Biochemical Pharmacology 82 (2011) 1930–1939

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Epigallocatechin-3-gallate induces cytokine production in mast cells by stimulating an extracellular superoxide-mediated calcium influx

Toshio Inoue, Yoshihiro Suzuki *, Chisei Ra

Division of Molecular Cell Immunology and Allergology, Nihon University Graduate School of Medical Science, Tokyo, Japan

ARTICLE INFO

Article history:
Received 27 July 2011
Accepted 9 September 2011
Available online 16 September 2011

Keywords:
Mast cell
EGCG
Calcium
Superoxide
Cytokine

ABSTRACT

The green tea polyphenol (−)-epigallocatechin-3-*O*-gallate (EGCG) has various biological activities, including anti-inflammatory, anti-neoplastic, anti- and pro-apoptotic, and neuroprotective effects. Although these are often associated with increased intracellular reactive oxygen species (ROS) and Ca^{2+} levels, their involvement in biological effects is poorly understood. Here we report that EGCG induces cytokine production in mast cells via Ca^{2+} influx and ROS generation. EGCG at concentrations of ≥50 μM induced interleukin-13 and tumor necrosis factor-α production in RBL-2H3 and bone marrow-derived mast cells. The effects were dependent on extracellular Ca^{2+}, and EGCG induced Ca^{2+} release from intracellular stores and Ca^{2+} influx. Ca^{2+} influx was suppressed by 2-aminoethoxydiphenyl borate, an inhibitor of store-operated Ca^{2+} (SOC) channels, including Ca^{2+} release-activated Ca^{2+} channels and transient receptor potential canonical channels. EGCG failed to induce Ca^{2+} influx through SOC channels. EGCG-activated Ca^{2+} channels were genetically and pharmacologically distinct from $Ca_v1.2$ L-type Ca^{2+} channels, another route of Ca^{2+} influx into mast cells. EGCG evoked release of superoxide ($O_2^{\bullet-}$) into the extracellular space. Exogenous superoxide dismutase, but not catalase, inhibited EGCG-evoked Ca^{2+} influx and cytokine production, indicating that extracellular $O_2^{\bullet-}$ regulates these events. EGCG can serve as a powerful tool for studying $O_2^{\bullet-}$-regulated Ca^{2+} channels, which may be selectively involved in the regulation of cytokine production but have yet to be elucidated.

1. Introduction

Polyphenolic compounds such as (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-*O*-gallate (ECG) and (−)-epigallocatechin-3-*O*-gallate (EGCG) are major green tea constituents that are known for their strong antioxidant activities. Among these catechins, EGCG is the most abundant (accounting for 40–60% of the catechins) and a powerful antioxidant [1–3]. Epidemiological studies have shown the effectiveness of green tea at preventing cancer, and cardiovascular and neurological diseases [4–6]. These biological activities of EGCG were initially proposed to be based on its antioxidant activity. However, studies on the structure–activity relationship for EC, EGC, ECG and EGCG have revealed no apparent correlation between the degrees of their biological effects and antioxidant activities. Recent evidence suggests that the action of EGCG is more complicated than previously thought. To date, EGCG has been shown to interact with membrane lipid rafts [7,8] and directly targets various signaling molecules, including receptor tyrosine kinases, and that these effects are crucial for cancer chemoprevention and neuroprotection by EGCG [9,10].

Catechins including EGCG have also been shown to suppress allergic reactions in laboratory animal models [11]. Mast cells play central roles in allergic, inflammatory reactions. Upon stimulation of the high-affinity IgE receptor (FcεRI) by IgE and a multivalent antigen, mast cells have remarkably diverse functional outputs. These outputs include degranulation (secretion of pre-formed chemical mediators stored in granules, such as histamine and serotonin, referred as to degranulation), synthesis and secretion of arachidonic acid-derived mediators such as leukotrienes (LTs) and prostaglandins, and the production of cytokines and chemokines [12]. These chemical mediators cause various pathophysiological events that contribute to acute and chronic allergic reactions. Therefore, inhibition of proinflammatory mediator release from mast cells is a potential mechanism for the anti-allergic effect of catechins. Indeed, catechins have been shown to suppress histamine release from mast cells *in vitro* in the order of EGCG > ECG > EGC, whereas EC has no effect [11]. Moreover, we have shown that EGCG, but not EC, inhibits mast cell degranulation, LTC_4 secretion and Ca^{2+} influx [13]. The effects are unlikely to be attributed to the anti-oxidant activity, because EC has been

* Corresponding author at: Division of Molecular Cell Immunology and Allergology, Nihon University Graduate School of Medical Sciences, 30-1 Oyaguchikamimachi, Itabashi-ku, Tokyo 173-8610, Japan. Tel.: +81 3 3972 8111; fax: +81 3 3972 8227.
E-mail address: suzuki.yoshihiro@nihon-u.ac.jp (Y. Suzuki).

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.09.011

shown to exhibit strong antioxidant and radical scavenging activities *in vitro* and *in vivo* [2,3,14]. In addition, we have found that EGCG, but not EC, has various biological effects, including generation of intracellular reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi_m$) collapse, and mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) release; all of which appear to be involved in the inhibitory effect [13]. However, EGCG appears to enhance the production of interleukin (IL)-13 in mast cells. These findings led us to study the effects of EGCG on cytokine production, with special attention to the role of ROS and Ca^{2+} signals.

In the present study, we found that EGCG induced production of IL-13 and tumor necrosis factor (TNF)-α by stimulating Ca^{2+} influx. The EGCG-induced Ca^{2+} influx appeared to occur via extracellular superoxide ($O_2^{\bullet-}$)-regulated channels, which differed from the three known routes of Ca^{2+} influx into mast cells, including Ca^{2+} release-activated Ca^{2+} (CRAC) channels, transient receptor potential canonical (TRPC) channels and L-type Ca^{2+} channels (LTCCs).

2. Materials and methods

2.1. Materials

EC and EGCG were obtained from Kurita Industries (Tokyo, Japan). These compounds were dissolved in phosphate buffered saline (PBS) and stored at −20 °C, and diluted with Hank's balanced salt solution (HBSS, pH 7.4) before use. Nifedipine, 2-aminoethoxydiphenyl borate (2-APB), superoxide dismutase (SOD), ebselen, cytochrome c, catalase and thapsigargin (Tg) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Diphenyleneiodonium chloride (DPI) was obtained from Enzo Life Sciences (San Diego, CA, USA). Fluo3-acetoxymethyl ester (Fluo3/AM) was purchased from Dojindo Laboratories (Kumamoto, Japan) and dissolved in dimethylsulfoxide (DMSO). Reagents were dissolved in DMSO and diluted with HBSS to a final concentration of <0.1% before use. DMSO alone at a concentration of 0.1% (vehicle) had no effects throughout this study. A monoclonal anti-2,4,6-trinitrophenyl (TNP) IgE antibody (clone IgE-3) was obtained from BD PharMingen Japan (Tokyo, Japan). A TNP-bovine serum albumin (BSA) conjugate (TNP:BSA conjugation ratio of 25:1) was purchased from Cosmo Bio (Tokyo, Japan).

2.2. Cells

Rat basophilic leukemia (RBL)-2H3 cells obtained from the National Institute of Health Sciences (Japan Collection of Research Bioresources, cell number JCRB0023) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA) in a 5% CO_2-containing atmosphere. The cells were harvested by incubation in HBSS containing 1 mM EDTA and 0.25% trypsin for 5 min at 37 °C. For IgE sensitization, cells suspended in DMEM were plated on 100-mm culture dishes (5×10^6 cells/5 ml) or in 24-well plates (2×10^5 cells/well) and incubated with anti-TNP IgE (0.1 μg) at 37 °C overnight. Bone marrow-derived mast cells (BMMCs) were prepared from the femurs of 4–8-week-old C3HeBFeJ mice as previously described [15]. All animal treatments were performed according to Nihon University guidelines. The cells were cultured in RPMI 1640 medium (Sigma–Aldrich) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), 5×10^{-5} M β-mercaptoethanol (Wako Pure Chemicals, Osaka, Japan), 100 μg/ml sodium pyruvate (Invitrogen), 1% MEM nonessential amino acid solution (Invitrogen) and 5 ng/ml recombinant IL-3 (Pepro Tech Inc., Rocky Hill, NJ, USA) in a 5% CO_2-containing atmosphere at 37 °C. After 4–6 weeks of culture, the cells were stained for cell surface expression of FcεRI, and BMMCs were used for experiments after 4–8 weeks of culture (>95% mast cells).

2.3. Degranulation assay

Mast cell degranulation was assessed by measuring β-hexosaminidase release. After stimulating IgE-sensitized cells with 30 ng/ml antigen or IgE-untreated cells with the agents to be tested at 37 °C for 30 min, supernatants were collected and β-hexosaminidase activity in supernatants was determined spectrophotometrically as previously described [15]. Cells were lysed with 0.1% (w/v) Triton X-100, and the β-hexosaminidese activity of the extracts was measured (total). The β-hexosaminidase activity in unstimulated cells (the spontaneous release <5% of total enzyme activity) was subtracted from the enzyme activity in stimulated cells (test). The percentage of β-hexosaminidase released into the supernatant was calculated by the following formula: release (%) = (test − spontaneous)/(total − spontaneous) × 100.

2.4. Cytokine production assay

After stimulating IgE-sensitized cells with 30 ng/ml antigen or IgE-untreated cells with the agents to be tested at 37 °C for 4 h, supernatants were collected and IL-13 and TNF-α contents in supernatants were determined by a solid-phase sandwich ELISA kit (Biosource International, Camarillo, CA, USA) for rat or mouse IL-13 and TNF-α, respectively, according to the manufacturer's protocols.

2.5. Calcium measurement

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) was measured using the Ca^{2+}-reactive fluorescent probe Fluo3/AM, as previously described [15]. Cell suspensions (1×10^6 cells/ml) were incubated with 4 μM Fluo3/AM for 30 min at 37 °C, washed with HBSS, and resuspened in HBSS supplemented with 1 mM $CaCl_2$. To study Ca^{2+} release from internal stores and Ca^{2+} entry separately, aliquots of the Fluo3/AM-loaded cells were resuspended in Ca^{2+}-free medium (HBSS supplemented with 1 mM EGTA instead of 1 mM $CaCl_2$). Fluorescence was monitored at 5-s intervals for 3 min using a microplate fluorometer (Fluoroskan Ascent CF, Labsystems, Helsinki, Finland; excitation and emission at 485 and 527 nm, respectively). $[Ca^{2+}]_c$ was calculated using the equation: $[Ca^{2+}]_c = K_d$ $((F - F_{min})/(F_{max} - F)$, where K_d is the dissociation constant of the Ca^{2+}-Fluo3 complex (450 nM). F_{max} represents the maximum fluorescence (obtained by treating cells with 5 μM A23187), and F_{min} represents the minimum fluorescence (obtained by treating cells with A23187 in the presence of 10 mM EGTA). F is the actual sample fluorescence. For quantitative analyses of inhibition of Ca^{2+} signals, the area under the curve (AUC) was calculated.

2.6. $Ca_v1.2$ LTCC knockdown cells

Expression of the α_{1C} subunit of $Ca_v1.2$ LTCCs was down-regulated at mRNA and protein levels ($Ca_v1.2$ knockdown cells) as previously described [16] with minor modifications. Adherent cells in six-well plates (2.5×10^5 cells/well) or 24-well plates (5.0×10^4 cells/well) were transfected with 30 nM siRNA targeting the α_{1C} subunit or a negative control siRNA with an irrelevant sequence (Ambion, Austin, TX) for 48 h using the siPORT™ *NeoFX* transfection agent (Ambion) according to the manufacturer's instructions. The efficacy for the knockdown was evaluated by measuring the α_{1C} transcript and surface α_{1C} protein expression using RT-PCR and flow cytometry, respectively as previously described [16].

2.7. Extracellular $O_2^{\bullet-}$ release measurement

Extracellular release of $O_2^{\bullet-}$ was measured using a SOD-dependent cytochrome c reduction assay as previously described

[17]. Cells (1×10^6 cells/ml) were stimulated and 20 μM cytochrome c was added. The reduction in absorbance at 540 nm (ΔOD_{540}) was measured immediately in a Model 680 Microplate Reader (Nippon Bio-Rad Laboratories, Osaka, Japan). To measure the SOD-dependent response, SOD was added to the reaction mixture in separate samples at a final concentration of 10 U/ml, and the reduction in absorbance at 540 nm (ΔOD_{540} [SOD]) was measured in the same way. The data are shown as SOD-dependent cytochrome c reduction, corresponding to $\Delta OD_{540} - \Delta OD_{540}$ [SOD].

2.8. Statistical analysis

The statistical significance of differences among values was analysed by one-way ANOVA with a post hoc Tukey test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. EGCG alone induces IL-13 and TNF-α production but not degranulation in mast cells

To determine whether EGCG stimulated production of cytokines in mast cells, RBL-2H3 mast cells were treated with varying concentrations of EGCG for 4 h at 37 °C, and IL-13 and TNF-α production were measured by ELISA. EGCG at concentrations of ≥50 μM induced IL-13 and TNF-α production with a maximal effect at 100 μM (Fig. 1A and B). The effect of EGCG on IL-13 production was almost comparable to the effect of the optimal antigen stimulation, whereas EGCG was significantly less effective than antigen stimulation for inducing TNF-α production (maximum of 60%). On the contrary, EGCG at concentrations of up to 200 μM induced no substantial degranulation, as measured by β-hexosaminidase release (Fig. 1C). EC at concentrations of up to 200 μM alone had no effects on cytokine production and degranulation (Fig. 1A–C). These results showed that EGCG, but not EC, induced cytokine production, but not degranulation, in mast cells. To examine whether the effects were specific for tumor mast cells like RBL-2H3 cells, we performed similar experiments using BMMCs. EGCG alone induced IL-13 and TNF-α production in the cells with the maximal effect at 100 μM, while EC had no effect, although EGCG was significantly less effective than the optimal Antigen stimulation (maximum of 40%) (Figs. S1A and B). On the contrary, EGCG at concentrations of up to 200 μM had no effects on degranulation (Fig. S1C). EGCG induction of cytokine production is also observed in the primary mast cells. Therefore, we investigated the mechanisms underlying the EGCG induction of cytokine production in RBL-2H3 cells.

3.2. EGCG-induced cytokine production is dependent on extracellular Ca^{2+}

Elevations of the intracellular Ca^{2+} concentrations are necessary for the multiple Ca^{2+}-dependent biochemical processes during mediator secretion. The entry of Ca^{2+} from the extracellular space mainly contributes to the increase in $[Ca^{2+}]_c$, thereby playing a crucial role in IgE-dependent degranulation and cytokine production [12]. Therefore, we examined whether EGCG induction of cytokine production was dependent on extracellular Ca^{2+}. Cells in Ca^{2+}-containing or Ca^{2+}-free medium were stimulated with EGCG and analysed for cytokine production. Consistent with their strict Ca^{2+} dependences, degranulation and IL-13 and TNF-α production that were observed after antigen stimulation were diminished in the cells in Ca^{2+}-free medium (Fig. 2). Similarly, IL-13 (Fig. 2A) and TNF-α production (Fig. 2B) in the EGCG-treated cells were completely abolished under Ca^{2+}-free conditions, indicating that they were also dependent on extracellular Ca^{2+}.

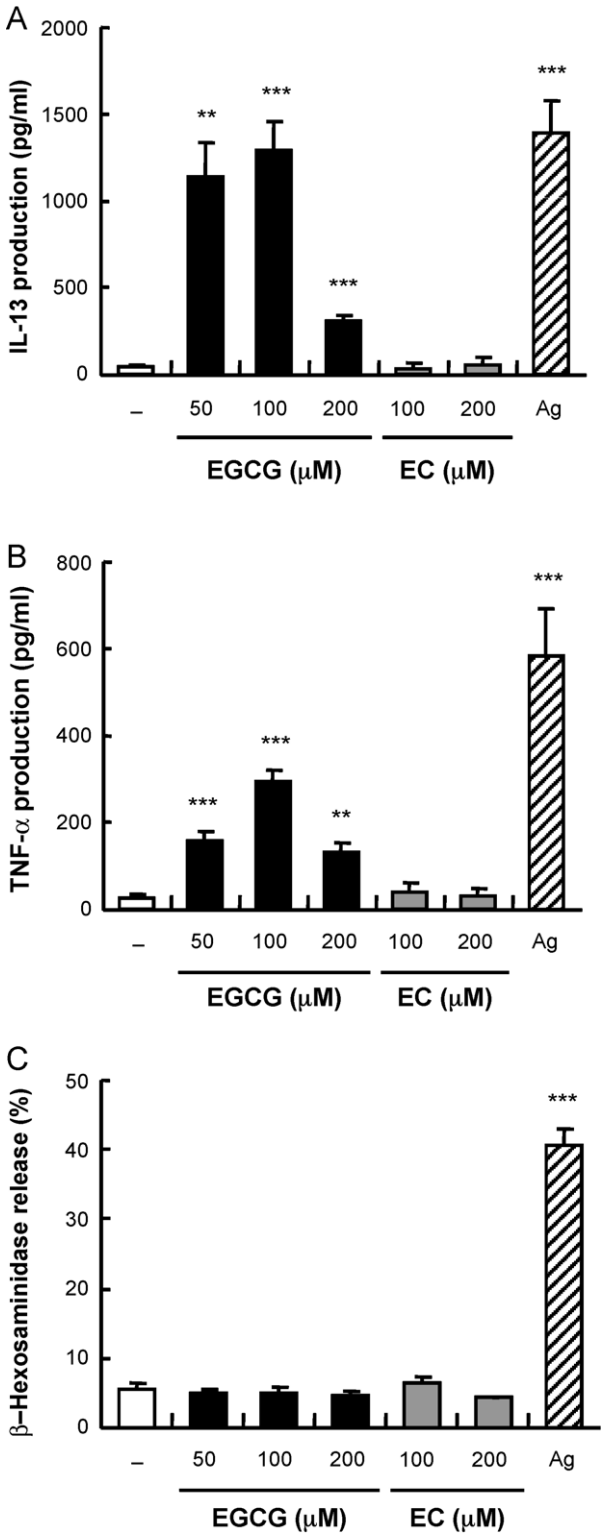

Fig. 1. EGCG, but not EC, induces cytokine production in mast cells without evoking mast cell degranulation. (A and B) RBL-2H3 cells (5×10^5 cells/200 μl) were treated with EGCG and EC at the indicated concentrations or sensitized with 0.1 μg/ml anti-TNP IgE overnight and stimulated with 30 ng/ml antigen (Ag). At 4 h after stimulation, the IL-13 (A) and TNF-α contents (B) in the supernatants were determined by ELISA. The data represent the means ± SE of nine independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$. (C) RBL-2H3 cells (5×10^5 cells/200 μl) were treated with EGCG and EC at the indicated concentrations or with stimulated with antigen for 30 min, and the β-hexosaminidase activities in the supernatants were determined enzymatically and the percentage of β-hexosaminidase released was calculated. The data represent the means ± SE of four independent experiments. $^{***}P < 0.001$.

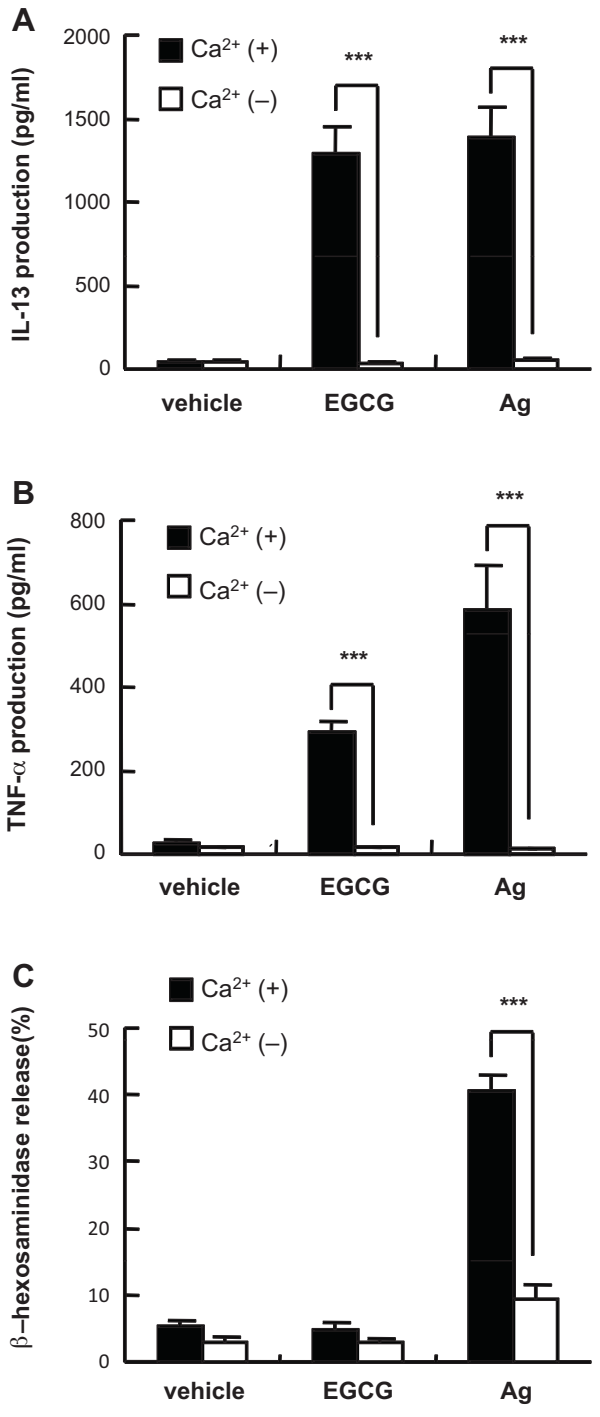

Fig. 2. EGCG induces cytokine production in a Ca^{2+}-dependent manner. (A and B) RBL-2H3 cells (5×10^5 cells/200 μl) were suspended in HBSS containing 1 mM $CaCl_2$ (Ca^{2+}-containing medium [Ca^{2+} (+)]) or HBSS containing 1 mM EGTA (Ca^{2+}-free medium [Ca^{2+} (−)]) and treated with 100 μM EGCG or with stimulated with antigen for 4 h, and the IL-13 (A) and TNF-α contents (B) in the supernatants were determined by ELISA. The data represent the means ± SE of four independent experiments. ***$P < 0.001$. (C) Cells suspended in Ca^{2+}-containing medium [Ca^{2+} (+)] or Ca^{2+}-free medium [Ca^{2+} (−)] were treated with 100 μM EGCG or with stimulated with Ag for 30 min, and the β-hexosaminidase activities in the supernatants were determined. The data represent the means ± SE of three independent experiments. ***$P < 0.001$.

3.3. EGCG induces Ca^{2+} mobilization from intracellular stores and the extracellular space

EGCG induced cytokine production in an extracellular Ca^{2+}-dependent manner. Therefore, we examined whether EGCG could affect Ca^{2+} mobilization. EGCG increased $[Ca^{2+}]_c$ at concentrations that induced cytokine production (Fig. 3A). The increase was observed more rapidly for higher concentrations of EGCG and sustained for at least 3 min. On the contrary, EC at concentrations of up to 100 μM had no such effects (Fig. 3B). As shown in Fig. 3C, EGCG at concentrations of ≥10 μM increased $[Ca^{2+}]_c$ in a concentration-dependent manner in Ca^{2+}-free medium, which indicated induction of Ca^{2+} mobilization from intracellular stores. However, this increase was less sustained compared with the $[Ca^{2+}]_c$ increase observed in Ca^{2+}-containing medium. Consequently, the $[Ca^{2+}]_c$ level returned to baseline within 3 min. As the concentration of EGCG increased, the time to the peak $[Ca^{2+}]_c$ level became shorter (50, 30 and 20 s for 10, 30 and 100 μM EGCG, respectively). On the contrary, EC at concentrations of up to 100 μM caused no increase in $[Ca^{2+}]_c$ (Fig. 3D). These data showed that EGCG, but not EC, induced Ca^{2+} mobilization from intracellular stores and the extracellular space.

3.4. EGCG activates 2-APB-sensitive non-SOC channels

Two different types of SOC channels, CRAC and TRPCs are found to mediate Ca^{2+} influx in mast cells and 2-APB at high concentrations above 10 μM inhibits their activities [18]. Therefore, to gain insight into Ca^{2+} channels involved in the EGCG-induced Ca^{2+} influx, we examined the effect of 2-APB on the Ca^{2+} influx. 2-APB (10 μM) had no significant effect on the EGCG-induced $[Ca^{2+}]_c$ increase until 30 s but substantially reduced the increase thereafter (Fig. 4A and B). Next, we examined whether EGCG could induce SOC channel activation. To this end, RBL-2H3 cells in Ca^{2+}-free medium were treated with 2 μM Tg or 100 μM EGCG to deplete intracellular Ca^{2+} stores, and then Ca^{2+} was added again. An increase in $[Ca^{2+}]_c$ was observed in the Tg-treated cells (Fig. 4C), but not in the EGCG-treated cells (Fig. 4D), which indicated that EGCG failed to activate SOC channels. So far, our data suggested that EGCG activated a store-independent Ca^{2+} influx. LTCCs are activated independently of Ca^{2+} store depletion and comprise another route of Ca^{2+} influx into mast cells [16,19]. Therefore, to elucidate the possible role of LTCCs, we examined the effect of the LTCC-specific inhibitor nifedipine on EGCG-induced Ca^{2+} influx and found that nifedipine (1 μM) had stimulatory rather than inhibitory effects on Ca^{2+} influx (Fig. 4A and B). Further analyses using the $Ca_v1.2$ knockdown cells [16] confirmed the independence of the EGCG-induced Ca^{2+} influx of this channel. Consistent with the role of $Ca_v1.2$ LTCCs in antigen-activated Ca^{2+} influx, the Ca^{2+} influx was significantly smaller in the $Ca_v1.2$ knockdown cells compared with control cells (Figs. S2A and B). Moreover, nifedipine reduced the Ca^{2+} influx in control cells, whereas nifedipine sensitivity was completely abolished in the $Ca_v1.2$ knockdown cells. These data confirm that $Ca_v1.2$ LTCCs activity was impaired in the $Ca_v1.2$ knockdown cells. On the contrary, EGCG induced a comparable level of $[Ca^{2+}]_c$ increase between the $Ca_v1.2$ knocked down cells and control cells (Fig. S2C), which indicated that the $[Ca^{2+}]_c$ increase was independent of $Ca_v1.2$ LTCC activity. Collectively, these data suggest that EGCG activates 2-APB-sensitive Ca^{2+} channels that are distinct from CRAC and TRPC channels and $Ca_v1.2$ LTCCs.

3.5. EGCG induces release of $O_2^{\bullet-}$ into the extracellular space

We have previously reported that EGCG, but not EC, induces the production of intracellular ROS [13]. Moreover, cytochrome c reduction assay revealed that EGCG at concentrations of ≥10 μM evoked release of $O_2^{\bullet-}$ into the extracellular space (Fig. 5A). $O_2^{\bullet-}$ release was observed within 1 min and kept at that level for further 5 min. On the contrary, EC at concentrations of up to 100 μM had no such effects (Fig. 5B). Fig. 5C shows the summarized data. The

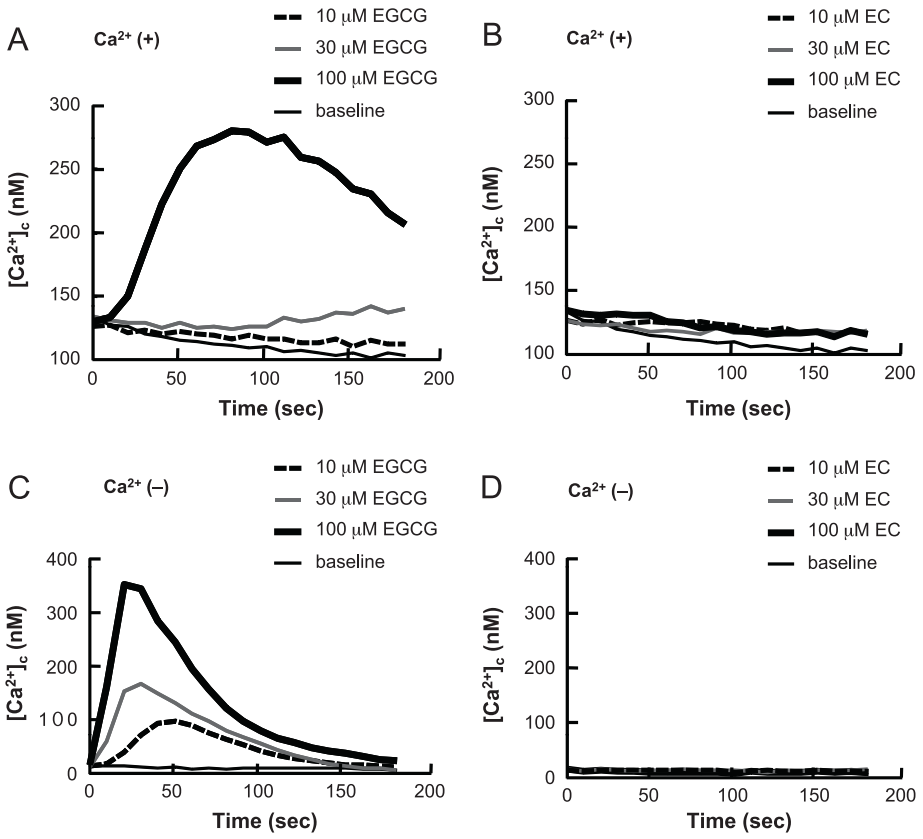

Fig. 3. EGCG, but not EC, induces Ca^{2+} mobilization. RBL-2H3 cells (1×10^6 cells/ml) were incubated with 4 μM Fluo3/AM for 40 min at 37 °C. The Fluo3/AM-loaded cells were suspended in Ca^{2+}-containing medium [Ca^{2+} (+)] (A and B) or Ca^{2+}-free medium [Ca^{2+} (−)]) (C and D) were treated with EGCG (A and C) or EC (B and D) at the indicated concentrations. The Fluo3 fluorescence was monitored for up to 3 min using a microplate fluorometer with excitation and emission at 485 and 527 nm, respectively. The data shown as the calculated $[Ca^{2+}]_c$ are representative of four (A and B) and three (C and D) independent experiments.

EGCG-induced $O_2^{\bullet-}$ release was highly sensitive to the treatment with DPI, a NADPH oxidase inhibitor. DPI at concentrations of ≥30 nM significantly inhibited the oxidative burst with almost complete inhibition at around 3 μM (Fig. 5D and E).

3.6. Extracellular $O_2^{\bullet-}$ mediates the EGCG-induced Ca^{2+} influx and cytokine production

We previously have reported evidence that ROS are involved in the regulation of Ca^{2+} signaling and cytokine production in antigen-activated mast cells [15,20,21]. Therefore, we hypothesized that ROS also played a role in the stimulatory effects of EGCG on cytokine production. To test the hypothesis, we examined the effects of various ROS scavengers on EGCG-induced cytokine production. Application of SOD at concentrations above 10 U/ml reduced the EGCG-induced IL-13 and TNF-α production in a dose-dependent manner (Fig. 6A and B). On the contrary, 10 U/ml SOD had minimal effects on the production of cytokines upon FcεRI stimulation (Fig. 6C). DPI also dose-dependently suppressed the production of both cytokines (Fig. 6D and E). In accordance with the increase in intracellular peroxide level [13], the glutathione peroxidase mimetic antioxidant ebselen also inhibited the cytokine production in a dose-dependent manner. On the contrary, catalase at concentrations of up to 100 U/ml had minimal effects on the production (Fig. S3). We also examined whether the EGCG-induced Ca^{2+} influx was ROS-dependent. In the cells treated with DPI and SOD, the $[Ca^{2+}]_c$ increase grew less sustained compared with that observed in untreated cells (Fig. 7A). Consequently, the profiles of the $[Ca^{2+}]_c$ increase in the presence of DPI and SOD resembled that observed under Ca^{2+}-free conditions. The AUC measurements revealed that SOD and DPI significantly suppressed the EGCG-induced Ca^{2+} influx at concentrations that reduced the EGCG-induced cytokine production (Fig. 7B and C). On the contrary, catalase had minimal effects on the Ca^{2+} influx (Fig. 7B). Collectively, these data suggest that extracellular $O_2^{\bullet-}$ plays an important role in the regulation of EGCG-induced Ca^{2+} influx and cytokine production.

4. Discussion

Ca^{2+} is unequivocally a common second messenger in the signal transduction pathways that lead to mast cell degranulation and cytokine production. Ca^{2+} influx is necessary for these outputs induced by FcεRI stimulation, since the removal of extracellular Ca^{2+} completely abolishes them [12]. We previously showed that Ag (I) ions induce Ca^{2+} influx and degranulation of mast cell but have a minimal effect on cytokine production [22]. Similar to FcεRI stimulation, Ag (I)-induced degranulation is strictly dependent on the extracellular Ca^{2+}. However, Ag (I)-induced Ca^{2+} influx is mediated by another mode of Ca^{2+} influx that is activated independently of Ca^{2+} store depletion [23]. These findings support the view that non-store-operated Ca^{2+} entry comprises another route of Ca^{2+} influx into mast cells. In addition, they suggested that Ca^{2+} introduced through different pathways (channels) leads to different types of outputs. The results of the present study clearly demonstrate that EGCG also activates mast cells in a manner that differs from FcεRI stimulation, because it can evoke IL-13 and TNF-α production but not degranulation. Moreover, EGCG stimulated Ca^{2+} influx that was necessary for cytokine production. Collectively, the present findings indicate that EGCG stimulates a Ca^{2+} influx pathway that is selectively involved in cytokine production and support the Ca^{2+} channel-specific regulation of mast cell outputs.

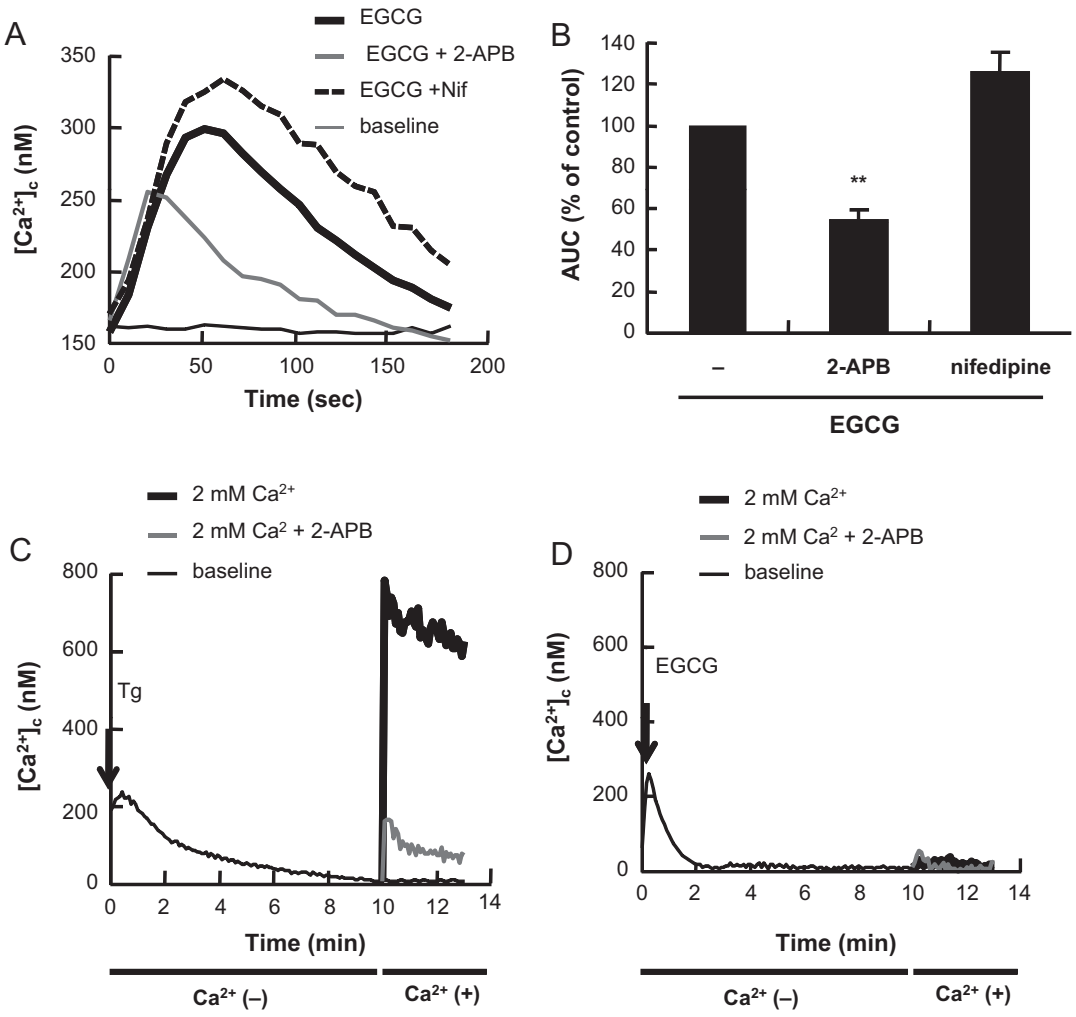

Fig. 4. EGCG induces 2-APB-sensitive non-SOC channels. (A) Fluo3/AM-loaded cells (1×10^6 cells/ml) were treated with 100 μM EGCG in the presence or absence of 10 μM 2-APB and 1 μM nifedipine (Nif), and monitored for their Fluo3 fluorescence for up to 3 min using a microplate fluorometer with excitation and emission at 485 and 527 nm, respectively. The data shown as the calculated $[Ca^{2+}]_c$ are a representative of four independent experiments. (B) The AUC was measured and expressed as percentages relative to the AUC in control cells treated with EGCG alone (set at 100%), and represent the means ± SE of four independent experiments. $^{**}P < 0.01$. (C and D) Fluo3/AM-loaded cells (1×10^6 cells/ml) were resuspended in medium containing 1 mM EGTA (Ca^{2+}-free medium) and treated with 1 μM Tg (C) or 100 μM EGCG (D) (indicated by the first arrow) for 10 min at 37 °C to empty the internal Ca^{2+} stores and activate SOC channels. Next, 2 mM $CaCl_2$ was added to the cells. The Fluo3 fluorescence was monitored for up to 3 min using a microplate fluorometer with excitation and emission at 485 and 527 nm, respectively. The data shown as the calculated $[Ca^{2+}]_c$ are representative of three independent experiments.

We found that EGCG induced Ca^{2+} release from intracellular stores but failed to evoke SOC activation. The findings appear to be inconsistent with the view that Ca^{2+} store depletion leads to SOC activation. There are several possible explanations for our observations. One is that EGCG induced Ca^{2+} release from intracellular stores other than the endoplasmic reticulum (ER). We previously have reported that EGCG induces Ca^{2+} release from the mitochondria [13]. Another possibility is that EGCG induced substantial Ca^{2+} store depletion in the ER but failed to activate SOCs. It is becoming increasingly evident that mitochondria are key regulators of CRAC channel activation in eukaryotic cells [24]. Respiring mitochondria rapidly take up the Ca^{2+} released from the ER stores, which results in more extensive store depletion and thus robust activation of CRAC channels. CRAC channel opening results in robust increase in $[Ca^{2+}]_c$, which feeds back to inactivate the channels. Mitochondria reduce the Ca^{2+}-dependent inactivation of the CRAC channels by sequestering the cytosolic Ca^{2+}, thereby leading to more prolonged Ca^{2+} influx. On the other hand, mitochondrial Ca^{2+} release close to the ER accelerates Ca^{2+} store refilling and thus promotes channel inactivation. Thus, mitochondria can reciprocally regulate CRAC channel gating by modulating the cytosolic and ER lumen Ca^{2+} levels. It is widely accepted that the Ca^{2+} unipoter, which is driven by $\Delta\Psi_m$, mediates rapid and massive Ca^{2+} entry into mitochondria [25]. Consistent with the essential role of mitochondrial Ca^{2+} uptake in CRAC channel activation in mast cells, the dissipation of the $\Delta\Psi_m$ results in severe impairment of store-operated Ca^{2+} entry, degranulation and LTC_4 secretion [26,27]. Therefore, it should be noted that EGCG, but not EC, induces strong $\Delta\Psi_m$ collapse and thus inhibits CRAC channel activation [13]. Taken together, our data suggest that the mitochondrial Ca^{2+} dysfunction mainly causes the failure of CRAC channel activation in EGCG-treated cells.

SOC channels are the major route of Ca^{2+} influx that leads to cytokine production in T cells and mast cells [18,28]. However, recent evidence suggests that, besides CRAC channels, other types of Ca^{2+} channels are also involved in Ca^{2+} signaling in mast cells and some of them are considered to be involved in the production of cytokines. The non-selective cation channels TRPC channels have been shown to be activated in response to FcεRI stimulation and are necessary for full Ca^{2+} influx and mast cell degranulation [29–31]. In addition, TRPC channels are 2-APB-sensitive channels and implicated to play a role in IL-2 production [30]. However,

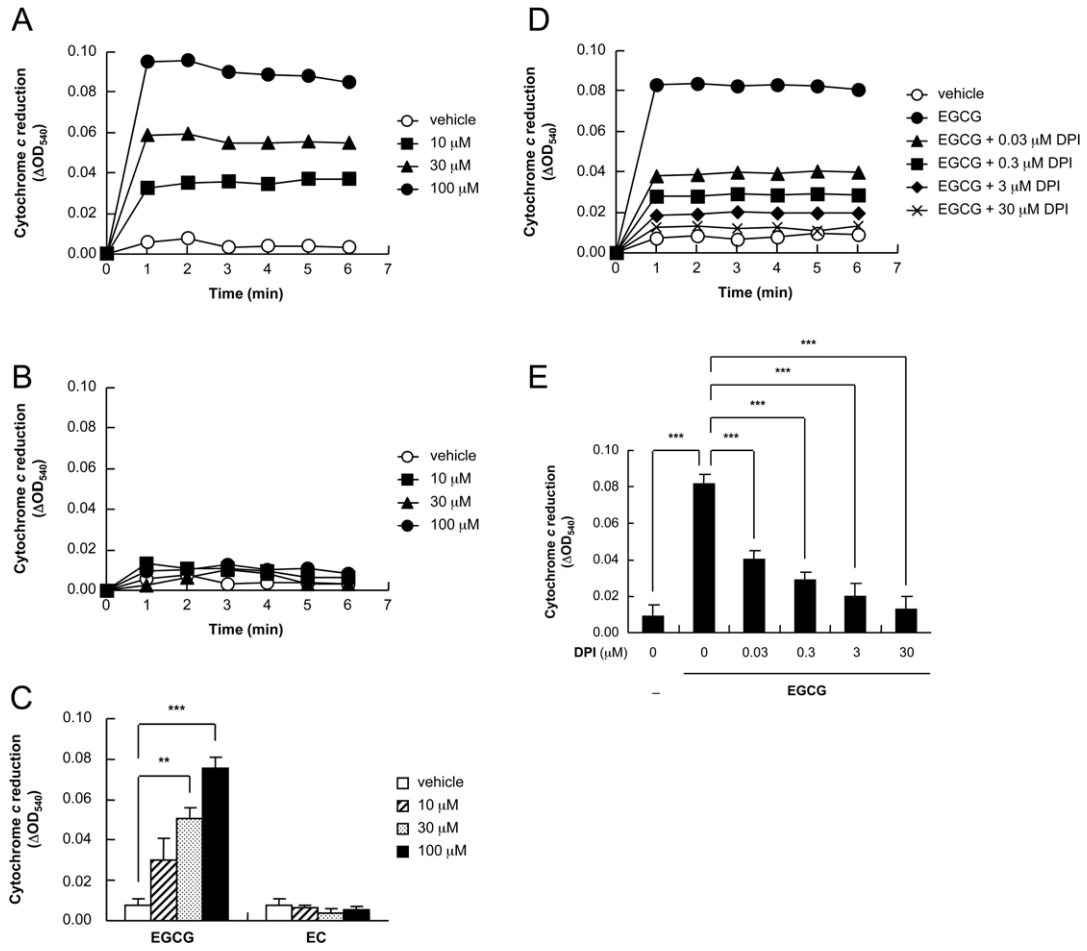

Fig. 5. EGCG, but not EC, induces the extracellular release of $O_2^{\bullet-}$. (A and B) RBL-2H3 cells (1×10^6 cells/ml) were treated with EGCG (A) or EC (B) at the indicated concentrations, and extracellular release of $O_2^{\bullet-}$ were measured by monitoring cytochrome c reduction (the reduction of absorbance at 540 nm) in a microplate reader. Panel C represents the summarized data of four independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$. (D) Cells (1×10^6 cells/ml) were treated with 100 μM EGCG in the presence or absence of DPI at the indicated concentrations, and extracellular release of $O_2^{\bullet-}$ were measured. Panel E represents the summarized data of four independent experiments. $^{***}P < 0.001$.

EGCG induced cytokine production, but not degranulation, which indicates that TRPC channels are unlikely targets of EGCG. Considering the crucial roles of SOC channels such as CRAC channels and TRPC channels in mast cell degranulation, the failure of EGCG to induce degranulation may be attributed to its inability to evoke SOC channel activation. $Ca_v1.2$ LTCCs are activated independently of Ca^{2+} store depletion and comprise another route of Ca^{2+} influx into mast cells and positively regulate production of cytokines such as IL-13 and TNF-α [16,19]. Therefore, there is the possibility that the channels are the targets of EGCG for inducing cytokines. However, the present pharmacological and genetic analyses revealed that this was not the case, because the LTCC-specific inhibitor nifedipine and downregulated $Ca_v1.2$ gene expression did not reduce the EGCG-induced Ca^{2+} influx. Collectively, EGCG appears to stimulate Ca^{2+} influx by a pathway, which differs from CRAC channels, TRPC channels and $Ca_v1.2$ LTCCs.

An increasing body of evidence suggests a close functional cross-talk between ROS and Ca^{2+} signals in mast cells. FcεRI stimulation results in the generation of intracellular ROS, which are involved in the regulation of Ca^{2+} influx, mast cell degranulation, LT secretion and cytokine production and survival [15,19,32]. FcεRI stimulation causes the production of intracellular $O_2^{\bullet-}$ and H_2O_2. The two oxidants appear to be discretely generated by separate intracellular signaling pathways, because the H_2O_2 generation is dependent on Src family kinase and phosphatidylinositol-3-kinase (PI3K) activities, but independent of extracellular Ca^{2+}. On the other hand, the $O_2^{\bullet-}$ generation is strictly dependent on extracellular Ca^{2+} but is negatively regulated by the Src family kinase and PI3K activities. Although the molecular entity of their sources remains unclear, the mitochondria and a NADPH oxidase appear to be involved in the generation of $O_2^{\bullet-}$ and H_2O_2, respectively [33]. In this regard, it is noteworthy that Ca^{2+} influx through SOC channels, most probably CRAC channels results in generation of $O_2^{\bullet-}$ within mitochondria in mast cells [21]. Collectively, the most likely source of the PI3K-independent $O_2^{\bullet-}$ generation is mitochondria whereas a NADPH oxidase is a likely source of the PI3K-dependent H_2O_2 generation. In addition, analyses using Tg revealed that the two oxidants may distinctly regulate mast cell degranulation and cytokine production. Tg can evoke the PI3K-independent mitochondrial $O_2^{\bullet-}$ generation, but not PI3K-dependent H_2O_2 generation, and can induce mast cell degranulation more strongly than FcεRI stimulation [21]. However, Tg is much less effective than FcεRI stimulation for evoking TNF-α and IL-13 production. Moreover, selective scavenging of H_2O_2 by ebselen has minimal effects on mast cell degranulation [15]. Therefore, intracellular $O_2^{\bullet-}$ may preferentially mediate the Ca^{2+} influx that is required for mast cell degranulation. This is consistent with the finding that $O_2^{\bullet-}$ generation is necessary for CRAC channel activation [32]. On the other hand, the PI3K-inhibitor wortmannin

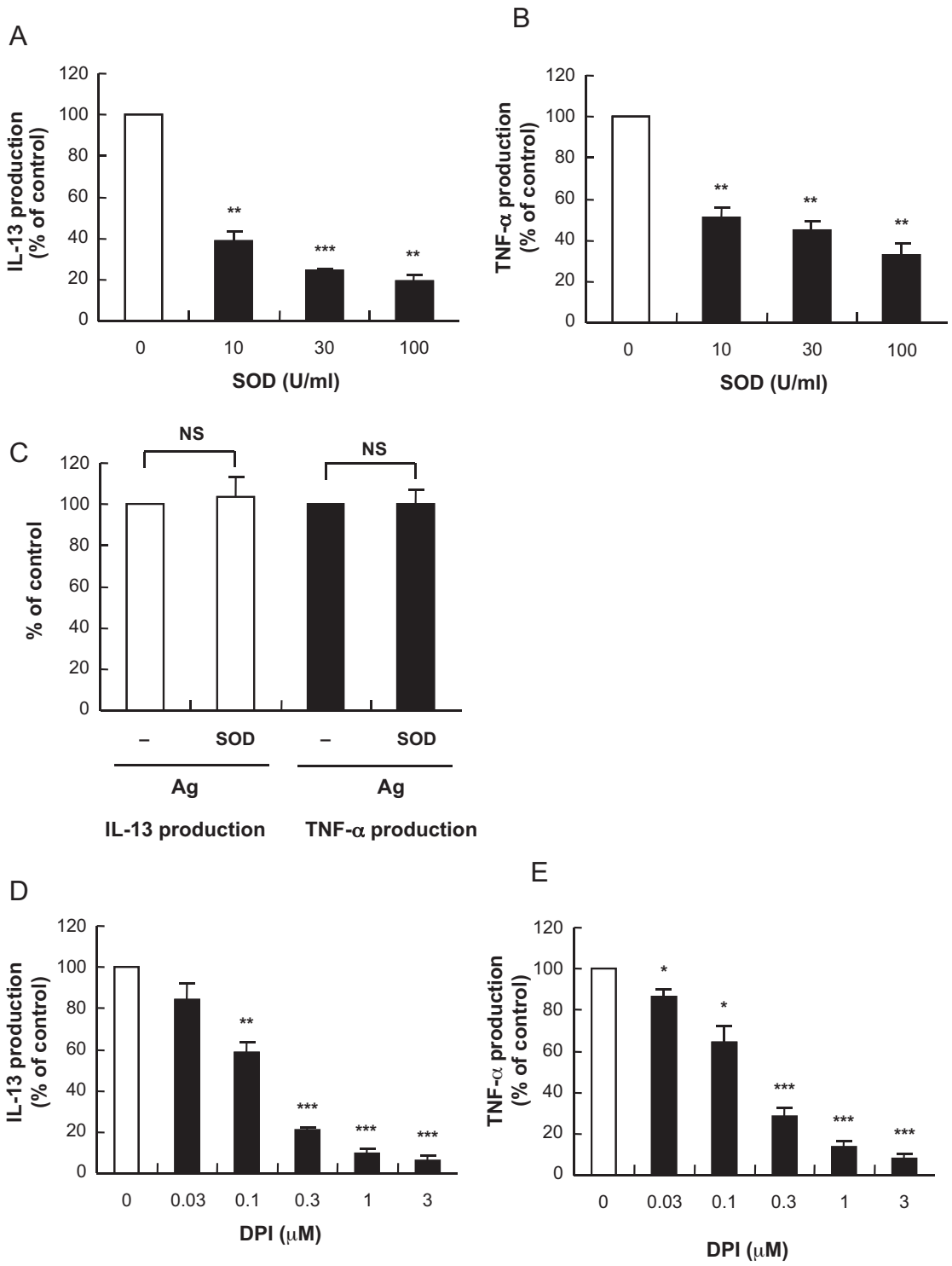

Fig. 6. Extracellular $O_2^{\bullet-}$ mediates the EGCG-induced cytokine production. (A, B, D and E) RBL-2H3 cells (5×10^5 cells/200 μl) were treated with 100 μM EGCG in the presence or absence of SOD (A and B) or DPI (D and E) at the indicated concentrations for 4 h, and the IL-13 (A and D) and TNF-α contents (B and E) in the supernatants were determined by ELISA. The data are expressed as percentages relative to the IL-13 and TNF-α contents in control cells stimulated with EGCG alone (set at 100%), and represent the means ± SE of three or four independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (C) RBL-2H3 cells (5×10^5 cells/200 μl) were sensitized with 0.1 μg/ml anti-TNP IgE overnight and stimulated with 30 ng/ml antigen (Ag) in the presence or absence of 10 U/ml SOD for 4 h, and IL-13 and TNF-α contents in the supernatants were determined by ELISA. The data are expressed as percentages relative to the IL-13 and TNF-α contents in control cells stimulated with antigen alone (set at 100%), and represent the means ± SE of three independent experiments. NS, not significant.

and ebselen strongly blocked the production of cytokines [21], which suggest that intracellular H_2O_2 generation is crucial for Ca^{2+} influx that leads to cytokine production. Recent studies have revealed that wortmannin and ebselen strongly inhibit $Ca_v1.2$ LTCC activity, which is involved in the production of cytokines but not degranulation [16,19]. The present study revealed that the EGCG-induced Ca^{2+} influx was partially dependent on intracellular H_2O_2. Taken together, it was possible that EGCG induced cytokine

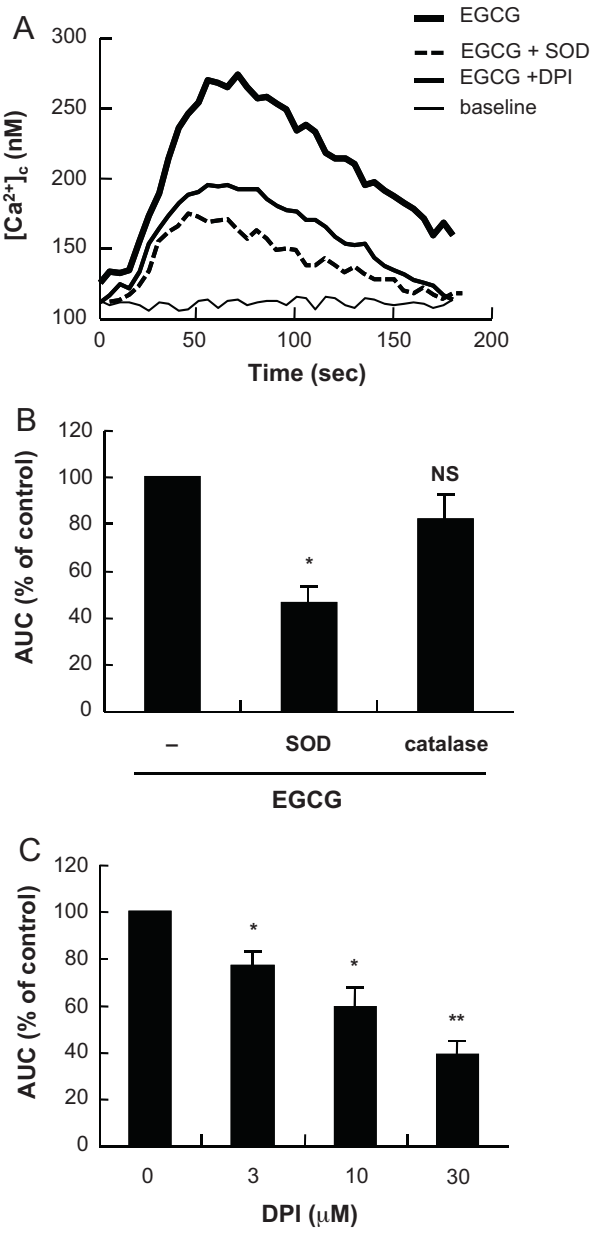

Fig. 7. Extracellular $O_2^{\bullet-}$ mediates the EGCG-induced Ca^{2+} influx. (A) Fluo3/AM-loaded cells (1×10^6 cells/ml) were treated with 100 μM EGCG in the presence or absence of 10 U/ml SOD or 30 μM DPI, and monitored for their Fluo3 fluorescence for up to 3 min using a microplate fluorometer with excitation and emission at 485 and 527 nm, respectively. The data shown as the calculated $[Ca^{2+}]_c$ are representative of three independent experiments. (B and C) Cells were treated with 100 μM EGCG in the presence or absence of 10 U/ml SOD or 10 U/ml catalase (B) or DPI (C) at the indicated concentrations, and monitored for Fluo3 fluorescence. AUC was measured and are expressed as percentages relative to the AUC in control cells treated with EGCG alone (set at 100%), and represent the means ± SE of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; NS, not significant.

production through $Ca_v1.2$ LTCCs. However, our data suggested that the Ca^{2+} influx occurred through pathways that are distinct from the known pathways including $Ca_v1.2$ LTCCs. In accordance with such unique properties, the EGCG-induced Ca^{2+} influx and cytokine production appeared to be mainly regulated by extracellular $O_2^{\bullet-}$, because scavenging of the $O_2^{\bullet-}$ release by exogenous SOD or inhibition of the production specifically blocked them.

It should be noted that EGCG, but not EC, evoked ROS generation. The most likely source of the ROS was mitochondria, because the former, but not the latter, increased $O_2^{\bullet-}$ level within mitochondria [13]. Previously, we reported that EGCG, but not EC, also induces depolarization of $\Delta\Psi_m$ [13]. Taken together that $\Delta\Psi_m$-depolarizing mitochondrial metabolic inhibitors, such as antimycin A and FCCP, increase $O_2^{\bullet-}$ level within mitochondria [21], it is possible that EGCG induces ROS generation by dissipating $\Delta\Psi_m$. In addition, NADPH oxidase also appears to play a role, because its specific inhibitor DPI strongly blocked the oxidative response.

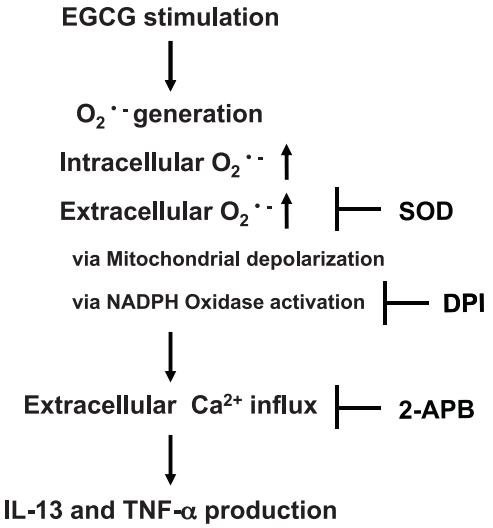

Fig. 8. A proposed model for EGCG-induced cytokine production in mast cells. Stimulation of mast cells with EGCG results in increased intra- and extracellular $O_2^{\bullet-}$ levels via mitochondrial depolarization and NADPH oxidase activation. The extracellular $O_2^{\bullet-}$ in turn stimulates Ca^{2+} influx through a 2-APB, store-independent Ca^{2+} channel, thereby inducing IL-13 and TNF-α production.

In conclusion, the present findings suggest that EGCG can stimulate extracellular $O_2^{\bullet-}$-regulated Ca^{2+} channels, which may be selectively involved in the regulation of cytokine production but have yet to be elucidated (Fig. 8). Further studies that attempt to identify the molecular entities of such $O_2^{\bullet-}$-regulated Ca^{2+} channels are underway.

Acknowledgments

We thank the National Institute of Health Sciences (Japanese Collection of Research Bioresources) for providing the RBL-2H3 cell line (cell number JCRB0023). This work was partially supported by a Grant-in-Aid from the High-Tech Research Center Project (2003–2007) for Private Universities: matching fund subsidy from MEXT, a Grant-in-Aid from MEXT (matching fund subsidy for Private Universities 2007–2009) and Grants-in-Aid from Nihon University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.09.011.

References

[1] Graham HN. Green tea composition, consumption, and polyphenol chemistry. Prev Med 1992;21:334–50.
[2] Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP, Rice-Evans C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidans. Arch Biochem Biophys 1995;322:339–46.
[3] Rice-Evans C. Plant polyphenols: free radical scavengers or chain-breaking antioxidant? Biochem Soc Symp 1995;61:103–16.
[4] Gao YT, McLaughlin JK, Blot WJ, Ji BT, Dai Q, Fraumeni Jr JF. Reduced risk of esophageal cancer associated with green tea consumption. J Natl Cancer Inst 1994;86:855–8.

[5] Mandel S, Youdim MB. Catechin polyphenols: neurodegeneration and neuroprotection in neurodegenerative diseases. Free Radic Biol Med 2004;37:304–17.
[6] Zaveri NT. Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. Life Sci 2006;78:2073–80.
[7] Patra SK, Rizzi F, Silva A, Rugina DO, Bettuzzi S. Molecular targets of (−)-epigallocatechin-3-gallate (EGCG): specificity and interaction with membrane lipid rafts. J Physiol Pharmacol 2008;59(Suppl. 9):217–35.
[8] Tachibana H. Molecular basis for cancer chemoprevention by green tea polyphenol EGCG. Forum Nutr 2009;61:156–69.
[9] Khan N, Afaq F, Saleem M, Ahmad N, Mukhtar H. Targeting multiple signaling pathways by green tea polyphenol (−)-epigallocatechin-3-gallate. Cancer Res 2006;66:2500–5.
[10] Shimizu M, Weinstein IB. Modulation of signal transduction by tea catechins and related phytochemicals. Mutat Res 2005;591:147–60.
[11] Suzuki M, Yoshino K, Maeda-Yamamoto M, Miyase T, Sano M. Inhibitory effects of tea catechins and *O*-methylated derivatives of (−)-epigallocatechin-3-*O*-gallate on mouse type IV allergy. J Agric Food Chem 2000;48:5649–53.
[12] Kinet JP. The high-affinity IgE receptor (FcεRI): from physiology to pathology. Annu Rev Immunol 1999;17:931–72.
[13] Inoue T, Suzuki Y, Ra C. Epigallocatechin-3-gallate inhibits mast cell degranulation, leukotriene C_4 secretion, and calcium influx via mitochondrial calcium dysfunction. Free Radic Biol Med 2010;49:632–40.
[14] Nanjo F, Goto K, Seto R, Suzuki M, Sakai M, Hara Y. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. Free Radic Biol Med 1996;21:895–902.
[15] Suzuki Y, Yoshimaru T, Matsui T, Inoue T, Niide O, Nunomura S, et al. FcεRI signaling of mast cells activates intracellular psoduction of hydrogen peroxide: role in the regulation of calcium signals. J Immunol 2003;171:6119–27.
[16] Yoshimaru T, Suzuki Y, Inoue T, Ra C. L-type Ca^{2+} channels in mast cells: activation by membrane depolarization and distinct roles in regulating mediator release from store-operated Ca^{2+} channels. Mol Immunol 2009;46:1267–77.
[17] Suzuki Y, Inoue T, Yoshimaru T, Ra C. Galectin-3 but not galectin-1 induces mast cell death by oxidative stress and mitochondrial permeability transition. Biochim Biophys Acta 2008;1783:924–34.
[18] Di Capite J, Parekh AB. CRAC channels and Ca^{2+} signaling in mast cells. Immunol Rev 2009;231:45–58.
[19] Suzuki Y, Inoue T, Ra C. L-type Ca^{2+} channels: a new player in the regulation of Ca^{2+} signaling, cell activation and cell survival in immune cells. Mol Immunol 2010;47:640–8.
[20] Suzuki Y, Yoshimaru T, Inoue T, Niide O, Ra C. Role of oxidants in mast cell activation. Chem Immunol Allergy 2005;87:32–42.
[21] Inoue T, Suzuki Y, Yoshimaru T, Ra C. Reactive oxygen species produced up- or downstream of calcium influx regulate proinflammatory mediator release from mast cells: role of NADPH oxidase and mitochondria. Biochim Biophys Acta 2008;1783:789–802.
[22] Suzuki Y, Yoshimaru T, Matsui T, Ra C. Silver activates calcium signals in rat basophilic leukemia-2H3 mast cells by a mechanism that differs from the FcεRI-activated response. J Immunol 2002;169:3954–62.
[23] Yoshimaru T, Suzuki Y, Inoue T, Niide O, Ra C. Silver activates mast cells through reactive oxygen species production and a thiol-sensitive store-independent Ca^{2+} influx. Free Radic Biol Med 2006;40:1949–59.
[24] Parekh AB. Mitochondrial regulation of store-operated CRAC channels. Cell Calcium 2008;44:6–13.
[25] Santo-Domingo J, Demaurex N. Calcium uptake mechanisms of mitochondria. Biochim Biophys Acta 2010;1797:907–12.
[26] Suzuki Y, Yoshimaru T, Inoue T, Ra C. Mitochondrial Ca^{2+} flux is a critical determinant of the Ca^{2+} dependence of mast cell degranulation. J Leukoc Biol 2006;79:508–18.
[27] Togo K, Suzuki Y, Yoshimaru T, Inoue T, Terui T, Ochiai T, et al. Aspirin and salicylates modulate IgE-mediated leukotriene secretion in mast cells through a dihydropyridine receptor-mediated Ca^{2+} influx. Clin Immunol 2009;13:146–56.
[28] Gwack Y, Feske S, Srikanth S, Hogan PG, Rao A. Signalling to transcription: store-operated Ca^{2+} entry and NFAT activation in lymphocytes. Cell Calcium 2007;42:145–56.
[29] Ma HT, Peng Z, Hiragun T, Iwaki S, Gilfillan AM, Beaven MA. Canonical transient receptor potential 5 channel in conjunction with Orai1 and STIM1 allows Sr^{2+} entry, optimal influx of Ca^{2+}, and degranulation in a rat mast cell line. J Immunol 2008;180:2233–9.
[30] Sanchez-Miranda E, Ibarra-Sanchez A, Gonzalez-Espinosa C. Fyn kinase controls FcεRI receptor-operated calcium entry necessary for full degranulation in mast cells. Biochem Biophys Res Commun 2010;391:1714–20.
[31] Suzuki R, Liu X, Olivera A, Aguiniga L, Yamashita Y, Blank U, et al. Loss of TRPC1-mediated Ca^{2+} influx contributes to impaired degranulation in Fyn-deficient mouse bone marrow-derived mast cells. J Leukoc Biol 2010;88:863–75.
[32] Sly LM, Kalesnikoff J, Lam V, Wong D, Song C, Omeis S, et al. G. IgE-induced mast cell survival requires the prolonged generation of reactive oxygen species. J Immunol 2008;181:3850–60.
[33] Suzuki Y, Yoshimaru T, Inoue T, Ra C. Discrete generations of intracellular hydrogen peroxide and superoxide in antigen-stimulated mast cells: reciprocal regulation of store-operated Ca^{2+} channel activity. Mol Immunol 2009;46:2200–9.