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Effect of Purified Green Tea Catechins on Cytosolic Phospholipase A_2 and Arachidonic Acid Release in Human Gastrointestinal Cancer Cell Lines

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Abstract Ingestion of green tea has been shown to decrease prostaglandin E_2 levels in human colorectum, suggesting that tea constituents modulate arachidonic acid metabolism. In the present study, we investigated the effects of four purified green tea catechins, (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG), and (-)-epicatechin-3-gallate (ECG), on the catalytic activity of cytosolic phospholipase A_2 ($cPLA_2$) and release of arachidonic acid and its metabolites from intact cells. At 50 μM, EGCG and ECG inhibited $cPLA_2$ activity by 19 and 37%, respectively, whereas EC and EGC were less effective. The inhibitory effects of these catechins on arachidonic acid metabolism in intact cells were much more pronounced. At 10 μM, EGCG and ECG inhibited the release of arachidonic acid and its metabolites by 50-70% in human colon adenocarcinoma cells (HT-29) and human esophageal squamous carcinoma cells (KYSE-190 and 450). EGCG and ECG also inhibited arachidonic acid release induced by A23187, a calcium ionophore, in both HT-29 and KYSE-450 cell lines by 30-50%. The inhibitory effects of green tea catechins on $cPLA_2$ and arachidonic acid release may provide a possible mechanism for the prevention of human gastrointestinal inflammation and cancers.

Keywords: green tea, catechins, cytosolic phospholipase A_2, arachidonic acid metabolism.

Introduction

Tea (*Camellia sinensis*) is one of the most widely consumed beverages in the world. Consumption of green tea has been suggested to have a number of beneficial health effects, and the mechanisms involved in these effects have been extensively investigated. Catechins, the major constituents of green tea, are believed to be important for the beneficial health effects of green tea (1). Green tea catechins include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC), and account for 30-42% of the dry weight of the water-extractable materials of green tea (2). EGCG is the most abundant catechin and is believed to be the most biologically active compound in green tea (3). The biological effects of tea catechins have been studied by many investigators, and among these effects, the anti-carcinogenic effects of tea have been studied extensively (1). A variety of mechanisms have been suggested for the anti-carcinogenic effect of green tea catechins in cell culture and animal models (4, 5).

The importance of arachidonic acid metabolites in carcinogenesis has been demonstrated in many studies (6-8). The major source of arachidonic acid in the cell is membrane phospholipids. Arachidonic acid is hydrolyzed and released from the membrane by phospholipase A_2 (PLA_2). Three major types of PLA_2 have been categorized, including secretory PLA_2 (sPLA_2), Ca^{2+}-independent PLA_2 (iPLA_2), and cytosolic PLA_2 ($cPLA_2$) (9). Among these PLA_2s, $cPLA_2$ has been reported to play a major role in releasing arachidonic acid from membrane phospholipids in most cells (10). The released arachidonic acid is further metabolized by three different types of oxygenases: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450s (11). Modulation of arachidonic acid metabolism through the inhibition of these enzymes using pharmaceutical agents or dietary compounds has been considered an important strategy for cancer prevention (12-15).

Previously, we reported that the administration of green tea significantly decreased prostaglandin E_2 levels in human rectal tissues (16). We also observed that green tea catechins inhibited COX and LOX activities from human colon mucosa and colon tumor tissues (17). However, few studies have been performed on the effects of tea catechins on $cPLA_2$. In the present study, we analyzed the effects of four purified green tea catechins, EC, EGC, EGCG, and ECG, on the catalytic activity of $cPLA_2$ and on the release of arachidonic acid and its metabolites in intact human gastrointestinal cells. Our results indicate that green tea catechins have much more potent inhibitory effects on the release of arachidonic acid and its metabolites from intact cells than on the catalytic activity of $cPLA_2$. This inhibitory effect may contribute to the anti-inflammatory and anti-carcinogenic actions of green tea.

Materials and Methods

Chemicals and cell lines [5,6,8,9,11,12,14,15-^{3}H](N)-arachidonic acid and 1-palmitoyl 2-[1-^{14}C] arachidonyl sn-glycero-3-phosphorylcholine were purchased from NEN Life Science (Boston, MA, USA). The purified green tea catechins, EC, EGC, EGCG, and ECG, were generous gifts

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from Thomas J. Lipton Company (Englewood, NJ, USA). All of the tea catechins were dissolved in dimethylsulfoxide (DMSO) at a concentration of 20 mM and stored at -80°C. The structures of these compounds are shown in Fig. 1. Bromoenol lactone and methyl arachidonyl fluorophosphate (MAFP) were acquired from Cayman Chemical Company (Ann Arbor, MI, USA). HT-29 human colon cancer cell line was obtained from American Type Culture Collection (Rockville, MD, USA). KYSE-190 and 450 cell lines were generous gifts from Dr. Yutaka Shimada (Kyoto University, School of Medicine, Kyoto, Japan). HT-29 cells were maintained in McCoy 5A medium supplemented with 10% fetal bovine serum, 100 unit/mL penicillin and 0.1 mg/mL streptomycin at 37°C in 95% humidified air and 5% CO_2. KYSE-190 and 450 cells were maintained in Ham's F-12 and RPMI-1640 (1:1 ratio) medium with 5% fetal bovine serum, 100 unit/mL penicillin and 0.1 mg/mL streptomycin at 37°C in 95% humidity and 5% CO_2. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Phospholipase A_2 assays For preparation of $cPLA_2$ source, approximately 2×10^6 HT-29 cells were plated into each cell culture dish (10 cm). After 24 hr, the cells were washed with ice cold phosphate-buffered saline twice and lysed using PLA_2 lysis buffer (2 M glycerol, 1 mM phenylmethylsulphonyl fluoride in 20 mM Tris, pH 7.4). The cell lysates were sonicated and centrifuged at 10,000 $\times g$ for 20 min at 4°C. The supernatants were then centrifuged at 105,000$\times g$ for 90 min at 4°C. The supernatants were collected as the cytosolic fraction and kept at -80°C for further analysis. The pellets for membrane fractions were resuspended in PLA_2 lysis buffer and stored at -80°C. The $cPLA_2$ assay was performed by following a previously reported method (18). In brief, 1-palmitoyl 2-[1-^{14}C] arachidonyl sn-glycero-3-phosphorylcholine (hot:cold, 1:3) and phosphoinositides were dried under a streaming of N_2 (g). To prepare the substrate solution, Triton X-100 (2 mM) in 100 mM Tris-HCl buffer, pH 7.4, was added to the dried lipids, and the substrate micelles were prepared by sonication in a bath-sonicator for 3 min. The effects of catechins on $cPLA_2$ activity were assayed at 37°C in a reaction mixture (100 μL) consisting of 20 μg microsomal protein from HT-29 cells, 40 μL substrate, 5 μg/mL phosphoinositides, 100 μM $CaCl_2$ and 200 μM Triton X-100 in 100 mM Tris-HCl buffer, pH 7.4. The reaction was initiated by adding 10 μL of the substrate solution after 5 min preincubation and terminated by the addition of 10 μL of 0.5 N HCl. Modified Dole's method was used for the extraction of free arachidonic acid (18, 19).

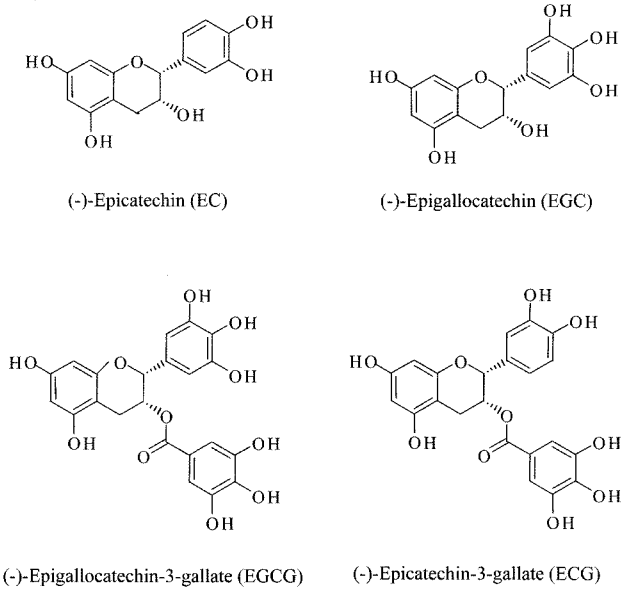

Fig. 1. Structures of tea catechins used in the present study.

Release of arachidonic acid and its metabolites in intact cell system HT-29, KYSE-190 and 450 cells were plated into a 24-well plate at approximately 2×10^5 cells/well in growth media. When the cells reached approximately 90% confluency, the media were removed and replaced with 1 mL of serum-free Ham's F-12 media containing 0.1 μCi/mL [5,6,8,9,11,12,14,15-^{3}H](N)-arachidonic acid. The cells were incubated for 16-18 hr, resulting in over 90% absorption of the arachidonic acid by the cells. The cells were washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin and incubated with 1 mL of serum free Ham's F-12 for 20 min at 37°C in order to remove background levels of arachidonic acid release. The media was then replaced with media containing tea catechins or vehicle (DMSO). For spontaneous release, the labeled cells were incubated with tea catechins or vehicle for 12 hr. For stimulator-induced arachidonic acid release, cells were incubated in 5-10 μM A23187 for 20 min before the treatment with tea catechins. After incubation of cells with tea catechins for 12 hr (spontaneous release) or 2 hr (A23187-stimulated release), the media were collected and centrifuged for 10 min at 10,000$\times g$. The radioactivity of the extracellular fluid was then measured.

Data analysis Statistical significance was evaluated using the Student's *t*-test. One-way analysis of variance and the Tukey's honestly significant difference (HSD) test were also used for comparing the effects of test compounds.

Results and Discussion

Characterization of phospholipase A_2 from HT-29 cells Arachidonic acid is normally hydrolyzed from membrane phospholipids by the action of PLA_2. There are three major types of PLA_2: $sPLA_2$, $iPLA_2$, and $cPLA_2$. $sPLA_2$s are a family of small molecular weight enzymes that are secreted extracellularly; these enzymes require a millimolar level of calcium ions for their catalytic activation (20). $iPLA_2$ does not show calcium-dependent activity or substrate specificity. It is known to be involved mainly in remodeling membrane phospholipids (21). $cPLA_2$ is an enzyme normally found in the cytosolic fraction of many tissues (10). Calcium ions are required for $cPLA_2$ catalytic activity, but at far less than millimolar levels. The binding of phosphatidylinositol 4,5-bisphosphate to $cPLA_2$ increases the activity of the enzyme by increasing the affinity of the enzyme for the membrane (22). Among the three types of PLA_2s, $cPLA_2$ is the most

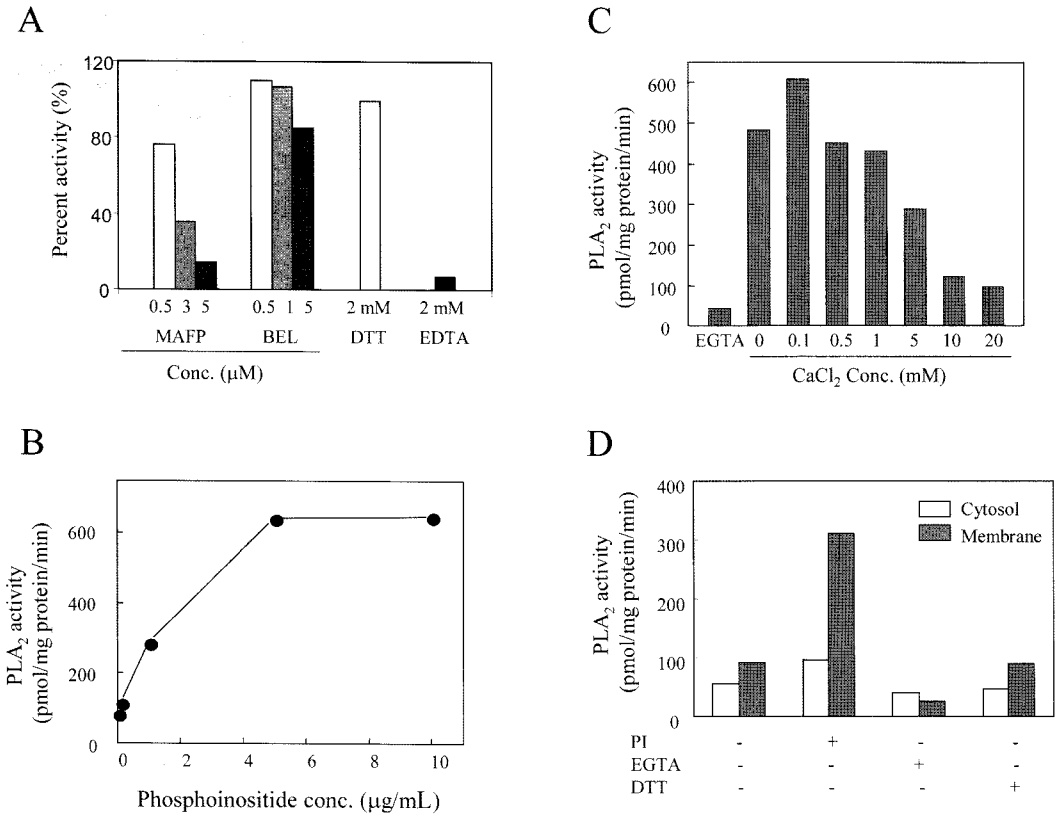

Fig. 2. Characterization of PLA$_2$ from fractions of HT-29 cells. (A) Effects of several agents on PLA$_2$ activity from membrane fraction. Effects of phosphoinositides (B) or $CaCl_2$ (C) on PLA$_2$ activity from membrane fraction. (D) Responses of cytosolic or membrane fraction from HT-29 cells to phosphoinositides (PI, 5 μg/mL), EGTA (2 mM) or DTT (2 mM). The data are the mean of duplicates.

important enzyme involved in releasing arachidonic acid in most tissues.

In order to evaluate the potential of tea catechins to inhibit cPLA$_2$, we developed an assay system for cPLA$_2$ using the microsomal fraction from HT-29 human colon adenocarcinoma cells. In the current assay system, the PLA$_2$ activity was not affected by 1 μM bromoenol lactone (a specific iPLA$_2$ inhibitor) or 2 mM dithiothreitol (DTT; a sPLA$_2$ inhibitor), whereas it was highly sensitive to MAFP (a cPLA$_2$ inhibitor), and ethylenediaminetetraacetic acid (EDTA; a metal ion chelator) (Fig. 2A). Phosphoinositides increased the PLA$_2$ activity in the microsomal fraction in a concentration-dependent manner, and the activity reached its peak at 5 μg/mL; the peak activity was about 8-fold higher compared to the activity obtained without phosphoinositides (Fig. 2B). The PLA$_2$ activity was fully activated with 100 μM calcium, yet 85% of the activity still remained when no calcium ions were added. Trace amounts of calcium in the assay system might account for the cPLA$_2$ activity. However, more than 95% of the activity disappeared in the presence of 2 mM ethyleneglycol-bis (beta-aminoethyl ether) tetraacetic acid (EGTA), a calcium ion chelator (Fig. 2C). The catalytic characteristics of the membrane fraction, including calcium and phosphoinositide dependence, as well as responses to several inhibitors, are closer to cPLA$_2$ than those of the cytosolic fraction (Fig. 2D). Although cPLA$_2$ is normally located in cytosol in the resting state, comparable portions of cPLA$_2$ activity were found in the microsomal fraction when the cells were harvested under growing conditions. Our result implies that significant portions of cPLA$_2$ are constantly translocated to the membrane fraction under growing conditions.

Effects of Tea catechins on cPLA$_2$ activity Since the PLA$_2$ activity found in HT-29 cell microsomal fractions was mostly derived from cPLA$_2$, the effects of individual tea catechins on the activity of cPLA$_2$ were investigated using these fractions. Among the tea catechins, ECG displayed the most potent inhibitory effects followed by EGCG. At 50 μM, ECG and EGCG inhibited cPLA$_2$ activity by 19 and 37%, respectively (Fig. 3A). EC and EGC, as well as other flavonoids including quercetin, kaempferol, and naringenin, were much less effective than EGCG and ECG, showing below 10% inhibition at 50 μM (Fig. 3A). EGCG and ECG inhibited cPLA$_2$ activity in a concentration-dependent manner, and their estimated IC_{50} values were 150.4 and 112.6 μM, respectively (Fig. 3B). The effects of tea catechins on iPLA$_2$ activity were also analyzed using HT-29 cytosolic fractions in the presence of 2 mM EGTA, a specific calcium ion chelator. EGCG, ECG, and EGC, however, did not show any appreciable inhibitory effects on iPLA$_2$ at 50 μM (Fig. 3C).

Regarding the structure and activity relationship, comparison of the inhibitory effect of EGCG *vs.* EGC, and ECG *vs.* EC (EGCG > EGC and ECG > EC; $p<0.01$) suggests that the gallate group (D ring) is important for the inhibitory action of catechins against cPLA$_2$ (Fig. 3A).

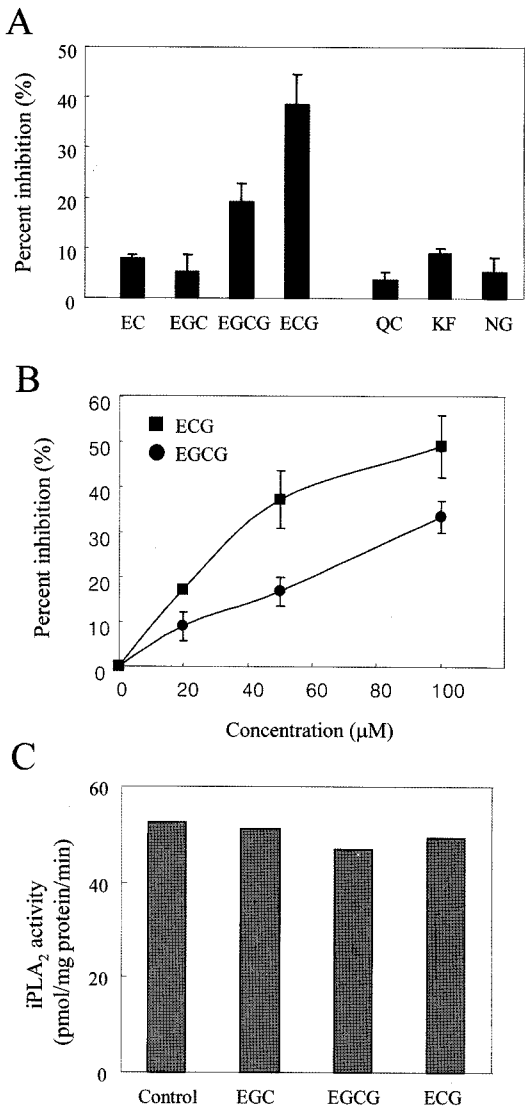

Fig. 3. Effects of purified green tea catechins on PLA_2 activity from HT-29 cells. (A) Effects of tea catechins or other flavonoids, quercetin (QC), kaempferol (KF), and naringenin (NG) (each 50 μM) on $cPLA_2$ activity. (B) Concentration-dependent effects of ECG and EGCG. (C) Effects of tea catechins on $iPLA_2$ activity of cytosolic fraction from HT-29 cells. The data are the mean±SD from 3 independent experiments (A and B) or mean of duplicates (C).

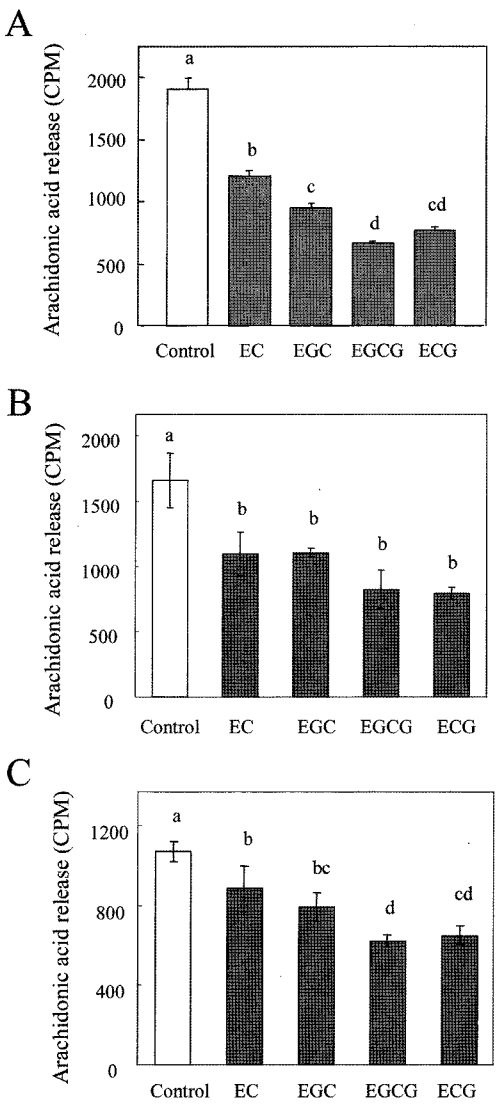

Fig. 4. Effects of green tea catechins on spontaneous release of arachidonic acid and its metabolites from gastrointestinal cells. HT-29 (A), KYSE-450 (B), or KYSE-190 (C) cells labeled with 0.1 μCi/mL [^{3}H]-arachidonic acid were treated with 10 μM of each catechin or vehicle. After 12 hr, the radioactivity in the extracellular fluid was analyzed. Each bar represents the mean±SD. Different letters indicate a significant difference ($p<0.05$) based on one-way analysis of variance and the Tukey's HSD test.

This is also supported by the weak inhibitory effects of quercetin, kaempferol, and naringenin, which do not have a gallate group (D ring). Comparing the effects of ECG *vs.* EGCG (ECG > EGCG; $p<0.01$), the hydrophobicity (one less hydroxyl group in ECG structures gives more hydrophobicity) of catechins is also important to their inhibitory action. The results are similar to those observed for EGGC *vs.* ECG on COX and LOX (17). Since $cPLA_2$, COX, and LOX require relatively hydrophobic environments for their respective activities, the hydrophobicity of ECG may be one of the important factors for interrupting these enzyme activities. The release of arachidonic acid from membrane phospholipids by PLA_2 is frequently the rate-limiting step in further arachidonic acid metabolism. Accordingly, inhibition of $cPLA_2$ and arachidonic acid release may be an effective target for cancer chemoprevention by diminishing the pathogenic potential of further metabolized eicosanoids.

Effects of tea catechins on the release of arachidonic acid and its metabolites in intact cells In order to test the effects of tea catechins on arachidonic acid metabolism in intact cells, HT-29 human colon adenocarcinoma cells and KYSE-190 and 450 human esophageal squamous carcinoma cells were used; these cells originate from the gastrointestinal tract. As the gastrointestinal tract is directly exposed to significant amounts of consumed dietary constituents such as tea catechins, chemopreventive action

in the mucosal layer of the gastrointestinal tract may be achieved even without absorption.

[^{3}H]-arachidonic acid was used for labeling cells. After 14-16 hr incubation with 0.1 μCi [^{3}H]-arachidonic acid in HT-29 and KYSE cells, over 90% of the arachidonic acid was taken up by the cells. Most of the arachidonic acid was incorporated into membrane phospholipids, which was confirmed by HPLC. The incorporated arachidonic acids are subsequently hydrolyzed from the membrane phospholipids by the action of phospholipases, mainly PLA_2, and transported out of the cells, predominantly as metabolites.

All green tea catechins tested (10 μM) significantly decreased the amount of released arachidonic acid and its metabolites found in extracellular fluids in all HT-29, KYSE-190, and KYSE-450 cells. Inhibition of the release by tea catechins was more pronounced in HT-29 cells than in KYSE cells (Fig. 4). EGCG and ECG (10 μM) inhibited the spontaneous release of arachidonic acid and its metabolites by 60-70% in colon cancer cells and by 40-50 % in esophageal cancer cells, whereas EC and EGC were slightly less effective (Fig. 4). Both EGCG and ECG showed concentration-dependent inhibitory effects. The inhibitory effect of EGCG was still significant even at 1 μM (Fig. 5A and 5B). Estimated IC_{50} values for EGCG and ECG were calculated to be 2.7 and 4.5 μM in HT-29 cells, respectively (Fig. 5B).

We also investigated whether tea catechins can actively inhibit arachidonic acid release when the release is stimulated. In order to find a possible stimulator for $cPLA_2$ and arachidonic acid release, the effect of A23187 was tested. A23187 is a calcium ionophore that causes an influx of calcium ion into cells. In response to A23187 treatment, an increase of intracellular calcium is known to activate $cPLA_2$ to induce membrane translocation (23). Indeed, A23187 consistently increased arachidonic acid release in both colon and esophageal cancer cells (Fig. 6A), while longer incubation and/or higher concentration of A23187 appeared to be toxic to HT-29 cells. All green tea catechins except EC (10 μM) significantly inhibited A23187- induced arachidonic acid release in both HT-29 and KYSE-450 cells (Fig. 6B and 6C). EGCG showed the most potent effect by inhibiting 40-50% of the release, especially in KYSE-450 cells (Fig. 6C).

All green tea catechins tested potently inhibited the release of arachidonic acid and its metabolites in the intact cell system. Compared to their inhibitory effects on the catalytic activity of $cPLA_2$, the effect in intact cells was even more substantial. Even though EGCG and ECG showed relatively weak inhibition on $cPLA_2$ activity from HT-29 cells (19 and 37% at 50 μM, respectively), EGCG and ECG at 10 μM inhibited the release of arachidonic acid and its metabolites by 50-70% (estimated IC_{50}: 2.7 and 4.5 μM, respectively) (Fig. 3B *vs.* Fig. 5B). The inhibitory effect of EGCG was still significant even at 1 μM (Fig. 5A). The discrepancy of results between the effects observed on enzyme activity and in intact cells implies that tea catechins inhibit the arachidonic acid release by mechanisms other than a direct inhibition of $cPLA_2$. The action of $cPLA_2$ is regulated by a complex signaling cascade in cells. $cPLA_2$ is an inducible enzyme that can be overexpressed in response to many stimulating

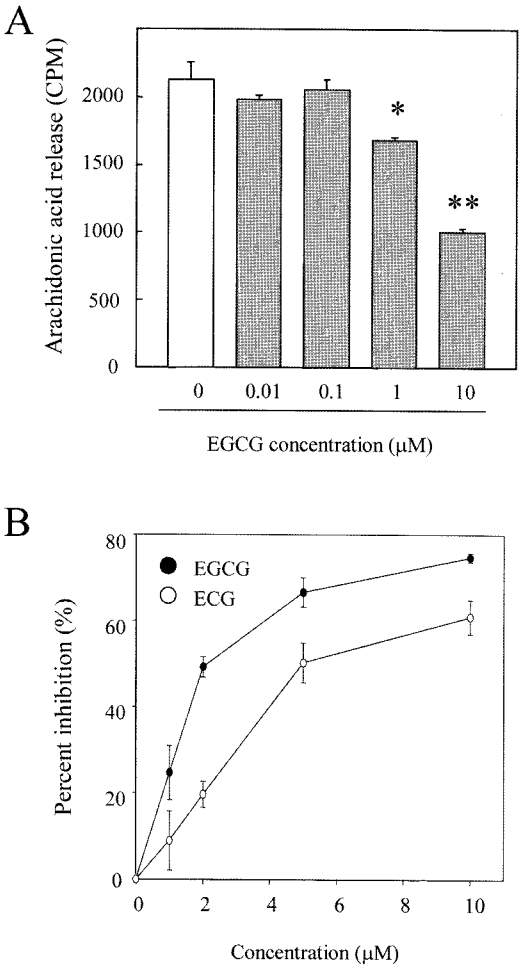

Fig. 5. Concentration-dependent effects of EGCG and ECG on release of arachidonic acid and its metabolites from gastrointestinal cells. KYSE-450 (A) and HT-29 (B) cells labeled with [^{3}H]-arachidonic acid were treated with different concentrations of EGCG and ECG for 12 hr (A) or 8 hr (B). The data represent the mean±SD (n=4). *,**Significantly different from control (*p< 0.05; **p<0.01).

signals. In the presence of micromolar levels of calcium ions, the enzyme is translocated into membrane fractions, mainly the nuclear envelope or the membrane of the endoplasmic reticulum (24). Activity of $cPLA_2$ is up-regulated by the phosphorylation of Ser^{505} by mitogen-activated protein kinases such as extracellular signal-regulated kinase and p38 (25, 26). Previously, we reported that curcumin inhibited the release of arachidonic acid and its metabolites by inhibiting phosphorylation of $cPLA_2$ in lipopolysaccharide-stimulated RAW264.7 macrophages (18). Our group also reported that EGCG inhibited the phosphorylation of extracellular signal-regulated kinases (27). This mechanism is likely applied to the inhibition of $cPLA_2$ up-regulation by EGCG, and may be involved in the inhibition of arachidonic acid release. Tea catechins may also inhibit the release of arachidonic acid and its metabolites in intact cells through other mechanisms, possibly affecting $cPLA_2$ expression levels and translocation of the protein onto membranes. The precise mechanism of tea catechins in inhibiting the release of arachidonic

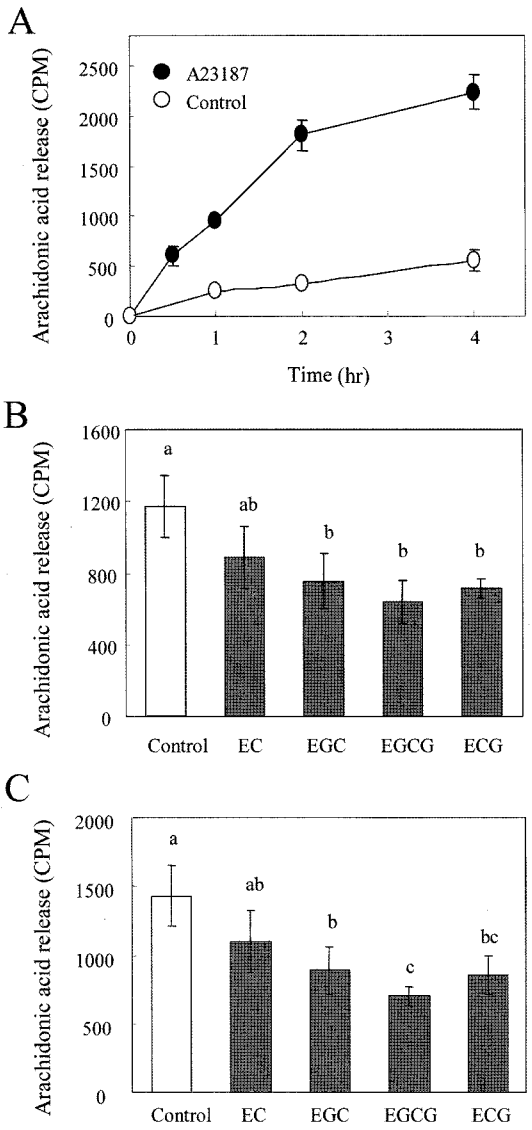

Fig. 6. Effects of A23187 on arachidonic acid release from gastrointestinal cells and its modulation by green tea catechins. (A) Stimulation of arachidonic acid release from HT-29 cells by A23187. Effects of green tea catechins on A23187-induced release of arachidonic acid from HT-29 (B) and KYSE-450 (C) cells. The data represent the mean±SD (n=4). Different letters indicate a significant difference ($p<0.05$) based on one-way analysis of variance and the Tukey's HSD test.

acid and its metabolites needs be further investigated.

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