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# Sealing effects of (−)-epigallocatechin gallate on protein kinase C and protein phosphatase 2A

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**Abstract**

(−)-Epigallocatechin gallate (EGCG) was reported to inhibit protein kinase C (PKC) activation by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and inhibit interaction of tumor promoter with its receptors, named 'a sealing effect'. In order to clarify the sealing effect of EGCG, we prepared liposomes and examined inhibition of PKC activation by various concentrations of EGCG dispersed in the liposome. EGCG added to a liposome dispersion existed either in a buffer solution as aggregates or in phospholipid bilayer membranes, and EGCG disturbed membrane structure. The potency of inhibitory effect of EGCG on PKC activation was dependent on the nature of liposomes, indicating that interaction of EGCG with phospholipid bilayer membrane affects PKC activation. Moreover, EGCG prevented the binding of adenosine 5'-triphosphate and TPA to PKC, resulting in inhibition of PKC activation. On the other hand, the activity of protein phosphatase 2A (PP2A) was suppressed in the presence of liposomes, but was not influenced by EGCG. Moreover, EGCG recovered phosphatase activity of PP2A in a buffer solution, the activity of which was inhibited by okadaic acid. All the results indicated that EGCG possesses sealing effects in terms of PKC and PP2A, by inhibiting interaction of various ligands with proteins. © 1997 Elsevier Science B.V.

*Keywords:* (−)-Epigallocatechin gallate; Protein kinase C; Protein phosphatase 2A; Lipid membrane; Tumor promotion; 12-*O*-tetradecanoylphorbol-13-acetate; Okadaic acid

## 1. Introduction

EGCG (Fig. 1) is the main polyphenolic constituent of Japanese green tea, and was reported to inhibit the tumor-promoting activity of teleocidin, one of the TPA-type tumor promoters in the two-stage carcinogenesis experiments [1–4]. TPA-type tumor promoters, such as TPA, teleocidin, and aplysiatoxin, induce activation of PKC which is involved in the pathway of tumor promotion in carcinogenesis [5–7].

Abbreviations: 2AS and 10AS = 2- and 10-anthroyloxystearic acid; ATP = adenosine 5'-triphosphate; CD = circular dichroism; CF = 5/6-carboxyfluorescein; DMPC = dimyristoylphosphatidylcholine; DOPC = dioleoylphosphatidylcholine; DPPC = dipalmitoylphosphatidylcholine; EGCG = (−)-epigallocatechin gallate; MLV = multilayer vesicle; PKC = protein kinase C; POPC = palmitoyloleoylphosphaddylcholine; PP2A = protein phosphatase 2A; PS = phosphatidylserine; SUV = small unilamellar vesicle; TPA = 12-*O*-tetradecanoylphorbol-13-acetate

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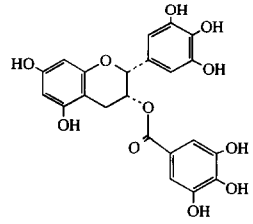

Fig. 1. Chemical structure of EGCG.

EGCG inhibited dose-dependently activation of protein kinase C by teleocidin in vitro [1]. EGCG also blocks the specific binding of $^{3}$H-TPA to the receptors in a particulate fraction of mouse skin [8]. It was, thus, suggested that EGCG inhibits carcinogenesis by blocking the interaction of tumor promoter and its receptors in cell membranes, and this effect was named 'a sealing effect' of EGCG. However, the mode of action of EGCG in terms of interaction of a tumor promoter with its receptors was not studied much.

Another pathway of tumor promotion is inhibition of protein phosphatases 1 and 2A by okadaic acid, dinophysistoxin-1, etc. [9]. The mechanism of tumor promotion by okadaic acid has been proven to be a general pathway for tumor promotion in various organs. In the pathway, mouse tumor necrosis factor-α (mTNFα) is released, which is now identified as endogenous tumor promoter in vivo [10,11]. EGCG also inhibits the specific binding of $^{3}$H-okadaic acid to the receptors in a particulate fraction of mouse skin [8]. It is, therefore, important to investigate the effect of EGCG on interaction of okadaic acid with protein phosphatases 1 and 2A.

PKC is a membrane-associating protein and requires lipidic environment for its activation. We prepared liposomes consisting of DPPC, DOPC, and DMPC, in which PKC was incorporated. This assay system was useful to evaluate inhibitory effects of EGCG on PKC activation directly or through bilayer membrane. Protein phosphatase 2A (PP2A) was extracted from plasma membrane as well as from the cytosol of the T-lymphoblast [12], indicating the partition of PP2A to lipid membrane. PP2A activity was, thus, determined in the presence of liposomes, and the effect of EGCG on inhibitory activity of okadaic acid toward PP2A was also investigated.

## 2. Experimental

### 2.1. Materials

2- and 10-Anthroyloxystearic acid (2AS and 10AS) were purchased from Molecular Probes, USA. TPA, DMPC, DPPC, DOPC, POPC, and CF were purchased from Sigma, USA. PS was obtained from Serdary Res. Lab., USA. Histone H1 and ATP were purchased from Gibco Brl., USA and Wako Pure Chem., Japan, respectively.

### 2.2. Methods

Fluorescence and CD spectra were measured on a Hitachi MPF-4 fluorophotometer and a JASCO J-600 spectropolarimeter, respectively. Lipid concentrations were determined by a calorimetric method using phospholipase D (Diacolor, Toyobo, Japan).

#### 2.2.1. CF leakage

CF leakage from CF-trapped vesicles was measured according to the method reported by Barbet et al. [13]. Excitation and monitoring wavelengths were 480 and 520 nm, respectively. Complete release of CF was determined by addition of Triton X-100 (0.3 wt%).

#### 2.2.2. Partition to liposome

Partition of EGCG to phospholipid bilayer membranes was determined by using MLV composed of DOPC, POPC, and DMPC. A dry thin film of lipids was dispersed in Tris buffer (10 mM, pH 7.4, 0.1 mM EDTA), and the dispersion was weakly sonicated above the phase transition temperature of the membrane for 5 min followed by freezing for 10 min. The freeze and thaw process was repeated six times to obtain MLV. EGCG at a concentration of 30 μM was incubated with MLV([lipid] = 25 mM) for 30 min at 20°C. The dispersion was centrifuged at 12000 g for 15 min, and concentration of EGCG in the supernatant was determined by HPLC (a reverse-phase column, 5C18, using acetonitrile/acetic acid/0.05% aqueous phosphate solution (12/2/86 v/v/v) as eluant).

*2.2.3. Inhibition of TPA-induced PKC activation by EGCG*

PKC was partially purified from bovine brain by elution through a DEAE-cellulose column followed by a Sephacryl S-300 column. The PKC was used throughout the present work, since the inhibitory effect of EGCG on the PKC was similar to that on PKC further purified by isoelectric electrophoresis using Rotofor (Bio-Rad Lab., USA). PKC activation was induced by TPA of 2.0 μM in the presence of various SUVs. SUVs were prepared by a sonication method. A dry thin film of phospholipid and PS was dispersed in a Tris buffer (50 mM Tris, pH 7.4, 5 mM $(AcO)_2Mg$, 1 mM $CaCl_2$) by using a probe-type sonicator. Various concentrations of EGCG, histone H1, and ATP, respectively, were added to the assay mixture to determine the PKC activation.

*2.2.4. Effect on protein phosphatase 2A (PP2A)*

PP2A was partially purified from bovine brain by elution through DEAE-cellulose column followed by elution through a Sephaeryl S-300 column and a Sephadex G-200 column according to the method reported by Mackenzie et al. [14]. Phosphatase activity was assayed at 30°C by measuring the reduction of radioactivity of $^{32}$P-labeled histone H1 as previously described [15].

## 3. Results and discussion

*3.1. Interaction of EGCG with phospholipid bilayer membrane*

EGCG fluoresces in methanol, but does not in a buffer solution. However, EGCG in a buffer solution becomes fluorescent by the addition of DOPC liposome (Fig. 2), indicating partition of EGCG to lipid bilayer membrane. Aggregation of EGCG in a buffer solution should quench EGCG fluorescence. When EGCG is transferred to lipid membrane, the aggregates should dissociate and EGCG becomes fluorescent.

EGCG shows a negative Cotton effect around 275 nm in a buffer solution (Fig. 3). The strong intensity suggests that EGCG forms aggregates where aromatic groups of EGCG molecules stack regularly [16]. The intensity decreases by the addition of

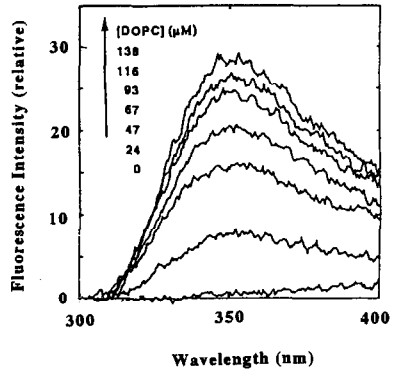


Fig. 2. Fluorescence spectra of EGCG in the presence of DOPC liposomes of various concentrations. [EGCG] = 10 μM. Excitation wavelength was 280 nm.

DMPC liposome, indicating binding of EGCG to DMPC bilayer membrane by dissociation of the aggregates.

The location of EGCG incorporated in DMPC liposome was investigated by fluorescence quenching of 2AS and 10AS with EGCG. The anthryl group of 2AS should be located in a surface region of the bilayer membrane, while that of 10AS in a hydrophobic core of the membrane. 2AS and 10AS should be molecularly dispersed in the membrane, because excimer emission was not detected, and the probes had a negative charge at the pH causing a repulsive force between them. The Stern–Volmer

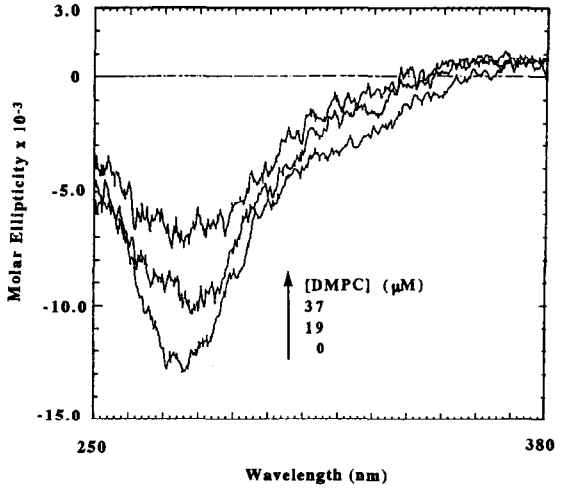


Fig. 3. CD spectra of EGCG in the presence of DMPC liposomes of various concentrations. [EGCG] = 50 μM.

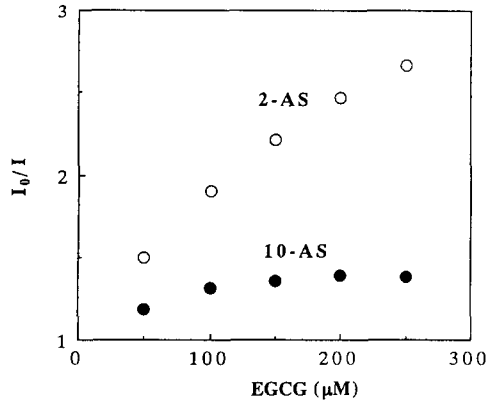


Fig. 4. Stem–Volmer plot of quenching of 2AS (○) or 10AS (●) with EGCG in the presence of DMPC liposome at 30°C. [2AS] or [10AS] = 4 μM. [DMPC] = 0.5 mM. Excitation wavelength was 365 nm.

plot (Fig. 4) shows that the emission of 2AS in the presence of DMPC liposome was quenched more strongly by EGCG than the emission of 10AS, implying that EGCG is located at the membrane surface. This interpretation is supported by the observation that DMPC liposomes aggregate in the presence of a large amount of EGCG above the phase transition temperature (Fig. 5). It is considered that the surface of liposomes becomes hydrophobic due to disturbance of membrane structure by accumulation of EGCG on the membrane surface.

### 3.2. Partition to different kinds of liposomes

Partition of EGCG to three kinds of phospholipid liposomes was investigated under the condition of [lipid]/[EGCG] = 2.5. The fractions of EGCG found in DMPC, POPC, and DOPC membranes were 13.8 ± 0.2, 19.8 ± 0.1, and 23.1 ± 3.7%, respectively, indicating that affinity of EGCG for phospholipid membrane increases in the order of DMPC < POPC < DOPC. Since the lipid packing in the membrane becomes looser in this order (DMPC, two saturated alkyl chains; POPC, one saturated chain and one unsaturated chain; DOPC, two unsaturated chains) [17,18], EGCG is suggested to sink into space among phospholipid molecules.

The amount of EGCG in the membrane was determined by varying concentrations of lipid molecules. The dissociation constant was evaluated from the slope of the plot of the partition rate ($\alpha$) against $\alpha/m$ ($m$ represents lipid concentration). The experimental data are on a straight line except that obtained at the highest concentration of DPPC (Fig. 6). The higher the concentration of EGCG in membrane becomes, the higher the $\alpha$ value is. This trend is explained by EGCG permeating through phospholipid bilayer membranes. The volume of inner aqueous phase of MLV is not negligible at high concentrations of lipid. When MLV is centrifuged, EGCG either encapsulated in the inner aqueous phase of

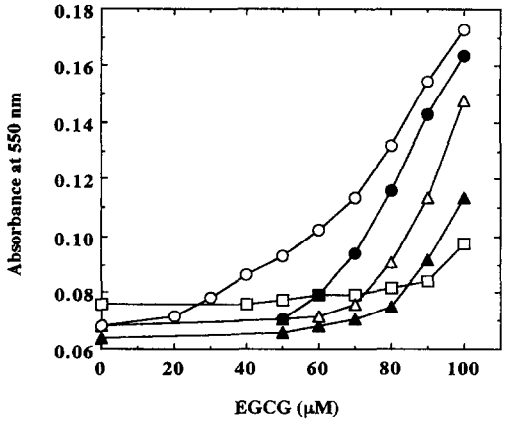


Fig. 5. Turbidity increase of DMPC liposome dispersion by EGCG addition at 50°C. [DPPC] = 54 μM (□); 109 μM (▲); 218 μM (△); 326 μM (●); 544 μM (○).

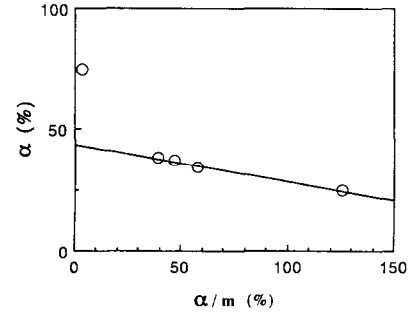


Fig. 6. Distribution of EGCG to DPPC MLV at 30°C. Circles are experimental values obtained at various concentrations of DPPC (0.2–25 mM). The straight line is drawn according to the equation, $\alpha = 1-(K_d/n)\times(\alpha/m)$, where $\alpha$, $K_d$, $n$, and $m$ represent the percentage of EGCG found in the membrane, the dissociation constant, the number of binding site, and the lipid concentration, respectively.

MLV or partitioned to bilayer membrane is included in the sediment and counted as those existing in the membrane.

### 3.3. Inhibition of PKC activation

EGCG inhibited PKC activation in the presence of DMPC liposome. $IC_{50}$, which is the concentration necessary to inhibit 50% activity of PKC, of EGCG was changed by varying amounts of DMPC liposome as follows; 36.1 μM for 254 μM DMPC + 20 μg $ml^{-1}$ PS, 20.9 μM for 127 μM DMPC + 10 μg PS, and 14.5 μM for 63.5 μM DMPC + 5 μg PS. $IC_{50}$ decreases with decreasing amount of lipids, suggesting that the inhibitory effect of EGCG on PKC activation occurs in the environment of phospholipid bilayer membrane. This interpretation is supported by the observation that the inhibitory effect of EGCG is also influenced by the lipid type. When the inhibition of PKC activation was examined in the presence of DPPC, DOPC, or DMPC liposome of the same concentration (63.5 μM) containing PS of 5 μg $ml^{-1}$, $IC_{50}$ was 59.6, 48.7, and 14.5 μM, respectively. The lower $IC_{50}$ in the presence of DMPC liposome than DPPC liposome may be explained by in terms of more abundant defects in DMPC bilayer membrane in a liquid–crystalline state than in DPPC bilayer membrane in a gel state. In order to examine this consideration, membrane perturbation induced by EGCG was examined by measuring CF leakage. CF leakage from DPPC liposome induced by EGCG was higher above the phase transition temperature of the membrane than below it (Fig. 7). It is, therefore, considered that EGCG disturbs the membrane structure more intensively at a liquid–crystalline state than at a gel state, and influences the PKC activation more strongly in a liquid–crystalline membrane. In addition, it may be considered that EGCG partition to the membrane may change the distribution of PS in the membrane and interaction of PS with PKC, resulted in change of PKC activity.

### 3.4. Effect of EGCG on ATP binding to PKC

Myricetin and quercetin, which are flavonoids, have been shown to inhibit PKC activation by competitive inhibition of ATP binding to PKC [19,20]. Effect of EGCG on ATP binding to PKC was examined. Fig. 8 shows the change of PKC activation under different concentrations of ATP and EGCG in the form of the Lineweaver–Burk plot. The inverse velocity of phosphorylation was dependent on the ATP concentration, and the value extrapolated to excess amount of ATP is independent of EGCG concentration, indicating competitive inhibition of EGCG by ATP for binding to the ATP-binding site of PKC. The ATP site is considered to be located at the membrane surface, which is very close to EGCG in the membrane, leading to a strong inhibition of ATP binding by EGCG.

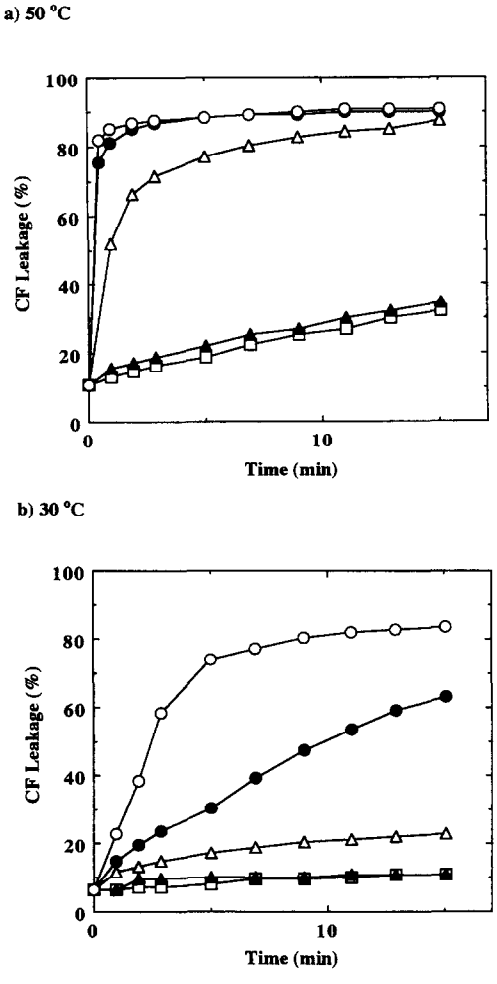


Fig. 7. CF leakage from DPPC liposome with the addition of EGCG of various concentrations at (a) 50°C and (b) 30°C. [EGCG] = 0 μM (□); 1 μM (▲); 10 μM (△); 50 μM (●); 100 μM (○).

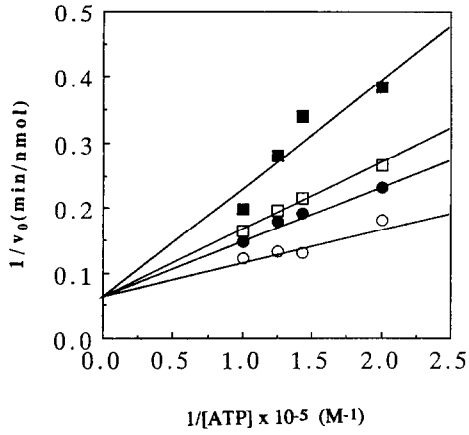


Fig. 8. Lineweaver–Burk plot of PKC activation against different concentrations of ATP. [EGCG] = 0 μM (○), 3.2 μM (●), 10 μM (□), 32 μM (■); [TPA] = 2 μM; [DMPC] = 63.5 μM; [PS] = 5 μg $ml^{-1}$.

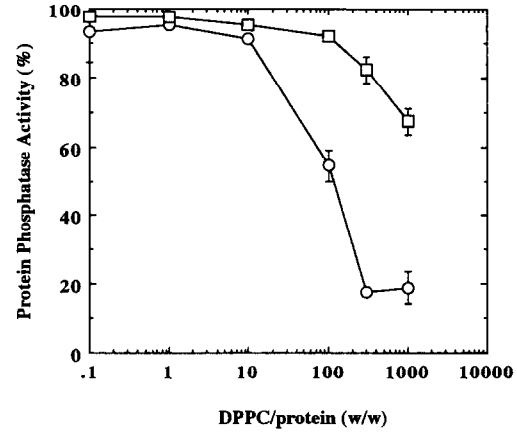


Fig. 10. Inhibitory effect of DPPC MLV (□) and DPPC SUV (○) on protein phosphatase activity. The protein (0.2 μg) was incubated with various amounts of DPPC vesicles, and the protein phosphatase activity was measured.

### 3.5. Effect of EGCG on TPA binding to PKC

PKC activation, which is inhibited by 100 μM EGCG, was investigated in the presence of varying concentrations of TPA, and the change of the activity is summarized in Fig. 9. TPA of 2 μM is enough to activate PKC in the absence of EGCG, but the activity was completely lost in the presence of EGCG of 100 μM. The activity was recovered by 50% with increasing amount of TPA, but a complete recovery of PKC activity was not attained even at higher concentrations of TPA. TPA has been pointed out to act as a boundary lipid [6]. It is, therefore, considered that TPA and EGCG bind competitively to PKC in lipid bilayer membrane. This could explain the incomplete recovery of PKC activity in the presence of high concentrations of TPA.

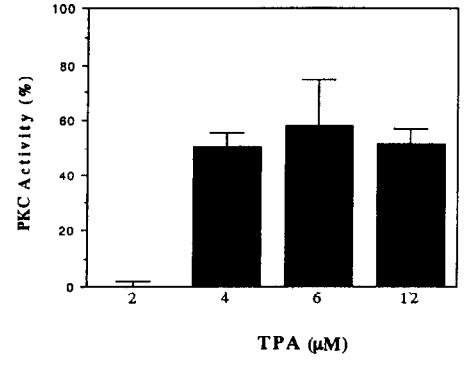


Fig. 9. Effect of TPA on PKC suppression by EGCG. [DMPC] = 63.5 μM; [PS] = 5 μg $ml^{-1}$; [EGCG] = 100 μM.

### 3.6. Effect of liposome on PP2A

The protein phosphatase activity was measured in the presence of DPPC liposome. The enzyme activity decreased depending on increase of phospholipid (Fig. 10). The inhibitory effect of SUV was more significant than that of MLV at the same lipid concentration. This should be due to larger total surface area of SUV than that of MLV, suggesting that larger amount of protein phosphatase 2A is associated with SUV than with MLV.

The binding of phosphorylated histone H1 to the lipid membrane was examined. The substrate was incubated with DPPC MLV or PS/DPPC MLV, and the suspension was centrifuged. The amount of the substrate in the supernatant was not changed by increasing amount of MLV, indicating the absence of interaction of the substrate with lipid membrane. Therefore, this rules out the possibility for lipid membrane to inhibit protein phosphatase activity by sequestering substrate into phospholipid bilayer membrane. It was also confirmed that the liberated phosphates were not bound to lipid membrane, and that spontaneous dephosphorylation of the phosphorylated substrate did not occur on addition of phospholipid vesicles.

The binding of PP2A was indicated by using MLV. The protein phosphatase was incubated with DPPC MLV. The mixture was centrifuged to precipitate membrane-associated PP2A, if it occurs. The activity of protein phosphatase remaining in supernatant decreased with increasing amount of MLV (Table 1), indicating precipitation of PP2A with lipid molecules.

It was reported that PP2B specifically interacts with PS or phosphatidylglycerol, but not with phosphatidylcholine [21]. On the other hand, in the present investigation, the addition of PS to phospholipid bilayer membrane did not significantly increase the binding of PP2A to lipid membrane. Since excess lipid membrane was required for binding all PP2A molecules, the affinity of PP2A for lipid membrane should not be so high as PP2B. This observation is consistent with the experimental results using T-lymphoblast that the amount of PP2A isolated from the plasma membrane was less than that from cytosol [12]. Therefore, the association of PP2A with lipid membrane is not specific compared with PP2B.

These experimental results indicate that protein phosphatase 2A binds to excess amount of lipid membrane to lose the phosphatase activity on phosphorylated histone H1.

### 3.7. Effect of EGCG on PP2A activity

The PP2A activity in a buffer solution was not affected by addition of EGCG, suggesting the absence of interaction between EGCG and PP2A. Protein phosphatase activity was lost in the presence of okadaic acid of 1 nM. However, EGCG recovered inhibition of protein phosphatase activity by okadaic acid (Fig. 11). Since EGCG forms aggregates in a buffer solution as described above, okadaic acid may be incorporated into the aggregates by hydrophobic interaction as well as hydrogen bonding. Thus, EGCG resulted in suppression of okadalic acid binding to PP2A.

Table 1
Association of PP2A with DPPC MLV.

| DPPC/protein (w/w) | Protein phosphatase activity (%) [a] |
|---|---|
| 200 | 93±6 |
| 1000 | 68±3 |
| 2000 | 61±4 |

[a] The membrane-associated protein phosphatase was precipitated and the activity remaining in supernatant was determined and shown in the table, the activity in the absence of DPPC MLV being taken as 100%.

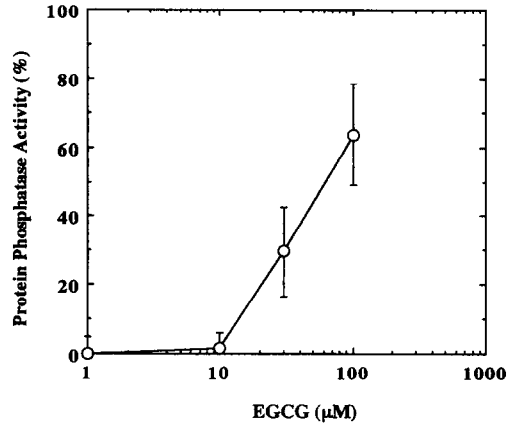


Fig. 11. Effect of EGCG on PP2A activity in the presence of okadaic acid (1 nM).

## 4. Conclusion

EGCG is distributed into the surface region of phospholipid bilayer membrane, and competitively inhibits binding of ATP and TPA to PKC, resulting in suppression of PKC activation. In addition, EGCG affects PKC activation through perturbation of the membrane structure. EGCG permeates phospholipid bilayer membrane, interacting easily with cytosolic proteins. Another interesting finding of EGCG is aggregate formation in aqueous solution. Okadaic acid might be incorporated into the aggregate, resulting in suppression of okadaic acid binding to PP2A. Thus, EGCG exhibits sealing effects on proteins directly and indirectly.

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