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

Yu-Mi Yang1*, Soohong Park2*, HyeWon Ji1, Tae-im Kim2, Eung Kweon Kim3, Kyung Koo Kang3, and Dong Min Shin1

1Department of Oral Biology, BK21 PLUS Project, Yonsei University College of Dentistry, Seoul 120-752, 2Institute of Vision Research, Department of Ophthalmology, Yonsei University College of Medicine, Seoul 120-752, 3Research Institutes, Dong-A Pharmaceutical Company, Yongin 446-905, Korea

INTRODUCTION

Calcium ($\text{Ca}^{2+}$) is the universal and pluripotent cellular signaling molecules that control widely cellular processes. Cytosolic $\text{Ca}^{2+}$ signals are essential for the control of fluid and enzyme secretion from exocrine glands. $\text{Ca}^{2+}$ signaling of these cells are initiated by $\text{Ca}^{2+}$ release from intracellular stores, inositol 1,4,5-triphosphate (IP$_3$) receptors (IP$_3$R) on the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) and the mitochondria. After rapid global $\text{Ca}^{2+}$ signals, the clearance of $\text{Ca}^{2+}$ from the cytoplasm is accomplished by activities of $\text{Ca}^{2+}$-ATPases localized to both the ER (SERCAs) and plasma membrane (PMCAs) and then it is contributed substantially to the deactivation of chloride currents by SERCA [1].

The secretion can be significantly enhanced when both $\text{Ca}^{2+}$ and cAMP signaling systems are activated, although the activation of $\text{Ca}^{2+}$-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 lipoxigenase activity [5-7]. DA-6034 (7-carboxymethyloxy-3',4',5-trimethoxy flavones monohydrate) is a synthetic derivative of eupatilin, a flavonoid derivate. Eupatilin is a main component of the extract of Artemisiae species, a Korean folk medicine used for the treatment of stomach and liver diseases [6]. DA-6034 is a derivative of eupatilin and has interesting activities and selective inhibitors of lipoxigenase activity [5-7]. DA-6034 increases intracellular calcium concentrations ($[\text{Ca}^{2+}]_i$) in primary cultured human conjunctival cells. 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 $\text{Ca}^{2+}$ stores. These results suggest that DA-6034 induces $\text{Ca}^{2+}$ signaling via extracellular $\text{Ca}^{2+}$ entry and RyRs-sensitive $\text{Ca}^{2+}$ release from internal $\text{Ca}^{2+}$ stores in epithelial cells.

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

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Corresponding to: Dong Min Shin, Department of Oral Biology, Yonsei University College of Dentistry, 50 Yonsei-ro, Seodaemon-gu, Seoul 120-752, Korea. (Tel) 82-2-2228-3051, (Fax) 82-2-364-1085, (E-mail) dmshin@yuhs.ac

*These authors contributed equally to this work.

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ABBREVIATIONS: CaCCs, $\text{Ca}^{2+}$-activated Cl$^{-}$ channels; $[\text{Ca}^{2+}]_i$, 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-κB down-regulation in gastric epithelial cells [10-12]. DA-6034 prevents gastric mucosal injury through increases of endogenous prostaglandin E2 (PGE2) 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 Ca2+-activated fluid secretion in epithelial cells. However, whether DA-6034 induces Ca2+ 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 Ca2+ signaling of various epithelial cells.

METHODS

Preparation of exocrine gland acinar cells

ICR strain mice (6~8 weeks) were sacrificed by cervical dislocation under CO2 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 MgCl2, 1 CaCl2, 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 on 6-well plates containing Transwell permeable supports with KGM first. After the junctival cells were cultured on 6-well plates containing 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 [17]. Briefly, cells were incubated with 2 μM 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=F490) using a Molecular Devices (Downtown, 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=F340/F380). 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). SKF96365 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±S.E from at least 3 independent experiments. Statistical significance was determined by using a paired or unpaired Student’s t-test.
DA-6034 Induces [Ca\(^{2+}\)]:\ Increase

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 activates the CaCCs and induced transient [Ca\(^{2+}\)]\(_{i}\) increases in primary cultured human conjunctival epithelial cells.

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

Fig. 4. Relationship with internal Ca\(^{2+}\) stores and DA-6034. (A) DA-6034 induced [Ca\(^{2+}\)] increases were disappeared after ER depletion by CPA. (B) However, DA-6034 induced Ca\(^{2+}\) influx not inhibited by the PLC inhibitor, U73122, and its close analogue, U73343. (C) Caffeine induced [Ca\(^{2+}\)] 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 [Ca\(^{2+}\)] increases also inhibited by ryanodine (bottom left panel) and RR (bottom right panel). (D) DA-6034 induced [Ca\(^{2+}\)] increases were not inhibited by bafilomycin A1 (Baf-A1, an inhibitor of vacuolar-type H\(^{+}\)-ATPase) (Fig. 4D). Taken together, these results suggest that DA-6034 induced [Ca\(^{2+}\)] increase is dependent on RyRs but not the PLC/IP\(_3\) pathway and lysosomal Ca\(^{2+}\) pools.

The role of intracellular Ca\(^{2+}\) in DA-6034 induced [Ca\(^{2+}\)] increase

In Fig. 3, [Ca\(^{2+}\)] increases by DA-6034 also showed the possibility which DA-6034 induced [Ca\(^{2+}\)] increases are related to the intracellular Ca\(^{2+}\) stores. To investigate whether DA-6034-induced [Ca\(^{2+}\)] increases were related to Ca\(^{2+}\) release from internal stores, we used 25 μM CPA to deplete the ER and then applied DA-6034. In SMG acinar cells, treatment with CPA in nominally Ca\(^{2+}\)-free buffered solution showed inhibitory effects for the [Ca\(^{2+}\)] increase by DA-6034 (Fig. 4A, left panel) and also disappeared the effect of ER Ca\(^{2+}\) depletion by CPA in order changing (Fig. 4A, right panel), suggesting that DA-6034 induced [Ca\(^{2+}\)] increases are related to Ca\(^{2+}\) release from the ER. Mostly ER Ca\(^{2+}\) release can be mediated through the IP\(_3\)Rs [20]. Therefore, we examined the effects of DA-6034 in the treatment of PLC inhibitor U73122 whether DA-6034 induced [Ca\(^{2+}\)] increase is evoked by activation of PLC/IP\(_3\) pathway. The [Ca\(^{2+}\)] 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 Ca\(^{2+}\) stores, such as RyRs and lysosomal 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 of DA-6034 induced [Ca\(^{2+}\)] increase by ryanodine and RR in SMG acinar cells (Fig. 4C, bottom panels). However, the [Ca\(^{2+}\)] increase by DA-6034 still remained by the treatment of bafilomycin A1 (Baf-A1, an inhibitor of vacuolar-type H\(^{+}\)-ATPase) (Fig. 4D). Taken together, these results suggest that DA-6034 induced [Ca\(^{2+}\)] increase is dependent on RyRs but not the PLC/IP\(_3\) pathway and lysosomal Ca\(^{2+}\) pools.
DISCUSSION

In this study, we demonstrated for the first time the [Ca\textsuperscript{2+}], increases by DA-6034 through the regulation of extracellular Ca\textsuperscript{2+} entry and intracellular Ca\textsuperscript{2+} release in human conjunctival/corneal epithelial cells and mouse salivary gland epithelial cells. These effects could be the result of the released Ca\textsuperscript{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\textsubscript{2} 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\textsuperscript{2+} signaling in fluid secretion of exocrine gland [21]. Usually, the mucin and fluid secretion are stimulated predominantly by Ca\textsuperscript{2+} dependent pathways that are activated by extracellular ATP and other stimulators. Then, released peptides, such as secretin and VIP etc., stimulate adenylyl 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-κB activation and inflammation [22,23]. DA-6034 suppressed iNOS induction, NF-κB activation, p65 nuclear translocation, and restored IκBα level in the cytoplasm and dissociates the IKK-γ-Hsp90 complex in gastric cancer models [11,12]. In addition, DA-6034 attenuated JNK and p38 MAPK and also inhibited NF-κB 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\textsubscript{2} synthesis which prevented gastric mucosal injury and dry eye [8,13,14].

We also examined the sources of [Ca\textsuperscript{2+}], increased by DA-6034. Our results demonstrated that the effects of DA-6034 could be dependent on extracellular and intracellular Ca\textsuperscript{2+} levels, however this [Ca\textsuperscript{2+}], mobilization was included the RyRs but not the PLC/IP\textsubscript{3} pathway. The [Ca\textsuperscript{2+}], mobilization of DA-6034 through the RyRs is a novel finding in our results. Ca\textsuperscript{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\textsubscript{3}Rs are mainly expressed the apical region and exhibit the initiation and rapid global Ca\textsuperscript{2+} signals. Rydnoine receptors (RyRs) also intracellular Ca\textsuperscript{2+} release channels that belong to a family related to IP\textsubscript{3}Rs. RyRs are predominantly localized basal regions. They are gated by elevations in cytoplasmic Ca\textsuperscript{2+} in a process termed calcium-induced calcium release (CICR) and contribute to the mechanisms of regenerative Ca\textsuperscript{2+} waves in a various cells. Several studies reported that it could be cAMP-induced Ca\textsuperscript{2+} release without elevation of IP\textsubscript{3} 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-relasing factor-induced adrenocorticotropic secretion in mouse corticotropic 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\textsuperscript{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\textsuperscript{2+}], increases by DA-6034 can mediate related responses between RyRs and CaCCs. Taken together, DA-6034 could increase intracellular Ca\textsuperscript{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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