Physiology and Pharmacology

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

Hun Lee,¹ Eung Kweon Kim,¹,² Ji Yeon Kim,¹ Yu-Mi Yang,³ Dong Min Shin,³ Kyung Koo Kang,⁴ and Tae-im Kim¹

¹The Institute of Vision Research, Department of Ophthalmology, Yonsei University College of Medicine, Seoul, Korea
²Cornea Dystrophy Research Institute, Department of Ophthalmology, Severance Biomedical Science Institute, and Brain Korea 21 Plus Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea
³Department of Oral Biology, Yonsei University College of Dentistry, Seoul, Korea
⁴Research Laboratories, Dong-A Pharmaceutical Company, Kyunggi-do, Korea

Correspondence: Tae-im Kim, Department of Ophthalmology, Yonsei University College of Medicine, 50 Yonseiro, Seodaemun-gu, Seoul 120-752, Korea; tikim@yuhs.ac.

Submitted: January 2, 2014
Accepted: September 3, 2014


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.⁹ Both secreted and membrane-tethered mucins (MUCs) of the corneal and conjunctival epithelial cells 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 epithelium 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. Other studies have shown that MUC1 and MUC16 levels are increased in patients with Sjogren’s syndrome or in postmenopausal women with non-Sjogren dry eye, which may represent compensatory mechanisms to maintain a healthy ocular surface.

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 adenyl cyclase. The P2Y2 receptor has been believed to be the major coordinator of mucociliary clearance in the lung. Activation of this receptor increases mucin and water secretion. In addition, activation of P2Y2 receptors leads to stimulation of PLC and inositol 1,4,5-trisphosphate (IP3) release, which ultimately leads to release of Ca2+ from the ER Ca2+ store. P2Y receptors, most likely P2Y2 receptor, coordinate regulation of epithelial Na+ channels and Ca2+-activated Cl− channels (CaCCs) in many secretory epithelium including conjunctival epithelium. Several studies reported the therapeutic possibility for P2Y receptor stimulation in rabbit conjunctiva. Several authors have suggested that P2Y2 receptor agonists can regulate tear film secretion. Diquafosol promoted tear and mucin secretion via elevated [Ca2+]i. Moreover, Diquafosol elicited increases in net Cl− transport through P2Y2 receptor stimulation in rabbit conjunctiva.

The DA-6034 (7-carboxymethylxyl-3′, 4′, 5′-trimethoxyflavone monohydrate), has been known to treat gastric ulcers by changing 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) and seeded onto 24-well plates containing transwell polyester membrane inserts (Corning, Union City, CA, USA). Subcultures were seeded on 100-mm culture dishes at 2 × 105 cells/cm2. After reaching 60% to 70% confluence (~5–6 days), passage 2 cells (1 × 105 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 (5-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 × 104 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% CO2 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 RTPCR. 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). MUC4 (ab60720, 1:1000; Abcam, Cambridge, MA, USA), MUC1 (sc-15520, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), MUC3 (ab60720, 1:1000; Abcam, Cambridge, MA, USA), MUC5AC (ab24070, 1:200; Abcam), MUC16 (ab10035, 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).

**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 MgCl₂, and 1 mM Mg-ATP, adjusted to pH 7.2. The composition of external solution was as follows: 140 mM NMDG-Cl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.4. 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.

**Measurement of [Ca²⁺]**

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 Ca²⁺ dependent pathways was used. The emitted fluorescent images at 490 nm (fluorescence intensity = F/F₀) 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 Ca²⁺ mobilization, at which inhibitors were

**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).
added 15 minutes before DA-6034. Suramin (P2Y antagonist, 50 μM), PPADS (P2Y4 antagonist, 100 μM), or MRS (P2Y6 antagonist, 5 μM) was used. DA-6034 induced changes of [Ca²⁺]i after treatment with cyclopiazonic acid (CPA, 25 μM; Tocris) in the absence or presence of extracellular Ca²⁺ were measured. Ionomycin (5 μM; Tocris) was added at the end of every experiment to verify the cell viability from an increase in [Ca²⁺]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

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession Number</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
<th>Product Size, bp</th>
<th>Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>NM001101.3</td>
<td>GGACTTCGACCAAGAGATGG</td>
<td>AGCAGTGTGGCAGTACAG</td>
<td>233</td>
<td>55</td>
</tr>
<tr>
<td>Actin*</td>
<td>NM001101.3</td>
<td>GGATCCTCCACTCTGAACTGA</td>
<td>AGGTGTGGTCGCAATTTTTC</td>
<td>65</td>
<td>60</td>
</tr>
<tr>
<td>MUC1</td>
<td>NM001204286.1</td>
<td>TCTCACCTCTCCCTAACATAC</td>
<td>GAAATGGGCAATCATACCTCA</td>
<td>368</td>
<td>55</td>
</tr>
<tr>
<td>MUC1*</td>
<td>Argueso et al.</td>
<td>GTGCCCTCATACGATCCCG</td>
<td>GACGTGGCCCTACAGTTTGG</td>
<td>123</td>
<td>60</td>
</tr>
<tr>
<td>MUC4</td>
<td>NM0018406.6</td>
<td>TTCTAAGAACCACCAAGCTCAGACG</td>
<td>GAGACACACCTGGAGAGAATGAGC</td>
<td>466</td>
<td>62</td>
</tr>
<tr>
<td>MUC4*</td>
<td>Argueso et al.</td>
<td>GCCCAAGCTACATGCTGACTCA</td>
<td>ATGGTGCCGTTGAATTTGTG</td>
<td>102</td>
<td>60</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>GenBank A058803</td>
<td>TCCACCATATAACCGCACAAGA</td>
<td>TGGACCCGACAGTCTAGTCAAC</td>
<td>103</td>
<td>62</td>
</tr>
<tr>
<td>MUC16</td>
<td>NM026490.2</td>
<td>GCCCTACTCTTAACGTACTACAATGAA</td>
<td>GATCAGTGGGCAACCAAGTACAG</td>
<td>293</td>
<td>62</td>
</tr>
<tr>
<td>MUC16*</td>
<td>NM026490.2</td>
<td>GCCCTACTCTTAACGTACCTAGTAAGA</td>
<td>GATACCCATGGCTTTTGGT</td>
<td>114</td>
<td>60</td>
</tr>
<tr>
<td>P2Y1</td>
<td>NM002563.3</td>
<td>TGGTAGTGATACCCTCTAACATTCCC</td>
<td>ATCCGTAACAGCCAGATACCGA</td>
<td>259</td>
<td>58</td>
</tr>
<tr>
<td>P2Y2</td>
<td>NM176072.2</td>
<td>GGACATGTCTGAGCTAGGAG</td>
<td>AGTGCATCAGACACACCGCC</td>
<td>279</td>
<td>58</td>
</tr>
<tr>
<td>P2Y4</td>
<td>NM002565.3</td>
<td>CCACCTGCGCTTGTCAGACACC</td>
<td>GATGTGACCGAGGGCACGC</td>
<td>424</td>
<td>64</td>
</tr>
<tr>
<td>P2Y6</td>
<td>NM176798.2</td>
<td>CGTCTCCTCCTCTATGCGCAACC</td>
<td>CATCCTGCGGCCACAGCGCC</td>
<td>365</td>
<td>63</td>
</tr>
</tbody>
</table>

* Real-time PCR.
**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 < 0.01, ***P < 0.001, *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.
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 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933256/) 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.

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933256/) 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.
DA-6034 and the P2Y Receptor

FIGURE 6. Changes in Cl− currents and [Ca2+]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/F₀). 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 [Ca2+]i after application of DA-6034 using Fluo-4 fluorescence dye.

Effects of DA-6034 on Cl− Channel Activities and Ca2+ Mobilization

DA-6034 (100 μM) activated Ca2+-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 [Ca2+]i, (Fig. 6B). Rapid transient increase of [Ca2+]i was also evoked by DA-6034, followed by a sustained plateau. Increased intracellular Ca2+ response (as shown by fluorescence changes) was observed after application of DA-6034 (Fig. 6B).

Effects of DA-6034 on Ca2+ Mobilization in P2Y Antagonist Pretreated Cultured Human Conjunctival Epithelial Cells

We confirmed that suramin, PPADS, or MRS blocked the [Ca2+]i increases induced by DA-6034. Adenosine triphosphatase-stimulated Ca2+ mobilization under each inhibitor was also reduced but not completely blocked (Figs. 7A, 7B). Ionomycin treatment led to an increased [Ca2+]i, in all cases.

Effects of Extracellular/Intracellular Ca2+ in DA-6034–Induced [Ca2+]i Increase

To determine the sources of increased [Ca2+]i, induced by DA-6034, experiments with Ca2+-free buffered solution and/or CPA were performed. DA-6034–induced [Ca2+]i increases were completely inhibited by Ca2+-free buffered solution. When cells were re-exposed to Ca2+-containing buffered solution, DA-6034–induced [Ca2+]i increases persisted, although they were reduced by 40% of the initial reaction (Fig. 8A). To investigate whether DA-6034–induced [Ca2+]i increases are related to Ca2+ 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 Ca2+-free buffered solution showed inhibitory effects against the increases in [Ca2+]i, induced by DA-6034 (Fig. 8B).

DISCUSSION

In the present study, we demonstrated a role for Ca2+ signaling in mucin secretion by DA-6034 in multilayer, cultured, human conjunctival epithelial cells. This signaling pathway may involve P2Y receptor activation, followed by Ca2+ entry from extracellular space or Ca2+ 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-xB, and matrix metalloproteinase-9 inhibition.36,38,40,45 Of these, DA-6034–induced activities, mucin secretion is important to treatment of dry eye syndrome. Intracellular Ca2+ plays an important role in Ca2+-regulated ocular physiologic processes, such as tear and mucin secretion.1,44 These processes are not only controlled by the release of Ca2+ from intracellular stores, but also by influx of Ca2+ into the cell through Ca2+ channels. It has been well established that activation of P2Y2 receptor induces an increase in [Ca2+]i, from release of intracellular Ca2+ stores via PLC/IP3 pathways. In rabbit conjunctival epithelial cells, P2Y2 agonist activated PLC, thereby triggering an increase in IP3 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 Ca2+ 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, Randell47 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

[Image 58x351 to 297x729]
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 \([\text{Ca}^{2+}]_i\) increases were effectively inhibited by P2Y antagonists such as suramin, PPADS, and MRS. Therefore, we concluded that P2Y receptor-mediated processes via increased \([\text{Ca}^{2+}]_i\) 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\(^-\)/C\(_0\) current across cells, as well as increases in \([\text{Ca}^{2+}]_i\).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\(^-\)/C\(_0\) 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 \([\text{Ca}^{2+}]_i\) increases to the effect of ATP on \([\text{Ca}^{2+}]_i\) increases by ATP-evoked \([\text{Ca}^{2+}]_i\) increases. We also demonstrated that DA-6034–stimulated \([\text{Ca}^{2+}]_i\) mobilization is dependent on extracellular and intracellular \([\text{Ca}^{2+}]_i\) based on the fact that \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) 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
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-phosphate dehydrogenase. They could interfere with the interpretation of results in vitro studies. On the contrary, intracellular Ca\(^{2+}\) and increases in [Ca\(^{2+}\)] from the extracellular space and intracellular Ca\(^{2+}\) stores.

**Acknowledgments**

The authors thank Dong-Su Jang for his excellent support with medical illustration, and Hye Jeong Hwang and Jong Min Lee for their technical support in performing the experiments.

Supported by the National Research Foundation of Korea (NRF; Daejeon, Korea) grant funded by the Korea government (MEST No. 2013R1A1A02058907), and by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (2012R1A2A1A01003487).

Disclosure: H. Lee, None; E.K. Kim, None; J.Y. Kim, None; Y.-M. Yang, None; D.M. Shin, None; K.K. Kang, None; T.-I. Kim, None.

**References**

26. Werry TD, Wilkinson GF, Willars GB. Cross talk between P2Y2 nucleotide receptors and CXC chemokine receptor 2 resulting in enhanced Ca\(^{2+}\) signaling involves enhancement of phospholipase C activity and is enabled by incremental Ca\(^{2+}\) release in human embryonic kidney cells. *J Pharmacol Exp Ther.* 2003;307:661–669.
29. Murakami T, Fujihara T, Horibe Y, Nakamura M. Diquafosol elicits increases in net Cl\(^{-}\) transport through P2Y2 receptor.


