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Effect of tea polyphenols on histamine release from rat basophilic leukemia (RBL-2H3) cells: the structure–inhibitory activity relationship

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N. Matsuo[1,2], K. Yamada[1], K. Shoji[1], M. Mori[1], M. Sugano[1]

[1]Laboratory of Food Science, Kyushu University School of Agriculture, Fukuoka 812-81; [2]Department of Biochemistry, Oita Medical University, Oita 879-55, Japan

We studied the effect of tea polyphenols on histamine release from rat basophilic leukemia (RBL-2H3) cells. Among tea polyphenols, (–)-epigallocatechin gallate (EGCG) most strongly and dose-dependently inhibited histamine release from cells stimulated with a calcium ionophore, A23187. (–)-Epigallocatechin (EGC) and (–)-epicatechin gallate (ECG) with a triphenol residue moderately inhibited histamine release, whereas diphenolic (+)-catechin (C) and (–)-epicatechin (EC) did not. The magnitude of the inhibitory effect was in the order EGCG>ECG>EGC. Among simple polyphenols, the triphenol compounds, pyrogallol (PG) and gallic acid (GA) exerted inhibitory activity, but the diphenols, pyrocatechol, hydroquinone, and resorcinol did not. In addition, the mixture of PG and GA inhibited histamine release as strongly as EGCG with two triphenol residues. Similarly, they inhibited histamine release induced by IgE-antigen complex stimulation more efficiently than that induced by A23187 stimulation. EGCG did not inhibit the increase of intracellular Ca^{2+} in RBL-2H3 cells stimulated with A23187 or IgE antigen. These results indicate that the triphenol structure plays an important role in the inhibitory activity of tea polyphenols. Their activity seemed to be exerted through the metabolic events occurring after the elevation of intracellular Ca^{2+} concentration.

Key words: histamine; intracellular Ca^{2+}; mast cells; tea polyphenols.

Noritaka Matsuo
Department of Biochemistry
Oita Medical University
Hasama-machi
Oita 879-55
Japan

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Recently, the incidence of allergic disorders has increased; in particular, hypersensitivity to food or airborne allergens (1–3). Type I allergy includes a series of events: production of antigen-specific IgE, binding of IgE to the $Fc_\varepsilon RI$ receptor on mast cells or basophils, cross-linking of IgE with newly absorbed allergens, and release of chemical mediators such as histamine and leukotrienes from the cells followed by the elevation of intracellular Ca^{2+} concentration (4, 5). Inhibition of any steps of these sequential reactions leads to the attenuation of allergic symptoms. In fact, several agents such as corticosteroids, epinephrine, histamine antagonists, and leukotriene synthesis inhibitors interfere with the above reactions (6).

Some food components have also been reported to affect the immune reactions. For example, egg-yolk lipoproteins and milk proteins enhanced the immunoglobulin production of human lymphocytes (7) or human-human hybridoma (8), and n-3 polyunsaturated fatty acids reduced the production of leukotriene, platelet-activating factor, and IgE (9–12).

Tea polyphenols also have been reported to inhibit chemical mediator release from mast cells (13, 14), in addition to a range of physiologic functions (15–18). In the present study, we examined the effect of various tea polyphenols on histamine release from rat basophilic leukemia (RBL-2H3) cells to determine their structure–activity relationship and mechanism of action.

Material and methods

Chemicals

Tea polyphenols, (+)-catechin (C), (–)-epicatechin (EC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), and (–)-epigallocatechin gallate

(EGCG), were purchased from Kurita Water Industries Ltd (Tokyo, Japan). Other polyphenols, such as pyrocatechol (PC), hydroquinone (HQ), pyrogallol (PG), and gallic acid (GA) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and resorcinol (RE) was obtained from Mitsuwa's Pure Chemicals (Osaka, Japan). The chemical structures of these polyphenols are shown in Fig. 1. A calcium ionophore A23187 and monoclonal anti-DNP IgE were purchased from Sigma Chemical Co. (St Louis, MO, USA) and DNP bovine serum albumin (BSA) from L.S.L. Co. (Tokyo, Japan). Histamine dihydrochloride and *o*-phthalaldehyde (OPT) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Fura2-AM from Dojindo Co. (Kumamoto, Japan). Fura2-AM was dissolved in dimethyl sulfoxide (DMSO) and stored at −30°C. BSA was purchased from Boehringer Mannheim GmbH (Germany). Tyrode buffer, containing 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, and 5.6 mM glucose (pH 7.2), was used for histamine release assay. Ca^{2+} buffer, containing 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml glucose, 1 mg/ml BSA, and 20 mM HEPES (pH 7.4), was used for the intracellular Ca^{2+} concentration assay.

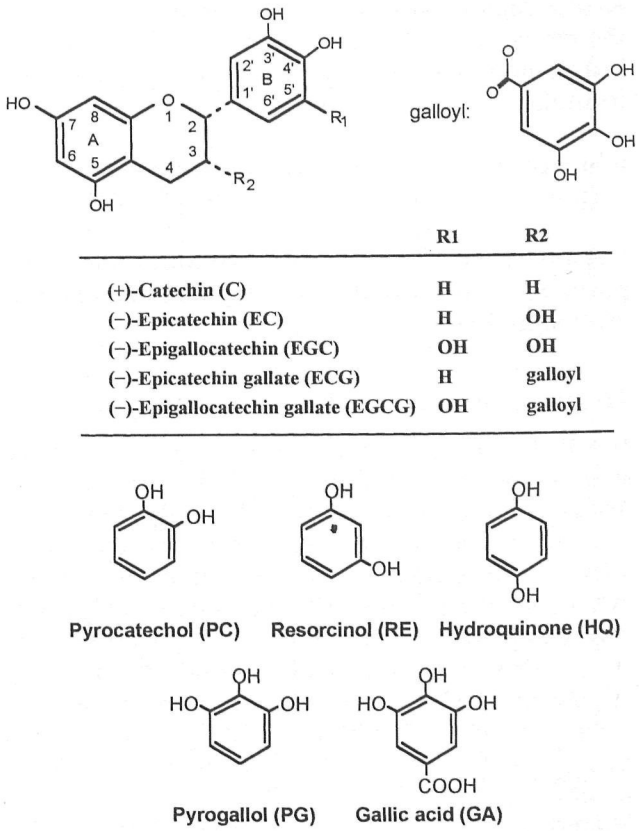

Fig. 1. Structures of polyphenols.

Cells and cell culture

RBL-2H3 cells were provided by the Japanese Cancer Research Resources Bank (Tokyo). Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) (Intergen Co., NY, USA) at 37°C in a humidified atmosphere of 10% CO_2/90% air. After dispersal with 0.05% trypsin dissolved in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 0.5 mM EDTA (trypsin-EDTA), cells were washed and suspended in Tyrode buffer and used for histamine release assay. In order to sensitize the $Fc_\varepsilon RI$ (IgE receptor), cells (1×10^6 cells) were cultured with the fresh medium with 1 μg anti-DNP IgE for 16 h. Cells were then dispersed with trypsin-EDTA and used for IgE-antigen-mediated histamine release assay. Cell viability was measured by trypan blue staining. In this study, viability of the cells treated with polyphenols was more than 95%, indicating the limited toxicity of polyphenols added.

Measurement of histamine

RBL-2H3 cells (1×10^6 cells) were suspended in 2 ml of Tyrode buffer containing 0.9 mM $CaCl_2$ in polypropylene tubes at 4°C. In the preliminary experiments, we examined the dose-response effect of A23187 (1 nM–100 μM) or DNP-BSA (0.1–250 μg/ml) on histamine release. Since 5 μM of A23187 and 100 μg/ml of DNP-BSA gave the maximum histamine release, we used these concentrations to examine the inhibitory activities of tea polyphenols. The cell suspension was mixed with 0.25 ml tea polyphenol solution, and then 0.25 ml of 50 μM (final, 5 μM) A23187 or 1 mg/ml (final, 100 μg/ml) of DNP-BSA solution was added. The mixture was incubated for 20 min at 37°C, and then the reaction was terminated by cooling it for 15 min at 4°C. The cell suspension was then centrifuged at 300 *g* for 10 min, and the histamine content in the supernatant was measured by fluorometric assay (19). To 2 ml of sample solution was added 0.75 g of NaCl and 0.5 ml of 1 N NaOH, followed by 5 ml of the mixture of *n*-butanol and chloroform (3 : 2, v/v), and this was mixed for 5 min. After centrifugation, 4 ml of the organic solvent layer was recovered and mixed with 2 ml *n*-heptane and 1.5 ml 0.1 N HCl. After centrifugation at 270 *g* for 5 min, 1 ml of the HCl layer was recovered, mixed with 0.15 ml of 1 N NaOH and 0.1 ml of 0.2% OPT, and left for 5 min at room temperature. The reaction was terminated by adding 0.14 ml of 0.5 N H_2SO_4, and then fluorescence intensity was measured with a spectrofluorophotometer (RF500, Simadzu Co.,

Kyoto, Japan) with the excitation at 360 nm and the emission at 450 nm. The percent inhibition of histamine release was calculated by the following formula:

inhibitory rate (%)=100 – (test – negative control)/(positive control – negative control)×100

Negative control was the value without stimulation, positive control was after stimulation, and test was after stimulation with sample.

Measurement of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration was measured with a spectrofluorometer (F2000, Hitachi Co., Tokyo, Japan) (20). RBL-2H3 cells were suspended in Ca^{2+} buffer and were then incubated with 2 μM Fura2 acetoxymethyl ester (Fura2-AM) for 40 min at 37°C. The cells (2×10^6 cells/ml) were washed three times, resuspended in fresh buffer in a quartz cuvet, and continuously stirred at 37°C. Cell suspensions were incubated for 15 min at 37°C and then EGCG (100 μM) was added to the cuvet with a microsyringe. After incubation for 5 min at 37°C, cells were challenged with DNP-BSA or A23187. Intracellular Ca^{2+} concentration was obtained from the ratio of fluorescences excited at two wavelengths, 340 and 380 nm. The emission wavelength was 510 nm.

Statistical analysis

Results were expressed as mean±SE (n=3). One-way analysis of variance followed by Duncan's new multiple range test (21) established the exact nature of the differences between the groups.

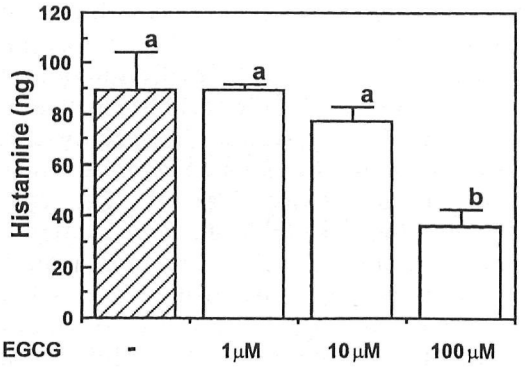

Fig. 2. Dose-dependent effect of EGCG on A23187-mediated histamine release from RBL-2H3 cells. Cells (1×10^6) were stimulated with A23187 for 20 min at 37°C in presence of various concentrations of EGCG (1–100 μM). Histamine content of supernatant was measured by fluorometric assay described in Material and methods. Results are mean±SE (n=3). a, b: Values not sharing common letter were significantly different at P<0.05.

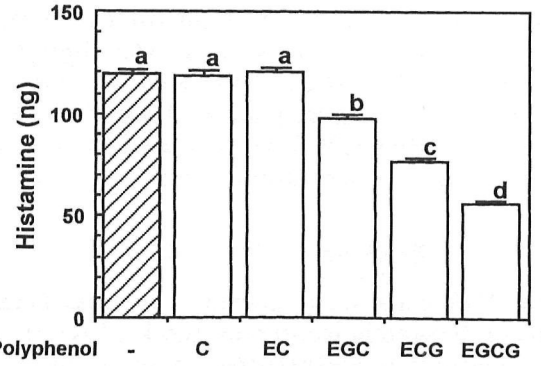

Fig. 3. Effect of various tea polyphenols on A23187-mediated histamine release from RBL-2H3 cells. Cells (1×10^6) were stimulated with A23187 for 20 min at 37°C in presence of various tea polyphenols (100 μM). See legend to Fig. 2. Results are mean±SE (n=3). a–d: Values not sharing common letter were significantly different at P<0.05.

Results

Effect of tea polyphenols on histamine release from RBL-2H3 cells stimulated with A23187

RBL-2H3 cells (1×10^6 cells) were stimulated with A23187 in the presence of 1–100 μM of EGCG to determine the dose-dependent effect of the most abundant tea polyphenol on histamine release. As shown in Fig. 2, EGCG exerted an inhibitory tendency at 10 μM (13% inhibition) and significant inhibition at 100 μM (59% inhibition).

To clarify the structure–activity relationship of tea polyphenols, RBL-2H3 cells were stimulated with A23187 in the presence of various tea polyphenols (100 μM). As shown in Fig. 3, EGC (18% inhibition) and ECG (35% inhibition) significantly inhibited the histamine release as effectively as EGCG (57% inhibition), while C and EC were not effective. These results suggest that the triphenol structure is essential for the expression of the inhibitory activity.

Effect of simple polyphenols on histamine release from RBL-2H3 cells stimulated with A23187

Thus, we examined the effect of simple polyphenols (100 μM) with diphenol or triphenol structure on histamine release from RBL-2H3 cells. As shown in Fig. 4, the diphenols PC, HQ, or RE exerted an inhibitory effect on histamine release from RBL-2H3 cells, but the effect was marginal. However, two triphenols, PG and GA, significantly inhibited histamine release (44% and 46% inhibition, respectively). When the cells were treated with

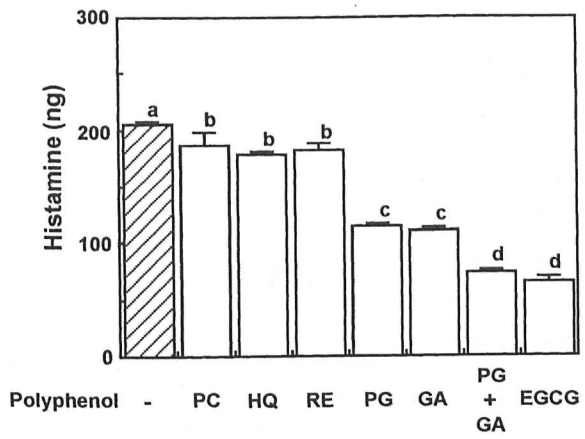

Fig. 4. Effect of various polyphenols on histamine release from RBL-2H3 cells. Cells (1×10^6) were stimulated with A23187 for 20 min in presence of polyphenols (100 μM). See legend to Fig. 2. Results are mean±SE (n=3). a–d: Values not sharing common letter were significantly different at $P<0.05$.

both PG and GA, the inhibitory effect increased (64% inhibition) to a level similar to that of EGCG (68% inhibition).

Effect of various tea polyphenols on histamine release from RBL-2H3 cells stimulated with IgE antigen

The effect of polyphenols on histamine release stimulated with IgE antigen complex was examined. As shown in Fig. 5A, EGCG dose-dependently inhibited histamine release, as seen in A23187-mediated histamine release (Fig. 2). The inhibition of histamine release by EGCG was 55% at 10 μM and 90% at 100 μM. Among tea polyphenols, EGC and ECG significantly inhibited the IgE-antigen-mediated histamine release (Fig. 5B). The inhibitory effects of histamine release by EGC and ECG were 45% and 59%, respectively. When the cells were stimulated with IgE-antigen complex, the inhibitory effect of tea polyphenols was stronger than that observed in A23187 stimulation.

Effect of EGCG on intracellular free Ca^{2+} concentration

For investigation of the inhibitory mechanism of EGCG, the effect of EGCG on intracellular free Ca^{2+} in RBL-2H3 cells was examined. After loading Fura2-AM, RBL-2H3 cells were incubated in the presence of 100 μM EGCG and challenged with DNP-BSA or A23187. As shown in Fig. 6, EGCG could not inhibit the increase of intracellular free Ca^{2+} concentration in RBL-2H3 cells after challenge not only by A23187 but also by DNP-BSA antigen.

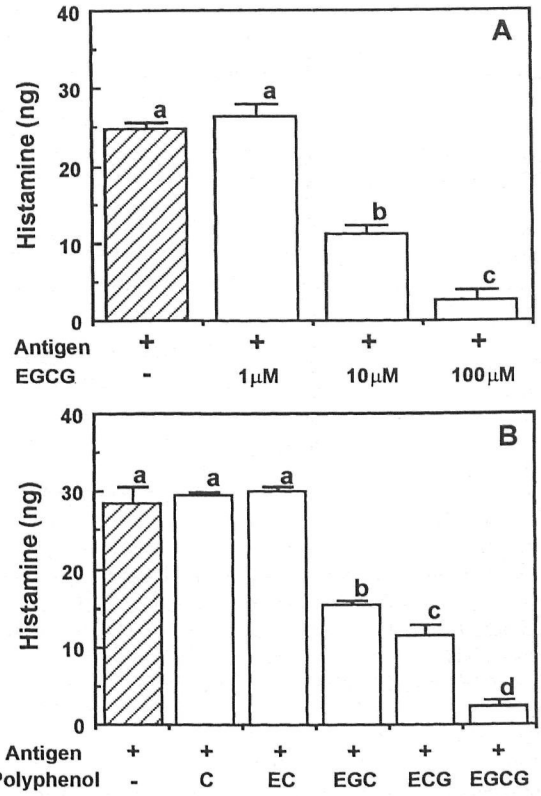

Fig. 5. Effect of tea polyphenols on IgE antigen-mediated histamine release from RBL-2H3 cells. Cells were cultured with anti-DNP-IgE antibody (1 μg/1×10^6 cells) for 16 h. After dispersal with trypsin-EDTA/PBS, cells (1×10^6) were resuspended in Tyrode buffer. Suspension was added to DNP-BSA antigen and incubated for 20 min in presence of tea polyphenols. A) Dose-dependent effect of EGCG on histamine release from RBL-2H3 cells. B) Effect of tea polyphenols (100 μM) on histamine release from RBL-2H3 cells. Results are mean±SE (n=3). a–d: Values not sharing common letter were significantly different at $P<0.05$.

Discussion

Mast cells play an important role in the development of allergic symptoms through the production and release of chemical mediators. When antigen-specific IgEs bound to the $Fc_\varepsilon RI$ receptor on mast cells were cross-linked with newly absorbed allergens, chemical mediators were produced and released from the cells (4). Chemical mediators cause various pathophysiologic events in acute allergic reactions, such as increase in vascular permeability, induction of bronchial smooth-muscle contraction or mucus production, and neutrophil chemotaxis (22–24). Thus, it is important to reduce the mediator release for the prevention and/or alleviation of allergic symptoms.

Tea polyphenols have flavan-3-ol structures with A and B rings (Fig. 1). Among them, triphenol compounds such as EGC, ECG, and EGCG inhibited histamine release, while C and EC with the

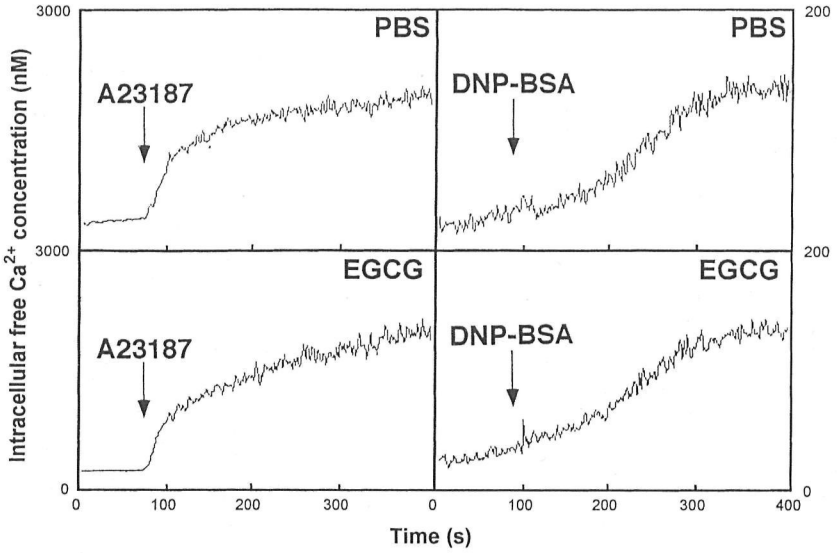

Fig. 6. Effect of EGCG on intracellular Ca^{2+} concentration in RBL-2H3 cells. Cells were cultured with anti-DNP-IgE antibody for 16 h. After dispersal with trypsin-EDTA/PBS, cells were resuspended with Ca^{2+} buffer in quartz cuvet. Cell suspensions were incubated for 15 min at 37°C, and then 100 μM EGCG was added to cuvet. After incubation for 5 min at 37°C, cells were challenged with DNP-BSA or A23187. Intracellular free Ca^{2+} concentration was obtained from ratio of fluorescence excited at two wavelengths, 340 and 380 nm. Emission wavelength was 510 nm.

diphenol structure did not. In addition, EGCG with two triphenol structures exerted stronger activity than EGC and ECG having one triphenol structure. A similar structure–activity relationship of tea polyphenols has been reported in the transformed cell-specific toxicity (25). EGCG and EGC exerted higher toxicity against rat diploid 3Y1 fibroblasts transformed by the E1A gene of human adenovirus than against parental 3Y1 cells. On the other hand, the toxicity of EC was much weaker than the triphenol derivatives. Agarwal et al. (18) also reported that the magnitude of the inhibitory effect of tea polyphenols on TPA-induced ornithine decarboxylase induction is in the order of EGCG>EGC=ECG>EC. In this study, the simple triphenols PG and GA inhibited histamine release, but the effects of the diphenols PC, HQ, and RE were negligible. In addition, PG when mixed with GA inhibited the release at a level similar to that of EGCG. These observations indicate that the flavan-3-ol structure and diphenol moiety of tea polyphenols are not responsible for inhibition of histamine release, while the triphenol compound or triphenol moiety are necessary for the expression of this inhibition.

Tea polyphenols inhibited histamine release in both nonspecific stimulation by A23187 and antigen-specific stimulation by the antigen-IgE complex with anti-DNP IgE and DNP-BSA. Their inhibitory effect on histamine release from RBL-2H3 cells stimulated by IgE antigen was much stronger than that observed in A23187-stimulation, suggesting their possible usefulness for the prevention of allergen-IgE-induced allergic reactions.

Mast cells produce and release two types of chemical mediators. Histamine is released from the intracellular secretory granules induced by the elevation of intracellular Ca^{2+} concentration and the activation of the protein kinase C (26–28). On the other hand, leukotrienes and prostaglandins are generated by the activation of phospholipase A_2, an intracellular, Ca^{2+}-dependent enzyme, an effect which is followed by the oxidation of fatty acids with lipoxygenase or cyclooxygenase (24, 29). Several lipoxygenase inhibitors inhibit the release of leukotriene from mast cells but have no effect on histamine release (30, 31). Since PG with a triphenol structure inhibits lipoxygenase activity and interferes with the release of newly generated mediators (32), tea polyphenols may suppress leukotriene release through the inhibition of lipoxygenase activity. In fact, we previously reported that tea polyphenols inhibit histamine and leukotriene B_4 release from rat peritoneal exudate cells (14). However, the inhibition of histamine release cannot be explained by the inhibition of lipoxygenase activity. It is possible that tea polyphenols affect common pathways involved in the release of these two types of mediators such as membrane fluidity and the increase of intracellular free Ca^{2+} concentration. However, EGCG did not inhibit the increase of intracellular free Ca^{2+} in RBL-2H3 cells induced either by A23187 or by cross-linkage of

IgE antigens. Since the intracellular Ca^{2+} concentration measured by intracellular Ca^{2+} indicators not only detects Ca^{2+} uptake but also reflects the intracellular free Ca^{2+} concentration, which can also be derived from stores, it is possible that intracellular events influence intracellular Ca^{2+} increase. Thus, EGCG may not interfere with the processes involved in the increase of intracellular free Ca^{2+} concentration, such as activation of phospholipase C, and generation of inositol 1,4-isophosphate, inositol 1,4,5-triphosphate, and 1,2-diacylglycerol. Therefore, tea polyphenols may affect the metabolic events that occur after increased intracellular free Ca^{2+} concentration.

Green tea is commonly drunk daily in Asian countries such as Japan, and this tea reduces or prevents a range of diseases such as atherosclerosis (17) and cancer (33–35). Fresh green tea leaf contains polyphenols (36%, in dry weight), carbohydrate (25%), protein (15%), amino acids (4%), and organic acids (1.5%) (36). Thus, tea polyphenols are the most abundant components, and they have diverse pharmacologic effects, antioxidative potential (15), antibacterial and antifungal activities (16), antiatherosclerotic effect (17), and antitumor activity (18, 33–35). Among tea polyphenols, EGCG is the most abundant component (40–60%), followed by EGC (10–20%), ECG (10–20%), EC (4–6%), and C (2–4%) (36). In the present study, EGCG, ECG, and EGC showed an inhibitory effect on histamine release from RBL-2H3 cells. A cup (200 ml) of green tea contains about 250 mg of tea polyphenols, especially about 140 mg of EGCG (35). Hackett & Griffiths (37) reported that orally administered catechin was absorbed and detected in serum, liver, lung, and skin in rats. In addition, EGCG was also detected in rat plasma after oral administration (38). In man, at least 10–20% of orally administered catechins was absorbed and finally excreted in urine and feces (39). Although it is difficult to correlate the doses needed *in vitro* to the concentrations after ingestion of green tea, the available information suggests that tea polyphenols, especially EGCG, exert antiallergic effects even *in vivo* through the inhibition of chemical mediator release from mast cells. Further studies on the antiallergic effect of tea polyphenols *in vivo* deserve attention.

Acknowledgments

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