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Inhibitory effects of epigallocatechin gallate on compound 48/80-induced mast cell activation and passive cutaneous anaphylaxis

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Abbreviation: C48/80, compound 48/80; EC, epicatechin; ECG, epicatechin gallate, EGC, epigallocatechin; EGCG, epigallocatechin gallate; PCA, passive cutaneous anaphylaxis; RPMC, rat peritoneal mast cells

Abstract

Epigallocatechin gallate (EGCG) is a principle phenolic antioxidant found in a variety of plants, including green and black tea. The anti-allergic effect of EGCG is unknown. The purpose of this study is to investigate the effects of EGCG on compound 48/80-induced mast cell activation and passive cutaneous anaphylaxis. For this, the influences of EGCG on the compound 48/80-induced cutaneous reaction were measured *in vivo* and the effects of EGCG on the compound 48/80-induced mast cell activations were examined *in vitro*. Results are below: as 1) EGCG significantly inhibited compound 48/80-induced passive cutaneous anaphylaxis, 2) the compound 48/80-induced degranulation, calcium influx and histamine release of rat peritoneal mast cells (RPMCs) were significantly inhibited by the pretreatment with EGCG, and 3) the compound 48/80-mediated inhibition of cAMP level in RPMCs was significantly increased by the pretreatment with EGCG. These results suggested that EGCG, the most abundant polyphenol in green tea, inhibits the compound 48/80-induced mast cell activation and the increase of vascular permeability, and potentially serve as effective therapeutic tools for allergic diseases.

Keywords: calcium signaling; cell degranulation; cyclic AMP; epigallocatechin gallate; histamine; mast cell

Introduction

Mast cells are known to play a crucial role in the development of many physiological changes during an immediate allergic response (Lantz *et al*, 1998). Activation of mast cells, the key cells of allergic inflammatory reactions, occurs in response to a challenge by a specific antigen against which the surface IgE is directed, or by other IgE-directed ligands. In IgE-mediated activation, a variety of biological substances (products of complement activation, neuropeptides and certain cytokines), pharmacological compounds (calcium ionophore, compound 48/80, and mellitin), and physical stimuli can elicit the release of mast cell mediators (Chai *et al.*, 2000, 2001; Platts-Mills, 2001). These mediators ultimately cause the various symptoms of allergy including dermatitis and asthma.

Green tea, derived from the plant *Camellia sinesis* (an evergreen shrub of the theaceae family), is one of the most popularly consumed beverages in the world. Green tea, specially green tea catechins, shows the pharmacological effects such as anticarcinogenic activity (Ogata *et al.*, 1995; Stoner and Mukhtar, 1995), antioxidant activity (Han, 2003; Kim *et al.*, 2004), anticarcinogenic and related dental activity (Hattori *et al.*, 1990; Saeki *et al.*, 1993), antimicrobial activity (Ikigai *et al.*, 1993), and prevention of cardiovascular disease (Uchida *et al.*, 1995; Yokozawa *et al.*, 1995). Most of the polyphenols in green tea are flavanols, commonly known as catechins: the major catechins in green tea are (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin-3-gallate (EGCG) (Ahmad *et al.*, 1998).

EGCG, the most abundant polyphenol in green tea, is known to induce apoptosis and cell cycle arrest in tumor cells (Ahmad *et al.*, 1997). Other studies showed that this compound inhibits urokinase activity (Jankun *et al.*, 1997), cell proliferation (Asano *et al.*, 1997), mitogen-activated protein kinase activation (Ahn *et al.*, 1999), and lipoxygenase and cyclooxygenase activities (Stoner an Mukhtar, 1995). ECG,

EGC, and EGCG have recently shown to inhibit antigen- or a calcium ionophore A23187-induced histamine release from RBL-2H3 (Matsuo *et al.*, 1996; 1997). However, the mechanism of the inhibition is poorly understood. Since mast cells are activated to release chemical mediators, and calcium and cAMP are shown to play a pivotal role in the mast cell activations, we assessed the effect of ECGC on the calcium uptake and change of intracellular cAMP in mast cells. In this paper we show that EGCG inhibits the compound 48/80-induced mast cell activations and the increased vascular permeability while cAMP level is increased.

Materials and Methods

Materials

Compound 48/80, bovine serum albumin (BSA), Evans blue, and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). Percoll solution was purchased from Pharmacia (Uppsala, Sweden). EGCG were purchased from Cyman Chemical (Ann Arbor, MI). ^{14}C, ^{45}Ca and cAMP kit were purchased from Perkin Elmer Life Science Inc. (Boston, MA). Male Sprague-Dawley rats aged 10 weeks purchased from Korean Damool Science (Taejeon, Chungnam, Korea). They were housed the experiments in a laminar flow cabinet and article lighting conditions with 12-hr d/night cycle and had access to food and water *ad libitum*.

Passive cutaneous anaphylaxis (PCA)

Evans blue dye was prepared as a 0.5% solution (w/v) in sterilized saline. Test compounds were prepared in saline. Male Sprague-Dawley rats were light anesthetized with ether, back skin hair was shaved, and rats were injected intradermally with 50 μl per site of the various concentrations of EGCG. After 10 min, rats were injected intradermally with 250 ng per site of compound 48/80. As a control, the dorsal skin site of these rats was injected with saline alone. Thirty min after the intravenous injection of Evans blue, all rats were sacrificed and the dorsal skin was removed to measure the pigment area. The amount of dye in the tissue was then determined colorimetrically after extraction with 1 ml of formamide, according to the method outlined by Fleming *et al*. (1997). The absorbent intensity of the extraction was measured at 620 nm in a spectrofluorometer (Spectra MAX PLUS, Molecular Devices, Sunnyvale, CA), and the amount of dye was calculated with the Evans blue standard curve.

Preparation of rat peritoneal mast cells (RPMCs) suspension and microscopic observation

RPMCs were isolated as previously described (Cochrane and Douglas, 1974). In brief, rats were anesthetized with ether and injected with 10 ml of calcium-free HEPES-Tyrode buffer (136 mM NaCl, 5 mM KCl, 11 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 2.75 mM $MgCl_2$, 5.4 mM HEPES, 1.0 mg/ml bovine serum albumin, 1.0 mg/ml glucose, 0.1 mg/ml heparin) into the peritoneal cavity, and the abdomen was gently massaged for 90s. The peritoneal cavity was opened, and the fluid was aspirated using a Pasteur pipette, and RPMCs were purified by a percoll density gradient as described in detail elsewhere (Hachisuka *et al.*, 1988). Purified mast cells (1×10^6 cells/ml) were resuspended in HEPES-Tyrode buffer. Mast cells were observed under phase contrast and photographed as described. RPMCs preparations were at least 95% pure and at least 98% of these cells were viable as assessed by trypan blue exclusion (Mascotti *et al.*, 2000).

Assay of histamine release

Mast cell suspensions (200 μl) were preincubated with the various concentrations of EGCG (25 μl) for 10 min at 37°C and then incubated with compound 48/80 (250 ng in 25 μl). The reaction was stopped by cooling the tubes in ice bath. The released histamine were separated from the cells by centrifugation at 150 *g* for 10 min at 4°C. Histamine present in the cells was released by disrupting the cells with boiling and centrifugation. Histamine content was measured by the radioenzymatic method described by Harvima *et al.,* (1988). The inhibition percentage of the histamine release was calculated using the following formula: % Inhibition = [(Histamine release without EGCG - Histamine release with EGCG) / Histamine release without EGCG] $\times$ 100.

Measurement of ^{45}Ca uptake

The calcium uptake of mast cells was measured according to the method described by Chai *et al.,* (2001). Mast cells were suspended in HEPES-Tyrode buffer containing ^{45}Ca (1.5 MCi/ml; 1 Ci = 3.7×10^{10} becquerels), and incubated for 10 min at 4°C. Mast cell suspensions (200 μl) were incubated with the various concentrations of EGCG (25 μl) for 10 min at 37°C and then with compound 48/80 (250 ng in 25 μl) for 20 min. The reaction was stopped by the addition of 1mM lanthanuim chloride and washed 3 times with HEPES-Tyrode buffer at 4°C for 10 min. The cells were disrupted with 10% Triton X-100 by vigorous shaking. Radioactivity in the solution was measured in a scintillation β-counter.

Measurement of cyclic adenosine-3',5' monophosphate (cAMP) level

The cAMP level was measured according to Holmegaard's method (1982). In brief, mast cell suspensions were added to an equivalent vol (200 μl) of prewarmed buffer containing the drug in an Eppendorf tube. The reaction was allowed to proceed for discrete time intervals, terminated by centrifugation, and then added 250 μl of 50 mM sodium acetate buffer (pH 6.2) under vigous vortexing, followed by snap frozen in liquid nitrogen. The frozen samples were thawed and vortexed, the debris was sedimented in a centrifuge (400 *g* at 4°C, for 10 min.). The cAMP level in the supernatant was determined by radioimmunoassay using a Rianen assay system (Boston, MA).

Statistical analysis

The data were expressed as mean ± SEM. Student's *t*-test was used to make a statistical comparison between the groups. Results with $P < 0.05$ were considered statistically significant.

Results

To assess the contribution of EGCG in passive cutaneous anaphylaxis, we first used the *in vivo* model of PCA. The PCA was best visualized by the extravasation of dye. As shown in Figure 1, compound 48/80 induced the extravasation of Evans blue. However the intradermal administration of EGCG resulted in the inhibition of PCA reactions in a dose-dependent manner, especially 50 μM EGCG showed the marked inhibition on compound 48/80-induced extravasation of Evans blue (Figure 1). In addition, trypan blue exclusion test revealed that the EGCG ranging from 10 μM to 50 μM had no cytotoxicity on RPMCs (Figure 2).

To investigate the inhibitory mechanism of EGCG on anaphylactic reactions, we examined compound 48/80-induced mast cell activation. First of all, inhibitory effects of EGCG on compound 48/80-induced mast cell degranulation were examined. Inverted microscopy technique showed that control RPMCs were generally spherical, or oval, and that had many fine granules surrounding a prominent nucleus (Figure 3A). The spherical shape is 93.8% of population, the degranulation rate of control RPMCs was 6.2%. After stimulation with compound 48/80, the cell became swollen and had many vacuoles and extruded granules near the cell surface and in the surrounding medium, which is interpreted as mast cell degranulation (Figure 3B). The degranulation rate of compound 48/80-treated RPMCs was 92%. When RPMCs were incubated with EGCG, RPMCs were showed similar to that seen with the control RPMCs (Figure 3C). The degranulation rate of RPMCs by EGCG was 6.5%. After the stimulation of EGCG-preincubated RPMCs with compound 48/80, the cells became swollen with an irregular boundary, but were not degranulated (Figure 3D). The degranulation rates of RPMCs treated with EGCG plus compound 48/80 was 7.2%. At 25 μM of EGCG, the inhibition rate of degranulation rate was 95%. Inhibitory effects of EGCG on compound 48/80-induced histamine release from RPMCs were also examined (Figure 4). Compound 48/80 induced the histamine release from RPMCs. EGCG alone had no effect on the spontan-

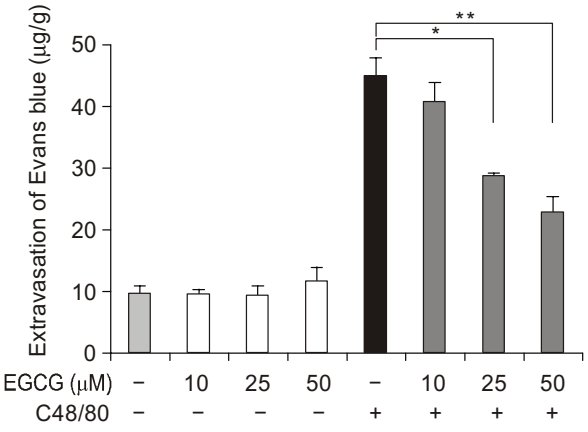

Figure 1. Inhibitory effect of Epigallocatechin Gallate (EGCG) on the compound 48/80 (C48/80)-induced passive cutaneous anaphylaxis. *$P < 0.05$, **$P < 0.01$.

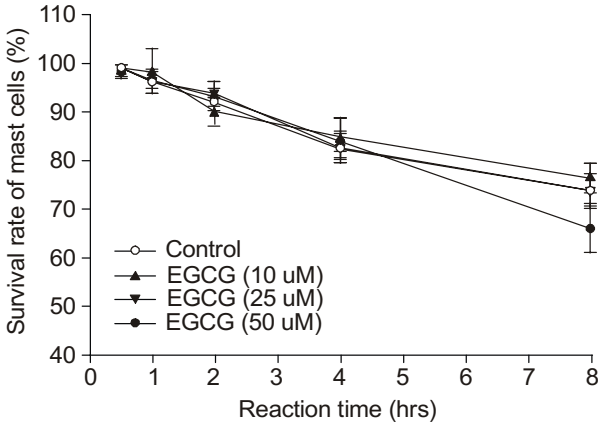

Figure 2. The cytotoxicity of Epigallocatechin Gallate (EGCG) in rat peritoneal mast cells by using trypan blue test. There were no significant differences in the survival rate of mast cells among all experimental groups.

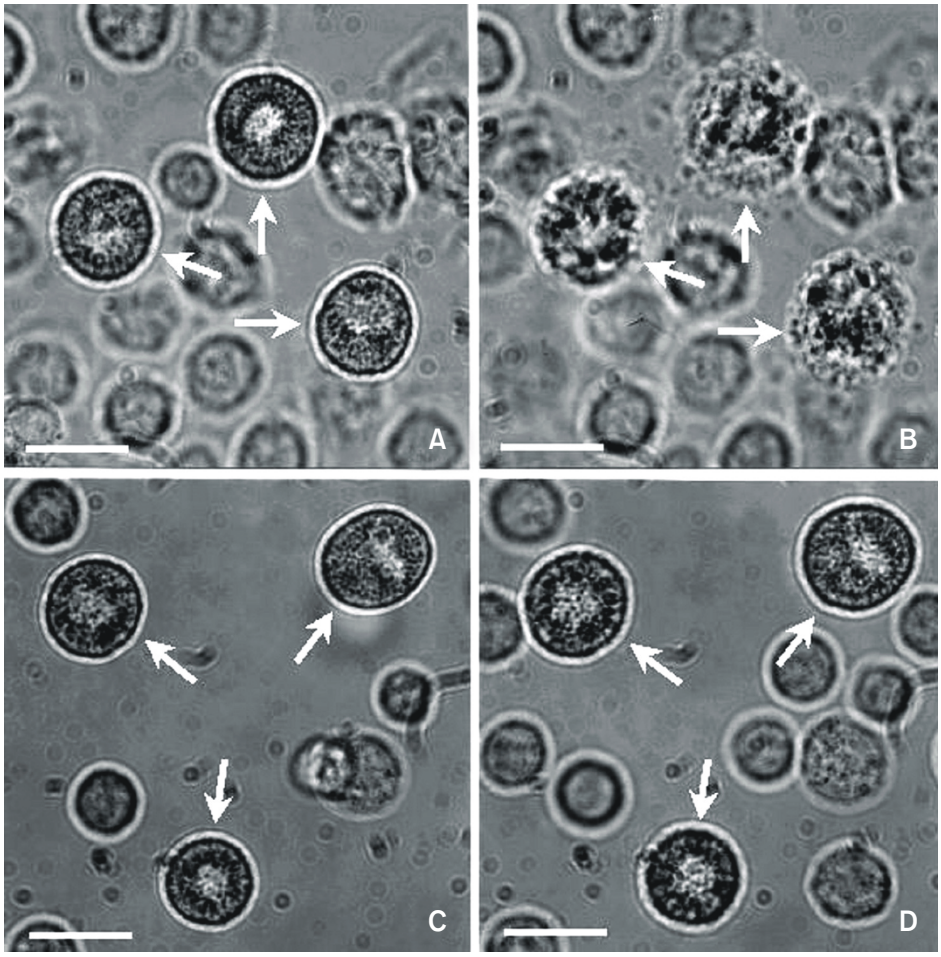

Figure 3. Light microphotographs using inverted microscopy of rat peritoneal mast cells (RPMCs) in HEPES-Tyrode buffer (A), after stimulation with 10 μg/ml compound 48/80 (B), 10 μM Epigallocatechin Gallate (EGCG) (C), and after being pretreated with 10 μM EGCG prior to the stimulation with 10 μg/ml compound 48/80 (D). Normal RPMCs are generally characterized by round shape with fine granules and regular surface. Degranulated mast cells become swollen and have many vacuoles, an irregular surface and extruded granules. However, compound 48/80 did not induce degranulation of RPMCs pretreated with EGCG. Bar = 10 μM.

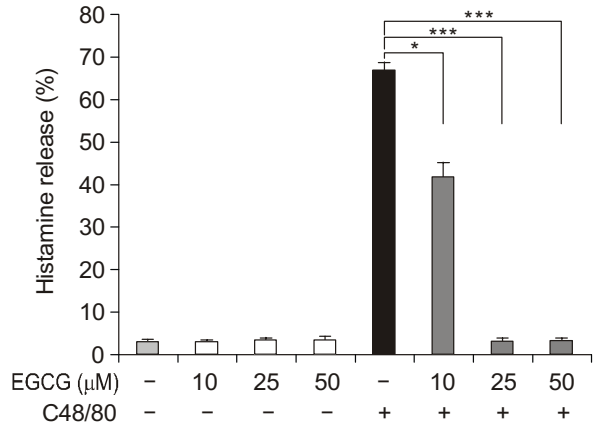

Figure 4. Inhibitory effect of Epigallocatechin Gallate (EGCG) on the compound 48/80 (C48/80)-induced histamine release from rat peritoneal mast cells. *$P < 0.05$, ***$P < 0.001$.

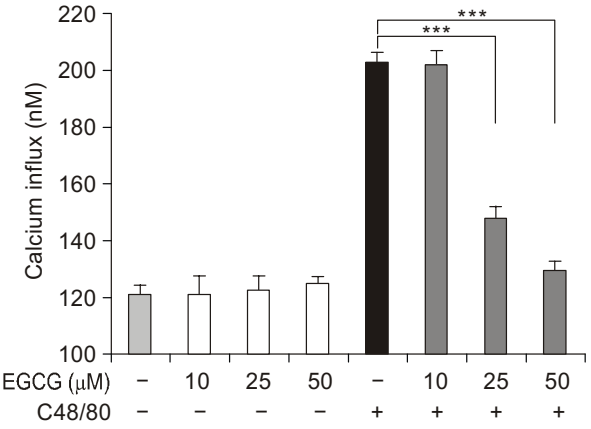

Figure 5. Inhibitory effect of Epigallocatechin Gallate (EGCG) on the compound 48/80 (C48/80)-induced calcium uptake into the rat peritoneal mast cells. ***$P < 0.001$.

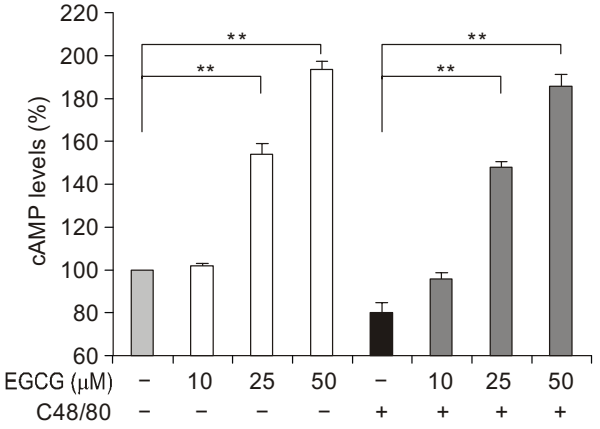

Figure 6. Inhibitory effect of Epigallocatechin Gallate (EGCG) on the compound 48/80 (C48/80)-induced decrease of cAMP levels in the rat peritoneal mast cells. $^{**}P < 0.01$.

eous histamine release. However, EGCG dose-dependently inhibited the compound 48/80-induced histamine release (Figure 4). The histamine release was reduced by 40% at 10 μM and 100% at 50 μM EGCG from RPMCs. These results indicated that EGCG significantly inhibits the compound 48/80-induced PCA via inhibiting the compound 48/80-induced mast cell degranulation and histamine release from RPMCs.

The close correlations among the concentration of the histamine-releasing stimulus, calcium ion influx, and the amount of released histamine suggest a cause- and effect-relationship between the influx of calcium and release of histamine. And the release of histamine is depressed by an increase in the intracellular cAMP. So, we next examined the effect of EGCG on second messenger such as calcium and cAMP. EGCG alone did not affect the calcium uptake into RPMCs, and compound 48/80 induced the calcium uptake into the RPMCs. However, EGCG inhibited the compound 48/80-induced calcium uptake into RPMCs in a concentration-dependent manner (Figure 5). The calcium uptake into RPMCs was inhibited significantly at 25 μM EGCG and almost completely inhibited by the administration of 50 μM EGCG.

EGCG itself increased the cAMP level in RPMCs, 50 μM EGCG increased more 2-fold increase in the cAMP level than those in control cells. RPMCs treated with compound 48/80 caused less than 2-fold decrease in the cAMP level as compared to those treated with buffer only. But EGCG inhibited the compound 48/80-induced cAMP reduction of RPMCs (Figure 6). These results suggest that EGCG contains an activity to inhibit the compound 48/80-induced mast cell activations and vascular permeability, via blocking of calcium uptake into RPMCs, or increasing of cAMP level in RPMCs.

Discussion

Green tea is the unprocessed dried young leaves of *Camellia sinesis*, also known as *Thea sinensis* L, which is widely consumed beverage in the world. Polyphenolic compounds such as catechins are ubiquitously found in green tea (Graham, 1992). The main polyphenolic compounds found in green tea are EC, ECG, EGC, and EGCG (Ahmad *et al.*, 1998). These polyphenolic compounds exhibit protective effects against bacterial infection, tumor promotion and progression (Gao *et al.*, 1994; Aucamp *et al.*, 1997). ECG, EGC, EGCG are recently shown to inhibit the antigen- or calcium-ionophore A23187-induced histamine release from RBL-2H3 (Matsuo *et al.*, 1996; 1997).

We examined the effect of EGCG on PCA induced by compound 48/80. Compound 48/80 induced extravasation of Evans blue. However intradermal administration of EGCG resulted in the inhibition of PCA reactions in a dose-dependent manner, especially 50 μM EGCG showed a marked inhibition on the compound 48/80-induced extravasation of Evans blue. In addition, Trypan blue exclusion test revealed that the EGCG ranging from 10 μM to 50 μM had no cytotoxicity on RPMCs.

To investigate the inhibitory mechanism of EGCG on PCA, we next examined the compound 48/80-induced mast cell activation. After the stimulation with compound 48/80, the cell became swollen and exhibited many vacuoles and extruded granules near the cell surface and in the surrounding medium. Compound 48/80 induced mast cell degranulation and histamine release from RPMCs. it is well known that the stimulation of mast cells with compound 48/80 initiates the activation of a signal-transduction pathway, which leads to histamine release (Mousli *et al.*, 1990). Compound 48/80 stimulates the activity of protein tyrosine kinases, leading to the enhanced tyrosine phosphorylation of a number of cellular proteins and to the activation of both the p42 and p44 mitogen-activated protein kinase. Activation of the protein tyrosine kinases pathway occurred via a mechanism that involves protein kinase, phosphatidylinositol 3-kinase, and Ca^{2+} as intermediates (Shefler and Sagi-Eisenberg, 2001). Thus, application of compound 48/80 and other cationic secretagogues transiently increase intracellular Ca^{2+} concentrations through G protein-mediated activation of phospholipase C. The calcium influx has two main results (Daniele, 1989). Firstly, there is an exocytosis of granule content with the release of preformed mediators, the major one being histamine. Secondly, there is the induction of synthesis of newly formed mediators from arachidonic acid leading to the production of prostaglandins and leukotrienes. EGCG alone had no effect on the mast

cell degranulation and the spontaneous histamine release. However, EGCG dose-dependently inhibited the compound 48/80-induced mast cell degranulation and the histamine release from RPMCs.

The close correlations among the concentration of the histamine-releasing stimulus, calcium ion influx (Raison *et al.*, 1999), and the amount of released histamine suggest a cause- and effect-relationship between the influx of calcium and release of histamine (Hachisuka *et al.*, 1988; Daniele, 1989; Lorenz *et al.*, 1998). And the release of histamine is depressed by an increase in the intracellular cAMP (Makino *et al.*, 1987; Alfonso *et al.*, 2000; Sarkar *et al.*, 2003). So, we examined the effect of EGCG on second messenger such as calcium and cAMP. EGCG alone did not affect the calcium uptake into RPMCs, and compound 48/80 induced the calcium uptake into RPMCs. However, EGCG inhibited the compound 48/80-induced calcium uptake into RPMCs in a concentration-dependent manner. EGCG itself increased the cAMP level in RPMCs, 50 μM EGCG increased more 2-fold increase in the cAMP level than those in control cells. RPMCs treated with compound 48/80 caused less than 2-fold decrease in the cAMP level as compared to these treated with buffer only. But EGCG inhibited the compound 48/80-induced cAMP reduction of RPMCs. The release of histamine is depressed by an increase in the intracellular cAMP content due to the activation of adenylate cyclase or inhibitor of cAMP phosphodiesterase (Makino *et al.*, 1987; Alfonso *et al.*, 2000; Sarkar *et al.*, 2003). The inhibitory effect of EGCG on mast cell degranulation and histamine release from mast cells may be related to the prevention of an increase of intracellular calcium content owing to elevation of the intracellular cAMP level by increase of adenylate cyclase activity or inhibition of the cAMP phosphodiesterase.

Drinking green tea daily would contribute to maintaining plasma catechin levels sufficient to exert antioxidant and anti-allergic activity in blood circulation system (Nakagawa *et al.*, 1997). Kang and colleagues reported that 121.3 mg of EGCG was obtained from 5 g of dry green tea by extracting in 50^{o}C water (Kang *et al.*, 1999). Also, Japanese epidemiologists reported that one cup of green tea infusion contained 100-200 mg of polyphenolic compounds (Kono *et al.*, 1988). Therefore, the suggested daily dosage of polyphenols is 240 to 320 mg, equal to about 3 cups of green tea.

In conclusion, we show that EGCG can inhibit the histamine release from RPMCs mainly by inhibiting calcium uptake and increasing of cAMP level, a critical event in the signal transduction leading to secretion. These findings suggest that EGCG, the most abundant polyphenol in green tea, potentially serve as effective therapeutic tools for allergic diseases.

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