

## DA-6034 Induces $[Ca^{2+}]_i$ Increase in Epithelial Cells

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DA-6034, a eupatilin derivative of flavonoid, has shown potent effects on the protection of gastric mucosa and induced the increases in fluid and glycoprotein secretion in human and rat corneal and conjunctival cells, suggesting that it might be considered as a drug for the treatment of dry eye. However, whether DA-6034 induces  $Ca^{2+}$  signaling and its underlying mechanism in epithelial cells are not known. In the present study, we investigated the mechanism for actions of DA-6034 in  $Ca^{2+}$  signaling pathways of the epithelial cells (conjunctival and corneal cells) from human donor eyes and mouse salivary gland epithelial cells. DA-6034 activated  $Ca^{2+}$ -activated  $Cl^-$  channels (CaCCs) and increased intracellular calcium concentrations ( $[Ca^{2+}]_i$ ) in primary cultured human conjunctival cells. DA-6034 also increased  $[Ca^{2+}]_i$  in mouse salivary gland cells and human corneal epithelial cells.  $[Ca^{2+}]_i$  increase of DA-6034 was dependent on the  $Ca^{2+}$  entry from extracellular and  $Ca^{2+}$  release from internal  $Ca^{2+}$  stores. Interestingly, these effects of DA-6034 were related to ryanodine receptors (RyRs) but not phospholipase C/inositol 1,4,5-triphosphate ( $IP_3$ ) pathway and lysosomal  $Ca^{2+}$  stores. These results suggest that DA-6034 induces  $Ca^{2+}$  signaling via extracellular  $Ca^{2+}$  entry and RyRs-sensitive  $Ca^{2+}$  release from internal  $Ca^{2+}$  stores in epithelial cells.

**Key Words:** Calcium signaling, DA-6034, Epithelial cells, Eupatilin

### INTRODUCTION

Calcium ( $Ca^{2+}$ ) is the universal and pluripotent cellular signaling molecules that control widely cellular processes. Cytosolic  $Ca^{2+}$  signals are essential for the control of fluid and enzyme secretion from exocrine glands.  $Ca^{2+}$  signaling of these cells are initiated by  $Ca^{2+}$  release from intracellular stores, inositol 1,4,5-triphosphate ( $IP_3$ ) receptors ( $IP_3$ Rs) and/or ryanodine receptors (RyRs) on the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) and the mitochondria. After rapid global  $Ca^{2+}$  signals, the clearance of  $Ca^{2+}$  from the cytoplasm is accomplished by activities of  $Ca^{2+}$ -ATPases localized to both the ER (SERCAs) and plasma membrane (PMCA) and then it is contributed substantially to the deactivation of chloride currents by SERCA [1].

The secretion can be significantly enhanced when both

$Ca^{2+}$  and cAMP signaling systems are activated, although the activation of  $Ca^{2+}$ -dependent ion channels is the primary mechanism. The secretion from salivary glands is typically a watery fluid containing electrolytes and complex mixture of proteins, however mucous gland secretions are often quite viscous because of the discharge of large molecular weight mucins [2]. Tears are also composed of mucins, lipids, proteins, electrolytes and various other metabolites which are involved in various functions like ocular surface wound [3]. The secretion dysfunctions induce the oral pain, increased dental caries, and infections by opportunistic microorganisms in salivary glands, parotid and submandibular glands (SMG), and cause blurred and fluctuating vision and discomfort in lacrimal gland. These glands dysfunction are most frequently observed in diseases such as cystic fibrosis, or Sjögren's syndrome [2,4]. Patients with Sjögren's syndrome suffer from a severe deficit in fluid production by salivary glands and other exocrine organs.

Flavonoids are polyphenols found in a wide variety of medical plants and are known to have anti-inflammatory activities and selective inhibitors of lipoxygenase activity [5-7]. DA-6034 (7-carboxymethoxy-3',4',5-trimethoxy flavones monohydrate) is a synthetic derivative of eupatilin, a flavonoid derivative. Eupatilin is a main component of the extract of *Artemisiae* species, a Korean folk medicine used

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**ABBREVIATIONS:** CaCCs,  $Ca^{2+}$ -activated  $Cl^-$  channels;  $[Ca^{2+}]_i$ , intracellular calcium concentrations; PLC, phospholipase C;  $IP_3$ , inositol 1,4,5-triphosphate; RyRs, ryanodine receptors; SMG, submandibular glands; CEC, human corneal epithelial cell line.

in the treatment of chronic diarrhea. Eupatilin has also the pharmacological activities including the induction of apoptosis in both human gastric cancer cells and promyelocytic leukemia cells, and cell cycle arrest in *ras*-transformed human mammary epithelial cells [8,9]. DA-6034 has anti-inflammatory action through NF- $\kappa$ B down-regulation in gastric epithelial cells [10-12]. DA-6034 prevents gastric mucosal injury through increases of endogenous prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis and gastric mucus secretion [8]. Recently, several reports showed the therapeutic possibility for effects of DA-6034 such as increases of mucin and lacrimal secretions, and down-regulation of mitogen-activated protein kinase (MAPK) signaling in the dry eye model [13-15]. It was suggested that DA-6034 can relate the mechanisms of the Ca<sup>2+</sup>-activated fluid secretion in epithelial cells. However, whether DA-6034 induces Ca<sup>2+</sup> signaling and its underlying mechanism in epithelial cells are not known. In this study, we aimed to investigate the physiological role of DA-6034 in Ca<sup>2+</sup> signaling of various epithelial cells.

## METHODS

### Preparation of exocrine gland acinar cells

ICR strain mice (6~8 weeks) were sacrificed by cervical dislocation under CO<sub>2</sub> anesthesia. Cells were prepared from the submandibular glands, parotids, and pancreases of ICR mice by limited collagenase digestion as previously described [16]. Following isolation, the acinar cells were suspended in an extracellular physiological salt solution (PSS), the composition of which was follows (in mM): 140 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 10 HEPES, 10 glucose, adjusted to pH 7.4 and 310 mOsm.

### Cell culture

During preparation for cornea buttons, human conjunctival tissue was saved and donated by the eye bank. The entire conjunctiva was dissected around the 2-mm lateral to limbus of the cornea. Conjunctival cells were isolated using the following method. First, conjunctiva tissue was incubated in phosphate-buffered saline with 1.4 U dispase (Sigma, St. Louis, MO, USA) for 1 hour in a 37°C incubator. Incubated tissues were scraped and washed by media. Isolated conjunctival cells were cultured with keratinocyte growth medium (KGM; Lonza Walkersville, Inc., Walkersville, MD, USA). For multilayer cell cultures, isolated conjunctival cells were cultured on 6-well plates containing Transwell permeable supports with KGM first. After the cells covered the bottom of Transwell plates, media was removed and replaced with differentiation media [1 : 1, KGM + 0.12% bovine serum albumin (BSA), 10 nM retinoic acid (RA): low glucose Dulbecco's Minimal Essential Medium (DMEM)]. During cell differentiation, medium was replaced every day. Human corneal epithelial cell line (CEC) was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37°C in humidified 5% CO<sub>2</sub> in air. CEC cells were grown in bronchial epithelial cell growth medium (BEGM; Lonza Walkersville, Inc., Walkersville, MD, USA). BEGM consists of bronchial epithelial basal medium (BEBM) supplemented with recombinant human epidermal growth factor (rhEGF), bovine pituitary extract (BPE), insulin, hydrocortisone, trans-

ferrin, tri-iodothyronine, epinephrine, pituitary extract (PE), GA-1000, and retinoic acid. Cells were plated onto glass coverslips for 4 days or until 75% confluence before calcium imaging.

### Electrophysiology

The method of perforated patch-clamp has been modified previously [17,18]. Briefly, Whole-cell voltage-clamp recordings were made using the perforated patch-clamp method at room temperature (22~25°C). Currents were recorded using a MultiClamp 700B amplifier (Axon Instruments, Union City, CA, USA), subsequently digitized with a sampling rate of 10 kHz, and analyzed using pCLAMP10 software (Axon Instruments). The pipette resistance varied between 3~5 M $\Omega$ . Whole-cell currents were elicited by voltage ramps from -100 mV to +100 mV (800-ms duration) applied every 10 s from a holding potential of -40 mV. Pipettes for recordings of Cl<sup>-</sup> currents were filled with an internal solution containing (in mM): 140 mM N-methyl-D-glucamine (NMDG)-Cl, 0.5 mM EGTA, 10 mM HEPES, 1 mM MgCl<sub>2</sub> and 1 mM Mg-ATP, adjusted to pH 7.2 [18]. Nystatin was diluted to a final concentration of 250  $\mu$ g/ml in the internal solution. The external solution containing (in mM): 140 mM NMDG-Cl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES and 10 mM glucose, adjusted to pH 7.4.

### [Ca<sup>2+</sup>]<sub>i</sub> measurement

The method of [Ca<sup>2+</sup>]<sub>i</sub> imaging has been modified previously [19]. Briefly, cells were incubated with 2  $\mu$ M Fluo-4/AM or Fura-2/AM and 0.05% Pluronic F-127 for 30 min in PSS at room temperature. Fluo-4/AM fluorescence was measured at 490 nm (fluorescence intensity=F/F<sub>0</sub>) using a Molecular Devices (Downingtown, PA, USA) imaging system. The emitted fluorescence was monitored with a charge-coupled device camera (Photometrics, Tucson, AZ, USA) attached to an inverted microscope. Fura-2 fluorescence intensity also was measured using excitation wave lengths of 340 and 380 nm, and emitted fluorescence 510 nm (Ratio=F<sub>340</sub>/F<sub>380</sub>). Fluorescence images were obtained at 2-s intervals. All data were analyzed using MetaFlour software (Molecular Devices).

### Reagents

Fluo-4/AM and fura-2/AM were purchased from Invitrogen Molecular Probes (Eugene, OR, USA). SFK96365 hydrochloride, ionomycin, cyclopiazonic acid (CPA), U73122, U73343, ryanodine, ruthenium red, and 2-aminoethoxydiphenyl borate (2APB) were from Tocris Bioscience (Bristol, UK). Bafilomycin A1 was obtained from Alexis Biochemicals (San Diego, CA, USA). Collagenase P was purchased from Roche (Indianapolis, IN, USA). All other chemicals were purchased from Sigma. Stock solutions of all drugs were made in distilled water, except, 2APB (made in ethanol), and DA-6034, fluo-4/AM, fura-2/AM, ionomycin, CPA, SKF96365, U73122, U73343, and ryanodine (made in DMSO).

### Statistics

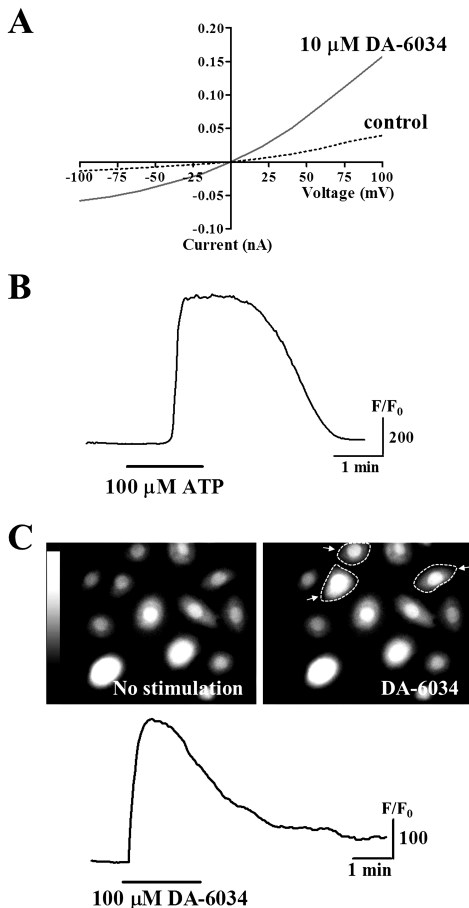
All data were expressed as the mean $\pm$ S.E from at least 3 independent experiments. Statistical significance was determined by using a paired or unpaired Student's *t*-test.

Statistical significance was set at  $p < 0.05$  level.

## RESULTS

### Effects of DA-6034 on $Cl^-$ currents and $[Ca^{2+}]_i$ in primary cultured human conjunctival cells

In the present study, we examined the effects of DA-6034 on  $Ca^{2+}$  signaling in primary cultured human conjunctival epithelial cells. We observed that DA-6034 (10  $\mu$ M) stimulation activated  $Cl^-$  transport across the  $Ca^{2+}$ -activated  $Cl^-$  channels (CaCCs) in conjunctival epithelial cells, when compared to control (Fig. 1A). Extracellular application of ATP (100  $\mu$ M) evoked transient increases of  $[Ca^{2+}]_i$  (Fig. 1B). Likewise, 100  $\mu$ M DA-6034 induced transient increases of  $[Ca^{2+}]_i$  in primary cultured human conjunctival epithelial cells (Fig. 1C). These results suggest that DA-6034 activate the CaCCs and induced transient  $[Ca^{2+}]_i$  increases in primary cultured human conjunctival epithelial cells.



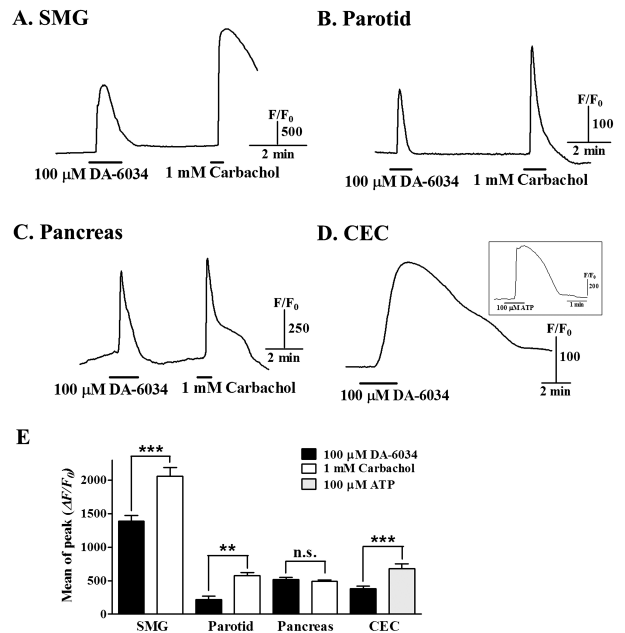
**Fig. 1.**  $Cl^-$  currents and  $[Ca^{2+}]_i$  after application of DA-6034 in primary cultured human conjunctival epithelial cells. (A)  $Cl^-$  currents were activated by 10  $\mu$ M DA-6034 in primary cultured human conjunctival epithelial cells. (B) Treatment with 100  $\mu$ M ATP and (C) 100  $\mu$ M DA-6034 induced increases of  $[Ca^{2+}]_i$  in human conjunctival cells (dotted cells in figure) using Fluo-4 fluorescence dye.

### Increases of $[Ca^{2+}]_i$ by DA-6034 in epithelial cells

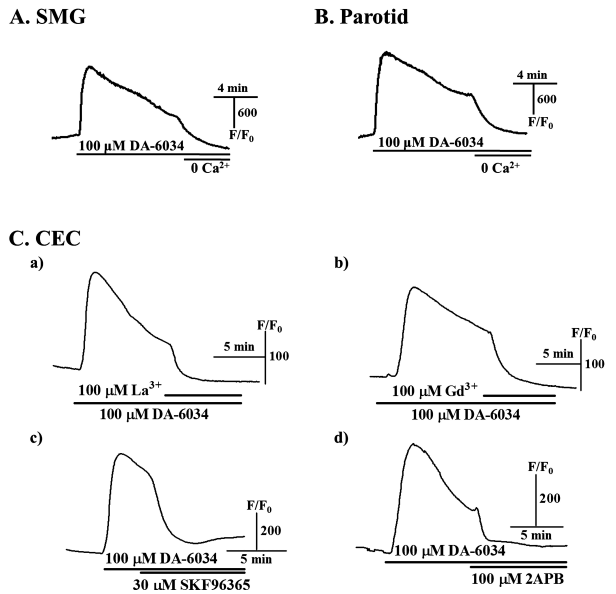
To confirm similar effects of DA-6034 on  $[Ca^{2+}]_i$  in various epithelial cells, we performed experiments for  $[Ca^{2+}]_i$  increases by DA-6034 in salivary gland epithelial cells and human corneal epithelial cell line (CEC). Application of 100  $\mu$ M DA-6034 induced an increase of  $[Ca^{2+}]_i$  in salivary gland (SMG and parotid) and pancreatic acinar cells (Fig. 2A~C). DA-6034 induced  $[Ca^{2+}]_i$  increase was also observed in CEC cells (Fig. 2D). The difference of DA-6034 induced  $[Ca^{2+}]_i$  increases was depending on cell types (Fig. 2E). These results suggest that DA-6034 induce the  $[Ca^{2+}]_i$  increases in various epithelial cells.

### Extracellular $Ca^{2+}$ entry and DA-6034 induced $[Ca^{2+}]_i$ increase

To determine sources of  $[Ca^{2+}]_i$  increased by DA-6034, the experiments with  $Ca^{2+}$ -free environment or extracellular  $Ca^{2+}$  entry blockers were performed. First, DA-6034 induced  $[Ca^{2+}]_i$  increase was completely inhibited by  $Ca^{2+}$ -free buffered solution in SMG and parotid acinar cells (Fig. 3A and B). We also observed these similar effects in CEC cells (data not shown). The  $[Ca^{2+}]_i$  increases by DA-6034 was also decreased by treatments with 100  $\mu$ M  $La^{3+}$  and  $Gd^{3+}$ , non-specific voltage-dependent  $Ca^{2+}$  channel blockers (Fig. 3Ca and 3Cb), and 30  $\mu$ M SKF96365, receptor/ store-operated  $Ca^{2+}$  channel blocker, and 100  $\mu$ M 2APB, store-operated



**Fig. 2.** Effects of  $[Ca^{2+}]_i$  increases by treatments of DA-6034 in cells. (A~C) Treatment of 100  $\mu$ M DA-6034 induced increases of  $[Ca^{2+}]_i$  and also showed  $[Ca^{2+}]_i$  increases by application of 1 mM carbachol in salivary gland (SMG and parotid) and pancreatic acinar cells using Fluo-4 fluorescence dye. (D) CEC cells were also increased  $[Ca^{2+}]_i$  by treatments DA-6034 and 100  $\mu$ M ATP (square box). (E) Bar graph presented the difference of  $[Ca^{2+}]_i$  increases by DA-6034 and carbachol/ATP in various cells. Data were expressed as the mean  $\pm$  SEM. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with carbachol or ATP. n.s., not significant.

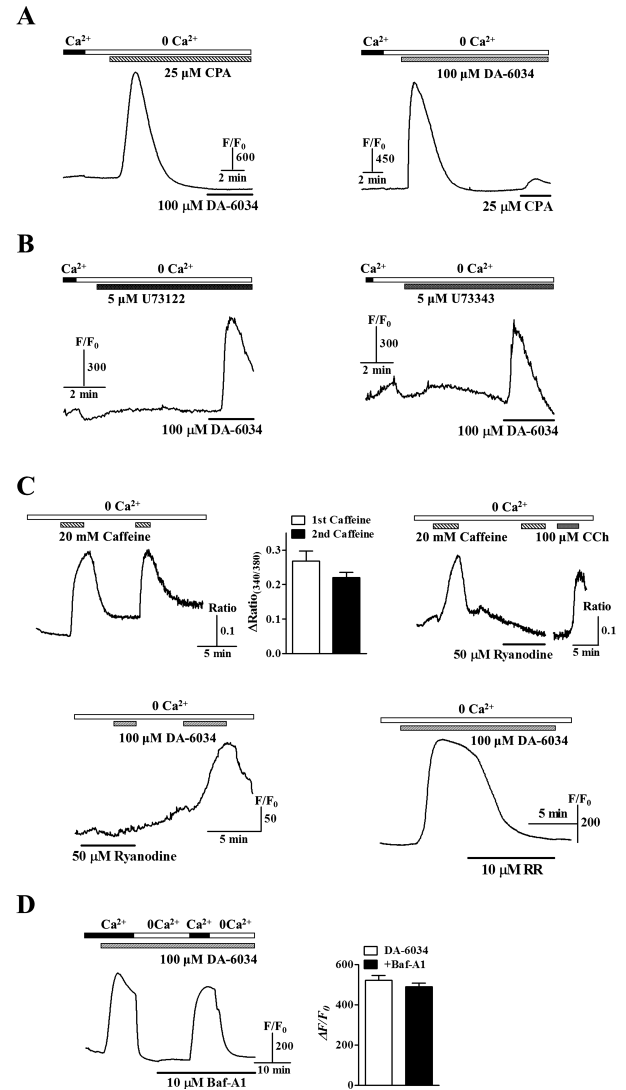


**Fig. 3.** Dependence on extracellular  $\text{Ca}^{2+}$  of DA-6034 induced  $[\text{Ca}^{2+}]_i$  increases. (A, B) The effects of  $[\text{Ca}^{2+}]_i$  increases by DA-6034 were inhibited by  $\text{Ca}^{2+}$ -free solution in SMG and parotid acinar cells. (C) The effects of  $[\text{Ca}^{2+}]_i$  increases by DA-6034 were inhibited by 100  $\mu\text{M}$   $\text{La}^{3+}$  (a) and 100  $\mu\text{M}$   $\text{Gd}^{3+}$  (b), which block a wide range of  $\text{Ca}^{2+}$ -permeable channels, in CEC cells. 30  $\mu\text{M}$  SKF96365 (c) and 100  $\mu\text{M}$  2APB (d) also blocked DA-6034 induced  $[\text{Ca}^{2+}]_i$  increases through the inhibition of the receptor/store-mediated  $\text{Ca}^{2+}$  influx.

ated  $\text{Ca}^{2+}$  entry blocker in CEC cells (Fig. 3Cc and 3Cd). These results suggest that the DA-6034 induced  $[\text{Ca}^{2+}]_i$  increase is related to  $\text{Ca}^{2+}$  entry from the extracellular space.

#### The role of intracellular $\text{Ca}^{2+}$ in DA-6034 induced $[\text{Ca}^{2+}]_i$ increase

In Fig. 3,  $[\text{Ca}^{2+}]_i$  increases by DA-6034 also showed the possibility which DA-6034 induced  $[\text{Ca}^{2+}]_i$  increases are related to the intracellular  $\text{Ca}^{2+}$  stores. To investigate whether DA-6034-induced  $[\text{Ca}^{2+}]_i$  increases were related to  $\text{Ca}^{2+}$  release from internal stores, we used 25  $\mu\text{M}$  CPA to deplete the ER and then applied DA-6034. In SMG acinar cells, treatment with CPA in nominally  $\text{Ca}^{2+}$ -free buffered solution showed inhibitory effects for the  $[\text{Ca}^{2+}]_i$  increase by DA-6034 (Fig. 4A, left panel) and also disappeared the effect of ER  $\text{Ca}^{2+}$  depletion by CPA in order changing (Fig. 4A, right panel), suggesting that DA-6034 induced  $[\text{Ca}^{2+}]_i$  increases are related to  $\text{Ca}^{2+}$  release from the ER. Mostly ER  $\text{Ca}^{2+}$  release can be mediated through the  $\text{IP}_3\text{Rs}$  [20]. Therefore, we examined the effects of DA-6034 in the treatment of PLC inhibitor U73122 whether DA-6034 induced  $[\text{Ca}^{2+}]_i$  increase is evoked by activation of PLC/ $\text{IP}_3$  pathway. The  $[\text{Ca}^{2+}]_i$  increase by DA-6034 still remained by the treatment of PLC inhibitor U73122 and U73343 (an inactive analog of U73122) in SMG acinar cells (Fig. 4B). These results suggest that effects of DA-6034 can be mediated by other intracellular  $\text{Ca}^{2+}$  stores, such as RyRs and lysosomal  $\text{Ca}^{2+}$  pools. Then, we confirmed the actions of RyRs using caffeine (an activator of RyRs), ryanodine (an inhibitor of RyRs), and ruthenium red (RR, an inhibitor of RyRs) (Fig. 4C, upper panels). Therefore, we showed inhibitory effects



**Fig. 4.** Relationship with internal  $\text{Ca}^{2+}$  stores and DA-6034. (A) DA-6034 induced  $[\text{Ca}^{2+}]_i$  increases were disappeared after ER depletion by CPA. (B) However, DA-6034 induced  $\text{Ca}^{2+}$  influx not inhibited by the PLC inhibitor, U73122, and its close analogue, U73343. (C) Caffeine induced  $[\text{Ca}^{2+}]_i$  increases were repeated by the second application of caffeine (upper left panel) and were blocked by ryanodine, which was an inhibitor of ryanodine receptors (upper right panel). DA-6034 induced  $[\text{Ca}^{2+}]_i$  increases also inhibited by ryanodine (bottom left panel) and RR (bottom right panel). (D) DA-6034 induced  $[\text{Ca}^{2+}]_i$  increases were not inhibited by Baf-A1, which was a specific inhibitor of vacuolar-type  $\text{H}^+$ -ATPase. Data were expressed as the mean  $\pm$  SEM.

of DA-6034 induced  $[\text{Ca}^{2+}]_i$  increase by ryanodine and RR in SMG acinar cells (Fig. 4C, bottom panels). However, the  $[\text{Ca}^{2+}]_i$  increase by DA-6034 still remained by the treatment of bafilomycin A1 (Baf-A1, an inhibitor of vacuolar-type  $\text{H}^+$ -ATPase) (Fig. 4D). Taken together, these results suggest that DA-6034 induced  $[\text{Ca}^{2+}]_i$  increase is dependent on RyRs but not the PLC/ $\text{IP}_3$  pathway and lysosomal  $\text{Ca}^{2+}$  pools.

## DISCUSSION

In this study, we demonstrated for the first time the  $[Ca^{2+}]_i$  increases by DA-6034 through the regulation of extracellular  $Ca^{2+}$  entry and intracellular  $Ca^{2+}$  release in human conjunctival/corneal epithelial cells and mouse salivary gland epithelial cells. These effects could be the result of the released  $Ca^{2+}$  accelerating activity during the stimulated fluid secretion by DA-6034, which is the previous reported physiological function of the DA-6034 such as increases of mucus content and endogenous PGE<sub>2</sub> synthesis in gastrointestinal tracts and tear secretion and mucin production in conjunctival goblet cells [8,13,14]. These results suggest that DA-6034 is a main regulator to modulate secretory activities in exocrine gland cells and also indicates possibilities to a DA-6034-mediated modulation on intracellular  $Ca^{2+}$  signaling in fluid secretion of exocrine gland [21]. Usually, the mucin and fluid secretion are stimulated predominantly by  $Ca^{2+}$  dependent pathways that are activated by extracellular ATP and other stimulators. Then, released peptides, such as secretin and VIP etc., stimulate adenyl cyclase activities via G-protein coupled receptors, and subsequently increases of cAMP, PKA, and PKC induce secretions of mucin, fluid, and anion [2,20,21]. However, potential redox state of epithelial cells leads to oxidative stress in the bacteria infection or cystic fibrosis. The distribution of unbalanced redox can evoke inflammatory signaling pathway and apoptosis, such as NF- $\kappa$ B activation and inflammation [22,23]. DA-6034 suppressed iNOS induction, NF- $\kappa$ B activation, p65 nuclear translocation, and restored I $\kappa$ B $\alpha$  level in the cytoplasm and dissociates the IKK- $\gamma$ -Hsp90 complex in gastric cancer models [11,12]. In addition, DA-6034 attenuated JNK and p38 MAPK and also inhibited NF- $\kappa$ B activation in dry eye model cells [15]. These results suggest that DA-6034 has anti-inflammatory and anti-apoptotic effects in epithelial cells. This is supported by previous reports showing that DA-6034 has increased mucin secretion and endogenous PGE<sub>2</sub> synthesis which prevented gastric mucosal injury and dry eye [8,13,14].

We also examined the sources of  $[Ca^{2+}]_i$  increased by DA-6034. Our results demonstrated that the effects of DA-6034 could be dependent on extracellular and intracellular  $Ca^{2+}$  levels, however this  $[Ca^{2+}]_i$  mobilization was included the RyRs but not the PLC/IP<sub>3</sub> pathway. The  $[Ca^{2+}]_i$  mobilization of DA-6034 through the RyRs is a novel finding in our results.  $Ca^{2+}$  mobilization is regarded as a key element in signal transduction and secretory process in exocrine cells [2,21]. In common with acinar cells from exocrine tissue, IP<sub>3</sub>R<sub>s</sub> are mainly expressed the apical region and exhibit the initiation and rapid global  $Ca^{2+}$  signals. Ryanodine receptors (RyRs) also intracellular  $Ca^{2+}$  release channels that belong to a family related to IP<sub>3</sub>R<sub>s</sub>. RyRs are predominantly localized basal regions. They are gated by elevations in cytoplasmic  $Ca^{2+}$  in a process termed calcium-induced calcium release (CICR) and contribute to the mechanisms of regenerative  $Ca^{2+}$  waves in a various cells. Several studies reported that it could be cAMP-induced  $Ca^{2+}$  release without elevation of IP<sub>3</sub> through the activation of RyRs in rat parotid acinar cells or microsomal vesicles [2]. Several reports showed that RyRs (RyR type 1 and type 3)-mediated CICR regulated the corticotropin-releasing factor-induced adrenocorticotropin secretion in mouse corticotroph cell line [24] and puerarin (a traditional Chinese medicine) facilitated CICR via RyRs and cAMP/PKA signaling pathway in

rat hippocampal neurons [25]. CaCCs are activated by both  $Ca^{2+}$  influx and CICR from internal stores. Treatments of caffeine activated CaCCs in DRG neurons [26]. In our results, DA-6034 activated CICR through RyRs and CaCCs in epithelial cells. Therefore, it suggests that activated  $[Ca^{2+}]_i$  increases by DA-6034 can mediate related responses between RyRs and CaCCs.

Taken together, DA-6034 could increase intracellular  $Ca^{2+}$  through RyRs and improve secretory process such as tear, mucin and salivary fluid in mouse salivary gland cells and human epithelial cells from eyes. Although further studies of mechanisms are necessary, these findings already suggest that DA-6034 may be a potential therapeutic agent for the dry eye and mouth diseases.

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