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Epigallocatechin Gallate Activates Phospholipase D in Glioma Cells

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Abstract

Epigallocatechin-3 Gallate (EGCG), a major constituent of green tea, has attracted increasing interest because of its many reported health benefits. Here we demonstrate for the first time that EGCG stimulates phospholipase D (PLD) activity in U87 human astroglioma cells. EGCG-induced PLD activation was abolished by the phospholipase C (PLC) inhibitor and a lipase inactive PLC-γ1 mutant, and was dependent on intracellular Ca^{2+}, and possibly involved Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II). Interestingly, EGCG induced translocation of PLC-γ1 from the cytosol to the membrane and PLC-γ1 interaction with PLD1. Taken together, these results demonstrate for the first time that in human astroglioma cells, EGCG regulates PLD activity via a signaling pathway involving a PLC-γ1 (inositol 1,4,5-trisphosphate-Ca^{2+})-CaM kinase II-PLD pathway.

Key words – EGCG, PLD, PLC-γ1, CaM kinase II

Introduction

Phospholipase D (PLD) catalyzes the hydrolysis of the most abundant membrane phospholipid, phosphatidylcholine, to generate phosphatidic acid (PA) and choline and is assumed to play an important function in cell regulation[3]. Signal-dependent activation of PLD was demonstrated in numerous cell types stimulated by various hormones, growth factors, cytokines, neurotransmitter, adhesion molecules, drugs, and physical stimuli [11]. Pathways leading to PLD activation include protein serine/threonine kinases, *e.g.* protein kinase C (PKC), small GTPases, *e.g.* ADP-ribosylation factor, RhoA and Ral, phosphatidylinositol 4,5-bisphosphate (PIP_2), and tyrosine kinases[5,11,13]. To date, two distinct isoforms of mammalian PLD have been cloned, specifically, PLD1 and PLD2. These isoforms share about 50% amino acid similarity, but exhibit quite different regulatory properties[2,6]. Both proteins appear to be complexly regulated, usually in an agonist- and cell-specific manner, and the molecular mechanisms underlying their functions have not been fully elucidated.

Green Tea (*Camellia sinensis*) is a popular beverage worldwide, and its possible health benefits have received a great deal of attention. Documented beneficial effects of green tea and its active components include cancer chemoprevention, inhibition of the growth, invasion and metastasis of tumor cells, as well as anti-viral and anti-inflammatory activities[22]. Green tea contains the characteristic polyphenolic compounds epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC). EGCG is considered to be the constituent that is primarily responsible for the green tea effect[16,23]. Although the activity of EGCG in some biological events has been investigated, its effect on the signal transduction cascade is not yet fully defined. Moreover, any information is not available regarding

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how EGCG effects PLD-mediated signaling pathway. Therefore, we undertook an investigation of PLD regulation by EGCG.

In the present study, we demonstrate for the first time that EGCG significantly stimulates PLD activity in U87 human astrocytoma cells, and that EGCG-induced PLD activation is mediated via a signaling pathway involving PLC-γ1 [inositol 1,4,5-trisphosphate (IP_3)-Ca^{2+}]-CaM kinase II-PLD pathway.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and LipofectAMINE were purchased from Invitrogen. EGCG, EGC, ECG, and EC were obtained from Sigma. Protein A sepharose were from Amersham Biosciences Biotech. Anti-PLC-γ1 antibody were from Upstate Biotechnology. U-73122 were purchased from Biomol (Biomol Research Laboratories, Inc., PA). KN-62 and pertussis toxin were obtained from Calbiochem. Other chemicals were purchased from Sigma. Rabbit polyclonal antibody that recognizes both PLD1 and PLD2 was generated as previously described [12]. Authentic phosphatidylbutanol (PtdBut) standard was from Avanti Polar Lipid. Myo-[2-^{3}H]inositol and[9, 10-^{3}H]myristate was purchased from PerkinElmer Life Sciences. AG 1-X8 anion exchange resin was bought from Bio-Rad. Silica gel 60 A thin layer chromatography plates were from Whatman. Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were from Kirkegaard & Perry Lab (Gaithersburg, MD). The ECL Western blotting detection kit was from Amersham Biosciences Biotech.

Cell culture and transfection

U87 human astroglioma and COS-7 cells were maintained in DMEM (Life Technologies, Inc) supplemented with 10% (v/v) fetal bovine serum under 5% CO_2. Cells were transiently transfected for 40 h with plasmids encoding empty vector, or a lipase inactive mutant PLC-γ1 (H335Q) expression vectors using LipofectAMINE (Invitrogen) according to manufacturer's instructions.

Measurement of phosphoinositides hydrolysis by PLC

The cells were labeled with *myo*-[2-^{3}H]inositol (2 μCi/ml) in inositol-free DMEM for 20 h. Subsequently, the labeled cells were pretreated with 20 mM LiCl for 15 min. After stimulation with EGCG, the reaction was terminated by the addition of ice-cold 5% $HClO_4$. The extracts were applied to Bio-Rad Dowex AG 1-X8 anion exchanger column. The column was then washed with 10 ml of distilled water followed by 10 ml of 60 mM ammonium formate containing 5 mM of sodium tetraborate. Total inositol phosphates were eluted with a solution containing 1 M ammonium formate and 0.1 M formic acid.

Phospholipase D Assay

PLD activity was assessed by measuring the formation of [^{3}H] phosphatidylbutanol (PtdBut), the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol. Cells were subcultured in 6-well plates at 2×10^5 cells/well and serum-starved in the presence of 1 μCi/ml [^{3}H]myristic acid. After overnight starvation, the cells were washed three times with 5 ml of phosphate-buffered saline (PBS) and pre-equilibrated in serum-free DMEM for 1 h. For the final 10 min of preincubation, 0.3% butan-1-ol was included. At the end of the preincubation, cells were treated with agonists for the indicated times. The extraction and characterization of lipids by thin-layer chromatography were performed as previously described[12].

Subcellular fractionation

Serum-starved cells were treated with 500 μM EGCG for 10 min, and washed with PBS and harvested by

microcentrifugation. The cells were then resuspended with lysis buffer (20 mM Hepes, pH 7.4, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM of dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin) and lysed by twenty passages through a 25-gauge needle. Trypan blue staining of the lysate indicate >95% disruption of the cells. The lysates were then spun at 100,000×g for 1 h at 4℃ to separate the cytosolic and membrane fractions. Membrane fractions were washed twice with the buffer to remove cytosolic proteins.

Immunoprecipitation

U87 cells were harvested and lysed with lysis buffer (20 mM Hepes, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.2% SDS, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenymethylsulfonyl fluoride). The cells were then centrifuged at 10,000×g for 1 h, and the resulting supernatant was incubated on anti-PLD or anti-PLC-γ1 antibody and protein A sepharose for 4 h at 4℃ with rocking. Protein concentrations were determined using the BioRad Protein Assay with bovine serum albumin as a standard. The immune complexes were collected by centrifugation and washed five times with a buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2 mM Na_3VO_4, 10% glycerol and 1% Nonidet P-40) and resuspended in sample buffer. The final pellet was loaded onto a polyacrylamide gel for immunoblot analysis.

Immunoblot analysis

Proteins were denatured by boiling for 5 min at 95℃ in Laemmli sample buffer[9], separated by SDS-PAGE, and transferred to nitrocellulose membranes. After blocking in TTBS containing 5% skim milk powder, the membranes were incubated with individual monoclonal or polyclonal antibodies and then further incubated with anti-mouse or anti-rabbit IgG coupled to horseradish peroxidase. Blots were detected using the enhanced chemiluminescence kit according to the manufacturer's instructions.

Results

EGCG stimulates PLD activity in U87 human astroglioma cells

We investigated whether green tea polyphenols activate PLD in U87 human astroglioma cells. Cells were treated for 30 min with 500 μM EGCG, ECG, EGC or EC. The data presented in Fig. 1A show these polyphenolic compounds stimulated significantly PLD activity, with EGCG being the most potent activator. EGCG-induced [^{3}H]PtdBut formation increased in a time- and concentration-dependent manner (Fig. 1B and C). Activation of PLD by EGCG continued up to 50 min and then remained constant up to 100 min; maximum activation was observed at 2 to 3 mM EGCG. Using PLD antibodies, we detected PLD1, but not PLD2, in U87 cells. Since EGCG (500 μM) as a hydrophobic compound can destabilize membranes in a non-specific fashion and induce PLD activation, we examined whether other phenolic compound causes comparable effects at the same concentration. We found that resveratrol (500 μM), a polyphenol found in grapes and grape wine, did not affect PLD activity (data not shown). Furhermore, it was reported that EGCG in the range of 40~400 μM had no effect on PLD activity in human neutrophils[19]. These results show that the action of EGCG are not simply secondary to such effects in U87 cells.

Role of PLC in EGCG-induced PLD activation

Numerous studies have implicated PLC in the activation of PLD[7,10]; however, the results of other studies have suggested that PLC is not involved[1,21]. To determine whether PLC activity or G-protein-mediated signaling was involved in EGCG-induced PLD activation in U87 cells, we examined the effects of pertussis toxin (PTX) and the phosphoinositide-specific PLC inhibitor,

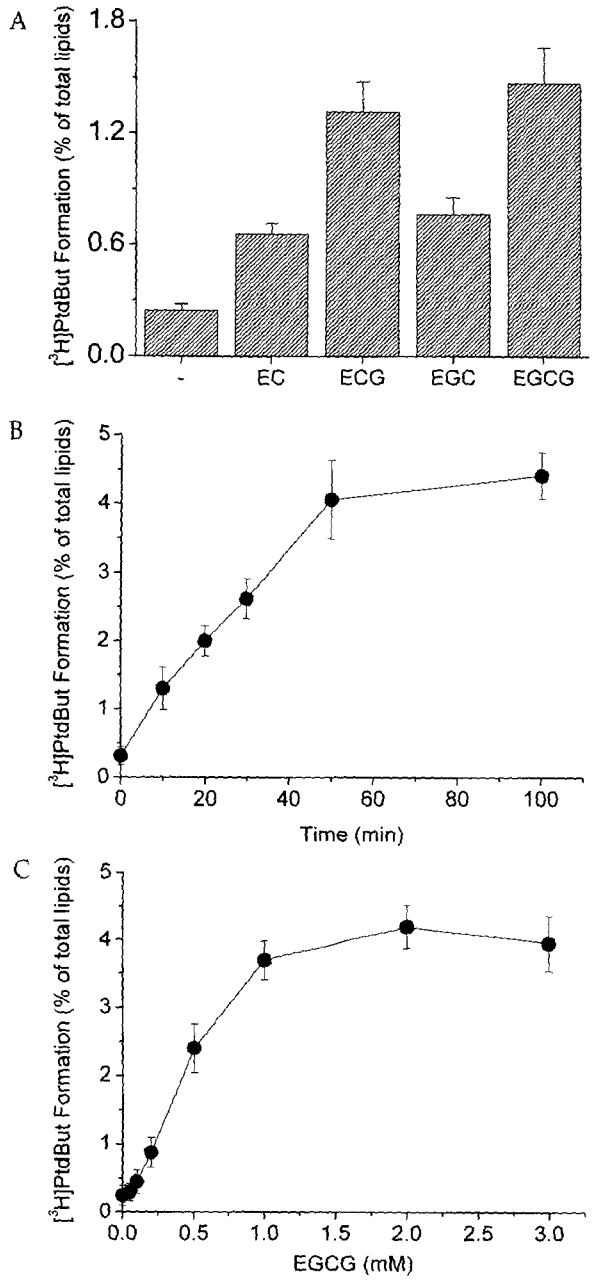

Fig. 1. Green tea polyphenols stimulate PLD activity in U87 human astroglioma cells.

Cells were cultured in six-well plates, labeled with [^{3}H]myristate, and treated for 30 min without or with 500 mM EGCG, ECG, EGC, or EC in the presence of 0.3 % butanol (A). [^{3}H]myristate-labeled cells were treated with 500 mM EGCG for the indicated time (B) or with the indicated concentration of EGCG for 50 min (C). The radioactivity incorporated into phosphatidylbutanol was measured as described under Experimental Procedures. Results are means±SD of three independent experiments.

U-73122. However, PTX had no effect on EGCG-induced PLD activation (Fig. 2A). EGCG-induced PLD activation was significantly attenuated by PLC-specific inhibitor U-73122, in a dose-dependent manner (Fig. 2B). These data suggest phosphoinositide-specific PLC activation, via a pertussis toxin-insensitive pathway plays a critical role in EGCG-induced PLD activity in these cells. We also investigated whether EGCG induces PLC activity in U87 cells. The data presented in Fig. 2C show that EGCG treatment stimulates PLC activity, as measured by formation of ^{3}H-inositol phosphates, which peaked after 10 min and was sustained for at least 50 min. Furthermore, we found that EGCG induced increase in intracellular calcium in the cells (data not shown). Using PLC isozyme-specific antibodies, we found PLC-γ1 was the predominantly expressed PLC in U87 cells, and PLC-β1, -β2, -β3, γ2 or -δ1 isozymes were not detectable (data not shown), indicating that the PLC activity shown in these cells may be due mainly to PLC-γ1. Therefore, we examined the involvement of PLC-γ1 in the PLD activation by EGCG in U87 cells. Interestingly, expression of the lipase inactive mutant PLC-γ1 (His $^{335}\rightarrow$ Gln)[14] significantly attenuated EGCG-induced PLD activation (Fig. 2D), suggesting PLC-γ1 is involved in this process.

EGCG induces translocation of PLC-γ1 and its interaction with PLD1

After growth factor stimulation, PLC-γ1 translocates from the cytosol to the membrane, where its substrate molecules reside[19]. We examined whether EGCG induced PLC-γ1 translocation. Incubation with EGCG for 10 min significantly increased the amount of PLC-γ1 associated with the membrane fraction in U87 cells (Fig. 3A). Using confocal immunofluorescence microscopy, we confirmed that PLC-γ1 translocation to membrane regions increased after EGCG treatment and co-localization of PLD1 and PLC-γ1 increased in the membraneous region after EGCG stimulation (data not shown). We

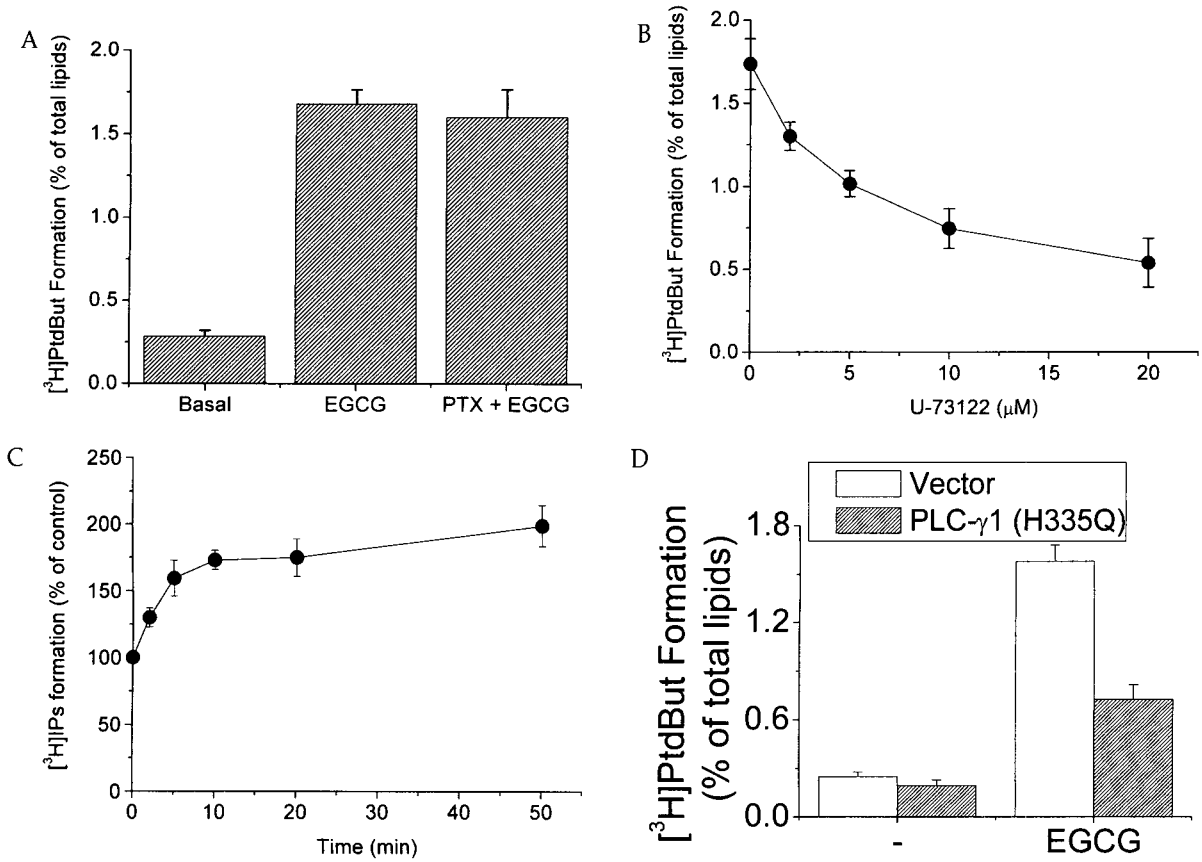

Fig. 2. PLC is involved in EGCG-induced PLD activation.

Quiescent U87 cells were pretreated with 200 ng/ml PTX for 24 h, labeled with [^{3}H]myristate, and stimulated with 500 mM EGCG for 30 min (A). [^{3}H]myristate-labeled cells were pretreated with the indicated concentrations of U-73122, and stimulated with 500 mM EGCG for 30 min (B). Quiescent cells were labeled with 1 mCi/ml myo-[2-^{3}H]inositol and stimulated with 500 mM EGCG for the indicated time and PLC activity were measured as described in Experimental Procedures (C). U87 cells were transiently transfected with a catalytically inactive mutant of PLC-γ1 (H335Q), labeled with [^{3}H]myristic acid and treated with EGCG (500 mM) for 30 min (D). *$p<0.05$ compared to cells transfected with vector and treated with EGCG. The radioactivity incorporated into phosphatidylbutanol was measured as described under Experimental Procedures. Results are means±SD of three independent experiments.

sought to confirm this apparent interaction between PLD1 and PLC-γ1 in EGCG-stimulated U87 cells. We found that PLD1 showed a mild interaction with PLC-γ1 in unstimulated cells and this association increased after treatment of EGCG for 10 min (Fig. 3B). These data suggest that PLD1 associates with PLC-γ1 during EGCG-induced PLD activation.

EGCG-induced PLD activation is dependent on intracellular Ca^{2+} and is mediated by CaM kinase II

Several examples of the participation of Ca^{2+} in the regulation of PLD activity have been reported, although the effector molecules involved have not been fully characterized[4,18]. We found that BAPTA 1,2-bis (2-aminophenoxy)ethane-N,N,N′,N′,-tetraacetic acid acetoxymethylester (BAPTA/AM), an intracellular chelator of Ca^{2+}, significantly reduced EGCG-induced PLD activity (Fig. 4A), indicating a role for intracellular Ca^{2+} in this process. The possible mechanisms by which intracellular Ca^{2+} regulates EGCG-stimulated PLD activity were investigated. We examined whether Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) mediates PLD activation in response to EGCG. As shown in Fig. 4B, KN-62, a specific CaM kinase II inhibitor inhibited

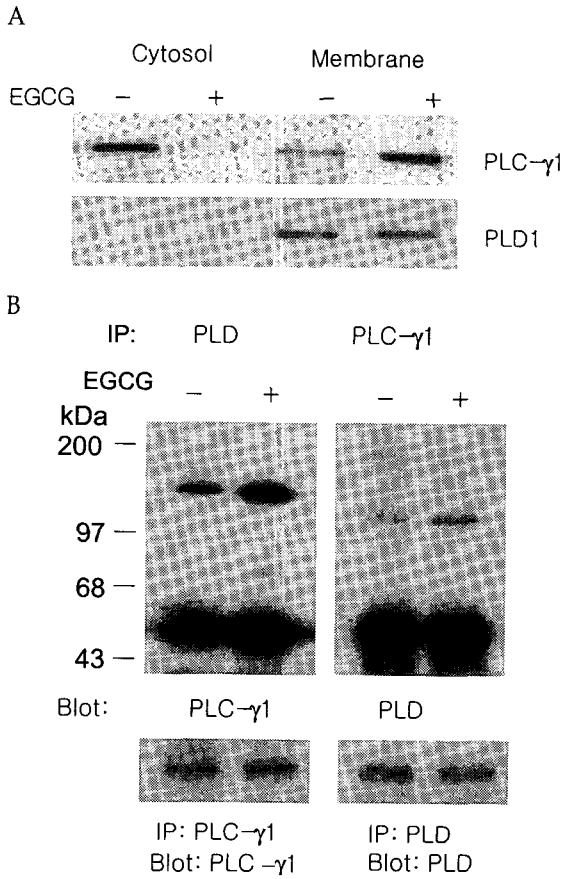

Fig. 3. EGCG induces translocation of PLC-γ1 and its interaction with PLD1 in U87 cells.
Serum-starved cells were treated with 500 mM EGCG for 10 min. Lysates were separated into cytosol and membrane fractions which were immunoblotted using anti-PLC-γ1 or PLD antibodies (A). Serum-starved cells were stimulated with 500 mM EGCG for 10 min, after which cell lysates were prepared and immunoprecipitated with anti-PLD or anti-PLC-γ1 antibodies and then immunoblotted using anti-PLC-γ1 or anti-PLD antibodies, respectively (B). Data are representative of three experiments.

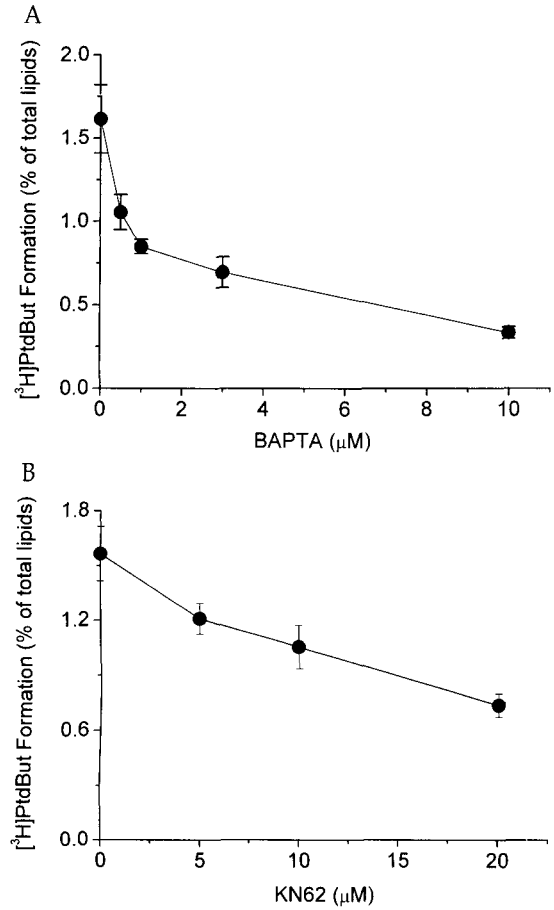

Fig. 4. EGCG-induced PLD activation is dependent on intracellular Ca^{2+} and is mediated by CaM kinase II.
(A) U87 cells were labeled with [^{3}H]myristate, preincubated with the indicated concentrations of BAPTA/AM, and then stimulated with 500 mM EGCG for 30 min. (B) [^{3}H]Myristate-labeled cells were pretreated with the indicated concentration of KN-62, and then stimulated with EGCG. PLD activity was measured as described. Results are means±SD of three independent experiments.

EGCG-induced PLD activation. These data suggest that EGCG-induced PLD activation is dependent on Ca^{2+} and possibly involves the Ca^{2+}-activated protein kinase, CaM kinase II.

Discussion

Many studies have provided evidence showing highly complex regulation of PLD by extracellular ligands. In the present study, we demonstrate for the first time that EGCG, a natural substance isolated from green tea, stimulates PLD activity via a network of signaling molecules in U87 human astroglioma cells.

PLD plays an important role in controling many biological functions, including exocytosis, phagocytosis, and secretion. PLD in mammalian cells can be activated by a range of extracellular signals[5]. The mechanisms underlying PLD activation are highly dependent on the

model system used, and are still under investigation in numerous laboratories. The recent cloning of the two mammalian PLD isozymes has led to an explosion of research in the field, principally driven by the availability of molecular tools.

During the last decade, green tea has received a great deal of attention regarding its possible health benefits. Despite a great deal of research on the biological properties of EGCG, until now nothing has been reported regarding its effects on PLD-mediated signal transduction.

In the present study, we demonstrated that EGCG significantly stimulated PLD activity, and induced inositol phosphates production and $[Ca^{2+}]_i$ in astroglioma cells. EGCG-induced PLD activation was suppressed by the phosphoinositide-specific PLC inhibitor. Only PLC-γ1 was the predominantly expressed PLC in U87 cells, indicating that the PLC activity shown in these cells may be due mainly to PLC-γ1. This led us to assume that PLC-γ1 might be involved in EGCG-induced PLD activation. Transfection experiment using a lipase-inactive PLC-γ1 mutant revealed significant attenuation of EGCG-induced PLD activation, suggesting that PLD lies downstream of PLC-γ1 in the signaling pathway. Expression of the inactive mutant of PLC-γ1 attenuated endogenous PLC activity (data not shown). In resting cells, PLC-γ1 is located predominantly in the cytosol, and translocates to the membrane fraction upon activation[14]; hence translocation is a widely accepted measure of PLC-γ1 activation. We observed that EGCG induced translocation of PLC-γ1 from the cytosol to membrane, where its substrate molecules reside. Furthermore, EGCG induced the interaction of PLC-γ1 with PLD1, as well as co-localization of these two molecules in membrane. In this study, we report for the first time two signaling phospholipase complex composed of PLC-γ1 and PLD1. Recently, it was reported that upon epidermal growth factor stimulation, PLC-γ1 directly interacts with PLD2[8]. Moreover, EGCG induced tyrosine phosphorylation of PLC-γ1 which was inhibited by pretreatment of anti-oxidant (data not shown). but also a PKC dependent pathway.

EGCG increased intracellular Ca^{2+} in U87 cells, and chelation of intracellular Ca^{2+} by BAPTA/AM abolished EGCG-induced PLD activation. It is therefore assumed that increase in intracellular Ca^{2+} may be due to EGCG-induced PLC activation and subsequent IP_3 production. Indeed, since CaM kinase II is activated via the PLC pathway in many cell types[15], and the inhibitor attenuated EGCG-induced increases in PLD activity, PLC probably regulates PLD through stimulation of this kinase. The EGCG-evoked increase in $[Ca^{2+}]_i$ was inhibited by the nonspecific Ca^{2+} channel inhibitor lanthanum, and the PLC inhibitor U73122, but not by pretreatment with the L-type Ca^{2+} channel blocker, nifedipine (data not shown). These results suggest that in U87 cells, EGCG-induced increases in $[Ca^{2+}]_i$ result from mobilization of IP_3-sensitive intracellular Ca^{2+} stores.

EGCG has been demonstrated to pass the blood-brain barrier and reach brain parenchyma in animal study, and detection of EGCG in rat brain suggests polyphenols can modulate neuronal activity[17].

In summary, we demonstrate for the first time that EGCG regulates PLD activity by PLC-γ1 (IP_3- Ca^{2+})-CaM kinase II-PLD. This study identifies PLD as a new target for EGCG in human astroglioma cells. The elucidation of the mechanisms underlying EGCG action, and its interaction with physiological factor(s) is essential for successful clinical use of EGCG. Further studies will be performed to gain a better understanding of the functional role of PLD in mediating a physiological responses by EGCG.

Acknowledgements

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Abbreviations

Epigallocatechin-3-gallate, EGCG; epigallocatechin, EGC; epicatechin-3-gallate, ECG; epicatechin (EC); phospholipase D, PLD; phospholipase C, PLC; inositol 1,4,5-trisphosphate, IP_3; protein kinase C, PKC; Ca^{2+}/calmodulin dependent protein kinase II, CaM kinase II; phosphatidylbutanol, PtdBut; pertussis toxin, PTX.

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초록 : 교세포에서 Epigallocatechin Gallate에 의한 포스포리파제 D의 활성화

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녹차의 주성분인 에피갈로 갈레이트 (EGCG)는 건강에 이로운 효과로 나타내고 있는것으로 알려져 있어 많은 주목을 받고 있는 실정이다. 본 연구에서는 처음으로 EGCG가, U87 사람의 교세포에서 포스포리파제 D의 효소활성을 증가시킨다는 사실을 규명하였다. EGCG에 의한 포스포리파제 D의 활성화는 포스포리파제 C효소의 특이적인 억제제와 지질분해효소 활성이 억제제 포스포리파제 γ1의 변이체를 이용하여 실험한 결과 세포내 칼슘에 의존적인 양상을 보였주었다. 이러한 포스포리파제 D의 활성화는 아마도 칼슘 의존성 단백질 키나아제 II (CaM kinase II)를 통하여 일어나는것으로 여겨진다. 흥미롭게도, EGCG는 포스포리파제 γ1를 세포질에서 세포막으로의 이동을 유도하였으며, 포스포리파제 γ1와 PLD1의 결합을 유도하였음을 관찰하였다. 이상의 결과들을 종합하여 볼 때, EGCG가 사람의 교세포에서 포스포리파제 γ1-칼슘-CaM kinase II의 신호전달 경로를 통하여 포스포리파제 D의 활성을 유도하는 것으로 사료된다.