, and fluids.

DA-6034–induced mucin and fluid secretion and $[Ca^{2+}]_i$ increases were effectively inhibited by P2Y antagonists such as suramin, PPADS, and MRS. Therefore, we concluded that P2Y receptor-mediated processes via increased $[Ca^{2+}]_i$ are related to mucin secretion. These are consistent with other studies that reported that P2Y2 receptor agonists stimulate the secretion of ocular mucin and Cl^- current across cells, as well as increases in $[Ca^{2+}]_i$.[24,34,45] One study reported that P2Y2 receptor is a common denominator in regulating ion channels on the luminal and basolateral membranes of secretory and absorptive epithelium.[28] P2Y4 receptors mediate a Cl^- secretory response in mouse intestinal epithelium.[48] P2Y4 agonists could be used to treat chronic constipation by activating Cl^- channels on the apical membrane of intestinal epithelial cells and enhancing intestinal fluid secretions.[49] On the other hand, in human bronchial cell lines, CaCCs were stimulated via P2Y6 receptors that are expressed luminally and basolaterally.[50] We demonstrated that DA-6034 stimulates Cl^- secretions in multilayer, cultured, human conjunctival epithelial cells. Considering the pivotal role of Cl^- channel opening in the initiation of epithelial secretions, DA-6034 could modulate fluid movement via Cl^- currents activation, which is also supported by the fact that action of DA-6034 is involved with P2Y receptor activation.

We compared DA-6034–induced $[Ca^{2+}]_i$ increases to the effect of ATP on Ca^{2+} ion transport, showing that $[Ca^{2+}]_i$ increases induced by DA-6034 were consistent with the ATP-evoked $[Ca^{2+}]_i$ increases. We also demonstrated that DA-6034–stimulated Ca^{2+} mobilization is dependent on extracellular and intracellular Ca^{2+} based on the fact that Ca^{2+} mobilization was obliterated in Ca^{2+}-free buffered condition and after treatment with CPA in Ca^{2+}-free buffered condition. Moreover, in our recently published study, we reported that DA-6034–induced $[Ca^{2+}]_i$ increases were dependent on the Ca^{2+} entry from extracellular space and Ca^{2+} release from internal Ca^{2+} stores in mouse salivary gland epithelial cells.[51] Thus, based on these studies, both extracellular and intracellular Ca^{2+} should be emphasized in the DA-6034 regulating mucin secretion.

Our results showed that various concentrations of DA-6034 did not affect cell proliferation. Considering that DA-6034 induced the expression of PAS-positive, mucin-secreting cells, we postulated that DA-6034 may have the potential to induce

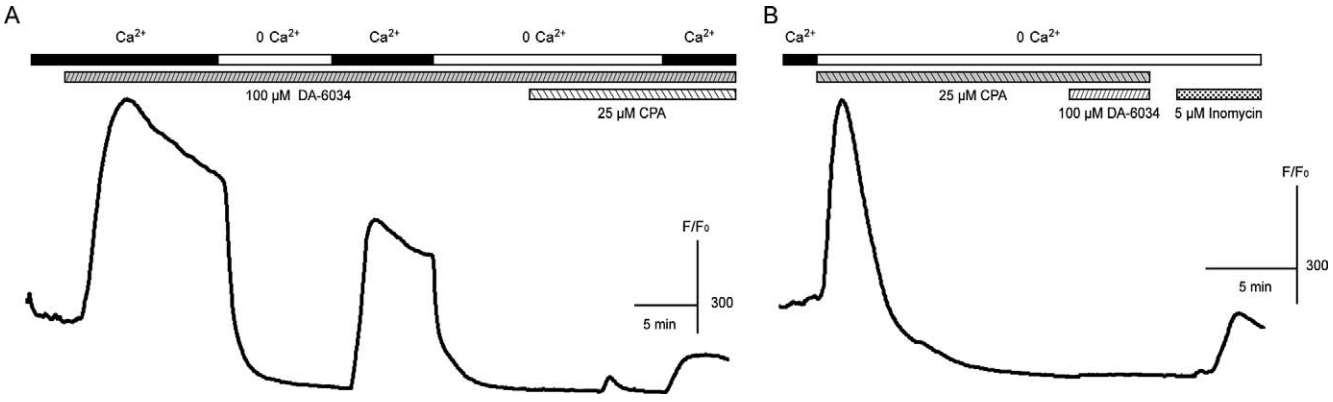

FIGURE 8. Changes in $[Ca^{2+}]_i$ after application of DA-6034 (100 μM) in CPA pretreated, multilayer, cultured, human conjunctival epithelial cells in the presence of Ca^{2+}-free or Ca^{2+}-containing buffered solution. (**A**) DA-6034–induced $[Ca^{2+}]_i$ increases were reduced in Ca^{2+}-free buffered solution. (**B**) DA-6034–induced $[Ca^{2+}]_i$ increases disappeared after depletion of internal Ca^{2+} stores by CPA.

the expression of PAS-positive cells without altering proliferation of conjunctival epithelial cells.

For our study, instead of Chang conjunctival cell line, we used primary, multilayer, cultured, human conjunctival epithelial cells. Compared with primary, cultured, human conjunctival epithelial cells, Chang conjunctival cells differed in certain features such as HeLa marker chromosome, fibroblastic phenotype, and the variant A of the enzyme glucose-6-phosphophate dehydrogenase.[52] They could interfere with the interpretation of results in vitro studies. On the contrary, primary, cultured, human conjunctival epithelial cells have been shown to exhibit the ability to differentiate goblet cells and to produce mucin-like glycoprotein, showing ultrastructural evidence of mucins.[53–55]

In summary, we investigated the action of DA-6034 in stimulating the production and secretion of ocular mucins. We confirmed that, in multilayer, cultured, human conjunctival epithelial cells, DA-6034–induced mucin secretion is associated with both P2Y receptor-activated signal transduction pathways and increases in $[Ca^{2+}]_i$ from the extracellular space and intracellular Ca^{2+} stores.

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