

# The Inhibitory Effect of (–)-Epigallocatechin Gallate on Activation of the Epidermal Growth Factor Receptor Is Associated with Altered Lipid Order in HT29 Colon Cancer Cells

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## Abstract

(–)-Epigallocatechin gallate (EGCG), a major biologically active constituent of green tea, inhibits activation of the epidermal growth factor (EGF) receptor (EGFR) and downstream signaling pathways in several types of human cancer cells, but the precise mechanism is not known. Because several plasma membrane-associated receptor tyrosine kinases (RTK) including EGFR are localized in detergent-insoluble ordered membrane domains, so-called “lipid rafts,” we examined whether the inhibitory effect of EGCG on activation of the EGFR is associated with changes in membrane lipid order in HT29 colon cancer cells. First, we did cold Triton X-100 solubility assays. Phosphorylated (activated) EGFR was found only in the Triton X-100-insoluble (lipid raft) fraction, whereas total cellular EGFR was present in the Triton X-100-soluble fraction. Pretreatment with EGCG inhibited the binding of Alexa Fluor 488-labeled EGF to the cells and also inhibited EGF-induced dimerization of the EGFR. To examine possible effects of EGCG on membrane lipid organization, we labeled the cells with the fluorescent lipid analogue 1, 1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, which preferentially incorporates into ordered membrane domains in cells and found that subsequent treatment with EGCG caused a marked reduction in the Triton X-100-resistant membrane fraction. Polyphenon E, a mixture of green tea catechins, had a similar effect but (–)-epicatechin (EC), the biologically inactive compound, did not significantly alter the Triton X-100 solubility properties of the membrane. Furthermore, we found that EGCG but not EC caused dramatic changes in the function of bilayer-incorporated gramicidin channels. Taken together, these findings suggest that EGCG inhibits the binding of EGF to the EGFR and the subsequent dimerization and activation of the EGFR by altering membrane organization. These effects may also explain the ability of EGCG to inhibit activation of other membrane-associated RTKs, and they may play a critical role in the anticancer effects of this and related compounds. [Cancer Res 2007; 67(13):6493–501]

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## Introduction

Members of the epidermal growth factor (EGF) receptor (EGFR) family of receptor tyrosine kinases (RTK) have been implicated in the abnormal growth of several types of human cancers, including cancers of the lung (1), head and neck (2), colon (3), prostate (4), and breast (5). Other RTKs, including platelet-derived growth factor receptor (PDGFR; ref. 6) and insulin-like growth factor-I receptor (IGF-IR; refs. 7, 8), are also implicated in various types of human cancer. There is evidence that the EGFR (9–13), the HER2 (14), and the IGF-IR (15) are associated with specific organized domains in the plasma membrane, sometimes called “lipid rafts,” although the precise organization of these RTKs in the plasma membrane is not known. These ordered domains contain relatively high levels of cholesterol and sphingolipids (16, 17), but the actual size, precise composition, and dynamics of these domains are not known (18). One method for analysis of incorporation in ordered membrane domains involves extraction of cells with cold Triton X-100 followed by isolation of a low buoyant density fraction by density gradient centrifugation (9). The detergent-resistant fraction is enriched in cholesterol and sphingolipids. These and other studies suggest that the plasma membrane contains coexisting cholesterol and sphingolipid-rich domains with a higher degree of lipid order, as well as more disordered domains enriched in phospholipids with unsaturated hydrocarbon chains (19). Lipids with long and saturated acyl chains, such as the synthetic fluorescent lipid analogue 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC<sub>16</sub>), preferentially partition into the ordered domains (20). In many studies, these and other lipid analogues have been used to observe the formation and dynamics of membrane domains in living cells (21, 22).

There is evidence that the amount of cholesterol, the status of lipid unsaturation, and the charge on lipid head groups are important determinants of the structure of membrane domains (23). Furthermore, cholesterol may play a crucial role in modulating signaling from plasma membrane-associated receptors. Thus, the cholesterol-depleting agent methyl β-cyclodextrin (MβCD) can cause ligand-independent activation of the EGFR, apparently by altering membrane lipid organization (11–13, 24). By contrast, depletion of cholesterol from vascular smooth muscle cells by treatment with MβCD led to inhibition of angiotensin II-stimulated phosphorylation of the EGFR (25), and treatment of prostate cancer cells with MβCD to reduce cellular cholesterol levels inhibited phosphorylation of EGFR and the downstream protein AKT (10). Thus, depending on the cell system and pathway, cholesterol depletion seems to have both positive and negative effects on RTK-mediated signaling.

Green tea contains several polyphenolic compounds, including the catechins (–)-epigallocatechin gallate (EGCG), (–)-epigallocatechin (EGC), epicatechin-3-gallate (ECG), and (–)-epicatechin (EC). Among the green tea constituents, EGCG is the major biologically active polyphenol in green tea; it has been shown to inhibit the growth of several types of cancer cell lines (2, 3, 5, 26). This is associated with inhibition of phosphorylation (i.e., activation) of the EGFR and inhibition of several downstream signaling pathways (2, 3, 27). EGCG can also inhibit activation of other RTKs, including HER2, HER3, HER4, IGFI-R, PDGFR, and fibroblast growth factor (2, 3, 6, 8, 28). Although there is evidence that EGCG may directly interfere with EGF binding to the EGFR (28), the ubiquitous effect of EGCG on several RTKs suggests that it might act on these plasma membrane-associated proteins by a more general mechanism. Indeed, it was reported recently that in the human basophilic cell line KU812, EGCG binds to the cell surface and preferentially associates with lipid rafts in these cells (29). Therefore, in the present study, we analyzed whether inhibition of activation of the EGFR in HT29 human colon cancer cells is associated with changes in plasma membrane lipid order in these cells. In the absence of EGCG, the activated form of EGFR [phosphorylated EGFR (p-EGFR)] was present in the Triton X-100-insoluble fraction of HT29 cells. Treatment with EGCG inhibited EGF binding as well as EGFR dimerization and activation. These effects were associated with depletion of the Triton X-100-resistant fraction of the plasma membrane. Similar inhibitory effects on activation of the EGFR and changes in plasma membrane lipid order were seen with the catechin mixture Polyphenon E (PolyE), but not with the biologically inactive compound EC. To explore whether EGCG could act through a general, bilayer-mediated mechanism, we also tested its effect on bilayer-incorporated gramicidin channels, where EGCG alters channel function at submicromolar concentrations, whereas EC is inactive. These findings suggest a novel mechanism by which EGCG, and perhaps other specific polyphenolic compounds, can inhibit the activity of specific RTKs and thereby exert anticancer effects.

## Materials and Methods

**Chemicals.** EGCG, EC, ECG, EGC, PolyE, and theaflavin-3 were kindly provided by Dr. Yukihiko Hara (Mitsui Norin Co., Shizuoka, Japan). Curcumin and resveratrol were purchased from Sigma Chemical Co. Cyanidin and delphinidin were provided by Dr. Edward J. Kennelly (The City University of New York, Bronx, NY). All compounds were diluted in DMSO. DiIC<sub>16</sub> was purchased from Invitrogen.

**Cell culture conditions.** The human colon cancer cell line HT29 was maintained in DMEM (Invitrogen), containing 10% fetal bovine serum. For the microscopy experiments,  $5 \times 10^4$  cells were plated 72 h before the experiments in 35-mm plastic tissue culture dishes with a 7-mm hole in the bottom covered by poly-D-lysine-coated coverslips (30). For Western blot analysis, the cells were starved in serum-free medium for 24 h before they were pretreated with the indicated compound for the indicated time. As indicated in the figure legends, 15 min before harvesting, 100 ng/mL EGF (Sigma) was added to activate the EGFR.

**Triton X-100 solubility assay.** Extraction of Triton X-100-soluble and Triton X-100-insoluble membrane constituents was done as described previously (10, 31). In brief, cells were resuspended in buffer A [25 mmol/L 2-(*N*-morpholino)ethanesulfonic acid, 150 mmol/L NaCl (pH 6.5)]. To this, an equal volume of the same buffer with 2% Triton X-100, 2 mmol/L Na<sub>3</sub>VO<sub>4</sub>, and 2 mmol/L phenylmethylsulfonyl fluoride (PMSF) was added, and the cells were incubated on ice for 30 min with no agitation. Insoluble fractions were pelleted in a microcentrifuge for 20 min at 4°C. The supernatant fraction was removed ["S" (soluble) fraction], and the insoluble

pellet was resuspended in buffer B [1% Triton X-100, 10 mmol/L Tris (pH 7.6), 500 mmol/L NaCl, 2 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 60 mmol/L β-octylglucoside (Sigma), 1 mmol/L PMSF] for 30 min on ice. Debris was pelleted in a microcentrifuge for 20 min at 4°C, and the supernatant fraction was collected. This fraction is referred to as "I" (insoluble) and represents the lipid raft fraction. The primary antibodies used in this study were anti-p-EGFR (Tyr<sup>1068</sup>; Cell Signaling), anti-EGFR (Santa Cruz Biotechnology), and anti-actin antibody (Sigma), respectively.

**Cross-linking of EGFR.** Cross-linking experiments were done as described (32) with minor modifications. Cells were washed with ice-cold PBS before incubation on ice for 30 min with the nonpermeable cross-linking reagent bis (sulfosuccinimidyl) suberate (BS<sup>3</sup>; 3 mmol/L in PBS; Pierce). In all experiments, a freshly prepared solution of BS<sup>3</sup> was used. The reaction was quenched for 5 min by the addition of 1 mol/L glycine (pH 8.0; final concentration of 250 mmol/L). The cells were then washed with cold PBS and lysed in buffer [50 mmol/L HEPES (pH 7.5), 10% glycerol, 0.5% Triton X-100, 1.5 mmol/L MgCl<sub>2</sub>, 1 mmol/L EDTA, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 2 mmol/L PMSF] and suspended with by aspiration through 23-gauge needle. The lysates were clarified at 14,000 rpm in an Eppendorf centrifuge for 10 min at 4°C. The supernatant fraction was collected and subjected to SDS-PAGE on a 5% polyacrylamide gel followed by immunoblotting with anti-EGFR antibody.

**EGF binding assay.** Twenty-four hours before the experiment, the medium was changed to serum-free DMEM. After pretreatment with the indicated compound in serum-free DMEM, the cells were treated with 100 ng/mL Alexa Fluor 488 EGF complex (Molecular Probes, Inc.) for 1 h at 4°C (on ice) to prevent EGF-induced internalization of the EGFR and then fixed with 2% paraformaldehyde. The cells were then examined using fluorescent confocal microscopy. To quantify, the cells were exposed to Alexa-EGF (100 ng/mL) for 1 h at 4°C after the indicated treatment at 37°C. They were then washed with cold PBS and harvested by the addition of trypsin and gentle scraping at 4°C followed by fixation with 2% paraformaldehyde for 15 min. The cells were then analyzed for cell surface-bound Alexa-EGF by flow cytometry using a FACS Calibur instrument with CellQuest computer program (Becton Dickinson).

**Cold Triton X-100 extractability of DiIC<sub>16</sub>.** Cold Triton X-100 extractability of DiIC<sub>16</sub> was done as described previously (18). To prepare the labeling solution, 750 nmol DiIC<sub>16</sub> was dissolved in 1 mL methanol. The cells were grown previously on coverslip-bottomed dishes and then taken out of the CO<sub>2</sub> incubator, rinsed several times with isotonic medium 1 [150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub>, and 20 mmol/L HEPES (pH 7.4); supplemented with 2 g/L glucose], and then labeled with a 1:1,000 dilution of the DiI labeling solution for 3 min at 37°C. The cells were then washed with ice-cold cytoskeleton stabilization buffer [CSB; 138 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub>, 2 mmol/L EGTA, 0.32 mol/L sucrose, 10 mmol/L MES (pH 6.1); ref. 33], extracted with 0.5% Triton X-100 on ice for 30 min, and then fixed with 2% paraformaldehyde.

**Confocal microscopy.** Confocal microscopy was done using an Axiovert 100M inverted microscope equipped with an LSM 510 laser scanning unit and a 63× 1.4 NA plan Apochromat objective (Carl Zeiss, Inc.). Cells labeled with DiIC<sub>16</sub> were excited with a helium/neon laser emitting at 543 nm, and a 560-nm long-pass filter was used for collecting emissions. Alexa Fluor 488-conjugated EGF was excited with an argon ion laser emitting at 488 nm, and a 505-nm long pass filter was used for emissions.

**Image analysis.** All image analyses were done using MetaMorph (Molecular Devices). Fixed threshold intensity was applied to F-actin fluorescence images, and the number of pixels above the threshold was defined as cell area. A threshold was then applied to the DiIC<sub>16</sub> fluorescence image such that only DiIC<sub>16</sub>-positive pixels were used in calculating the percentage of the DiIC<sub>16</sub>-positive staining per cell area, using the following formula: DiIC<sub>16</sub>-positive area = (the number of DiIC<sub>16</sub>-positive pixels) / (cell area). At least, 30 cells were measured at each sample.

**Studies with synthetic lipid bilayers.** The bilayer-forming lipid was 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids), which was used without further purification and dissolved (2% w/v) in *n*-decane (99.9% pure from ChemSampCo). The gramicidin analogues [Ala<sup>1</sup>] gA (AgA) and [D-Ala<sup>1</sup>] gA<sup>-</sup> (AgA<sup>-</sup>) were synthesized and purified as described previously (34).

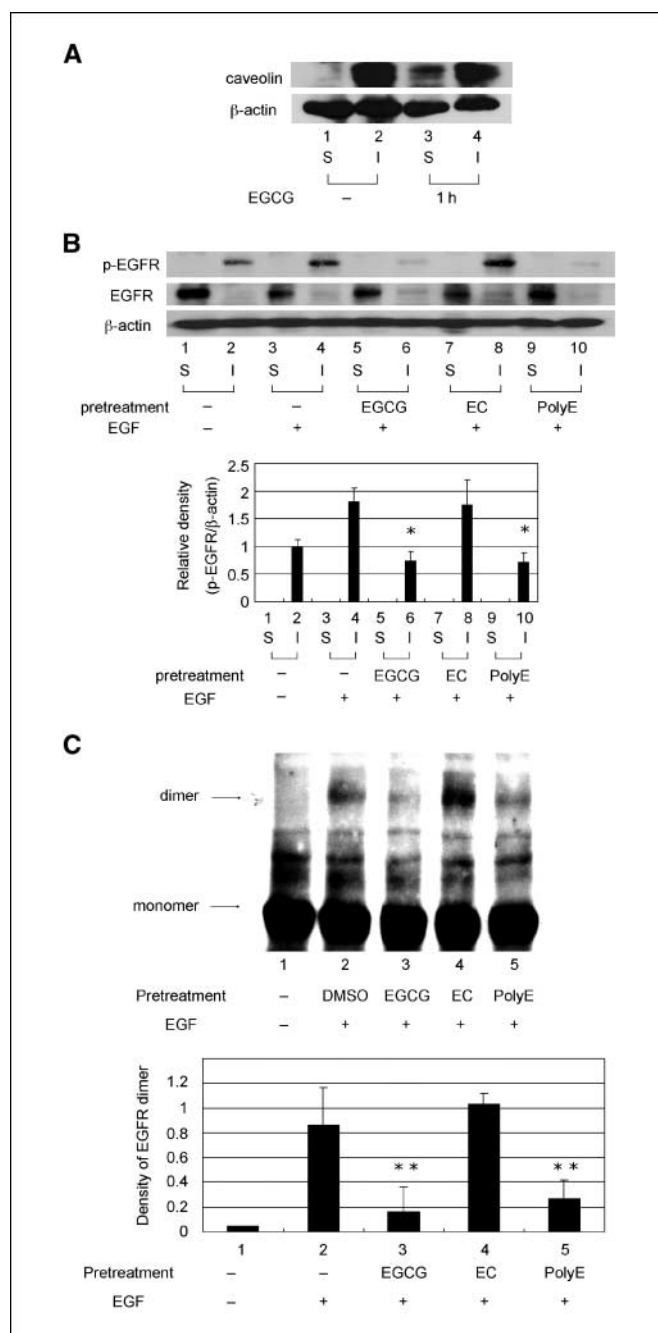
Planar lipid bilayers were formed across a 1.6-mm diameter hole in a Teflon partition separating two electrolyte solutions (1 mol/L CsCl, buffered to pH 7.0 using 10 mmol/L HEPES from Sigma). Gramicidin single-channel experiments were done using the bilayer punch method (35) at  $25 \pm 1^\circ\text{C}$ . All measurements were done using a Dagan 3900A Integrating patch clamp, with a 200 mV applied potential. The current signal was filtered with a low-pass Bessel filter at 1,000 Hz and digitized at 20 kHz.

## Results

**p-EGFR is localized in the Triton X-100-insoluble fraction, and total EGFR is localized in the Triton X-100-soluble fraction of HT29 cells.** In previous studies, we found that EGCG and PolyE suppressed phosphorylation of the EGFR in HT29 cells, but the precise mechanism was not apparent (3). There is evidence that activation of EGFR occurs within ordered domains (lipid rafts) in the plasma membrane (10). Therefore, we examined by Western blot analysis with the appropriate antibodies the effects of EGCG and PolyE on the distribution of total EGFR and p-EGFR, the activated form of EGFR, in the cold Triton X-100-insoluble (lipid raft) fraction, designated "I," and in the cold Triton X-100-soluble (non-lipid raft) fraction, designated "S," of lipid extracts of HT29 cells (see Materials and Methods). To validate the extraction method, we first studied the extraction of caveolin-1 in untreated HT29 cells. Western blot analysis indicated that caveolin-1 partitioned, almost completely, into the I fraction (Fig. 1A, lanes 1 and 2), which is consistent with previous studies (10, 13). Pretreatment of HT29 cells with 20  $\mu\text{g}/\text{mL}$  EGCG for 1 h caused a partial shift of caveolin from the I to the S fraction (Fig. 1A, lanes 3 and 4). These results suggest that EGCG might directly influence plasma membrane organization.

We next examined by cold Triton X-100 extraction and Western blot analysis, whether the EGFR and p-EGFR were present in the I or S lipid fractions of HT29 cells (Fig. 1B). When serum-starved HT29 cells were not treated with EGF, most of the total cellular EGFR was found in the S fraction (Fig. 1B, lane 1). We also detected a small amount of p-EGFR, and this was present only in the I fraction (Fig. 1B, lane 2). Presumably, this reflects partial activation of the EGFR by the autocrine production of transforming growth factor  $\alpha$  (TGF $\alpha$ ) in these cells (3). Treatment of the cells with 100 ng/mL EGF for 15 min resulted in an increase in the amount of p-EGFR, which was again confined to the I fraction (Fig. 1B, lane 4). Pretreatment of the cells with 20  $\mu\text{g}/\text{mL}$  EGCG or PolyE for 1 h (Fig. 1B, lanes 6 and 10, respectively) caused a marked decrease in the amount of p-EGFR in the I fraction, whereas treatment with EC, a biologically inactive catechin present in green tea (3), had no effect (Fig. 1B, lane 8). These results provide evidence that the bulk of the EGFR protein is present in the disordered lipid domains of these cells. With activation by EGF, a portion of these molecules is associated with detergent-resistant membranes, and both EGCG and PolyE inhibit this activation process. We also found that when the cells were treated with EGCG in the absence of added EGF, the small amount of p-EGFR found in Fig. 1B (lane 2) could not be detected in the Triton X-100-soluble fraction (data not shown), presumably because EGCG also inhibits activation of the EGFR by endogenous TGF $\alpha$ .

**EGCG and PolyE, but not EC, inhibit EGFR dimer formation.** Structural studies of the EGFR led to the proposal of a novel mechanism for ligand-induced receptor dimerization (36). In this model, the binding of EGF to the extracellular domain of the EGFR induces a dramatic conformational change in the EGFR protein, exposing a dimerization site in the cytoplasmic domain that is



**Figure 1.** The effects of EGCG, EC, and PolyE on phosphorylation and dimerization of the EGFR. HT29 cells were seeded at  $8 \times 10^5$  per 100-mm dish and incubated for 48 h in normal growth medium followed by incubation in serum-free medium for 24 h. As indicated, the cells were then treated at  $37^\circ\text{C}$  with the DMSO solvent and 20  $\mu\text{g}/\text{mL}$  EGCG, EC, or PolyE for 1 h at  $37^\circ\text{C}$ . Before harvesting, the cells were stimulated at  $37^\circ\text{C}$  with EGF (100 ng/mL) for 15 min. The cells were then harvested and extracted with cold Triton X-100 into a soluble (S) and an insoluble (I) fraction, as described in Materials and Methods. The soluble and insoluble fractions were then analyzed by Western blotting, using the respective antibody. **A**, distribution of caveolin. **B**, distribution of EGFR and p-EGFR. **Bottom**, quantification data of the relative levels of p-EGFR (*top*), after normalization with respect to  $\beta$ -actin, as determined by densitometry. \*,  $P < 0.05$ , significant decrease with respect to lane 4. **C**, cells were treated with the indicated compound as described above and then cell surface receptors were cross-linked in the presence of BS<sup>3</sup>, a membrane-impermeable cross-linker, for 30 min at  $4^\circ\text{C}$ . Cell lysates were subjected to electrophoresis on SDS-PAGE gels (5% polyacrylamide) followed by Western blotting with an anti-EGFR antibody. The positions of the EGFR dimer and monomer are indicated. **Bottom**, quantification data of the amount of the EGFR dimer (*top*). \*\*,  $P < 0.01$ , significant decrease with respect to lane 2. For additional details, see Materials and Methods.

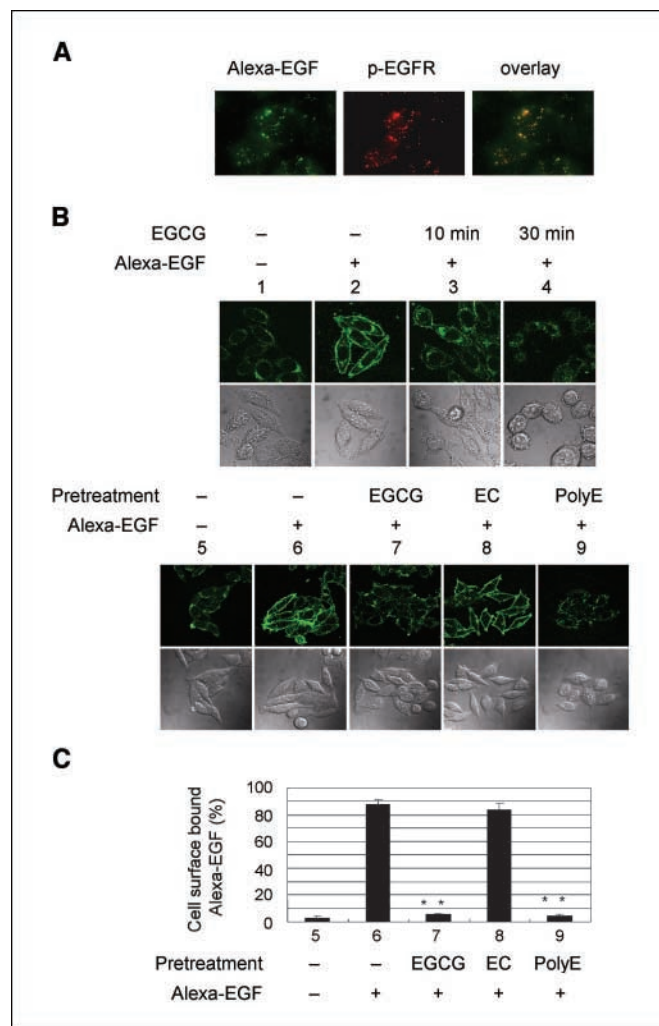
normally occluded in the inactivated conformation, thus promoting dimer formation. The above studies and our previous studies in HT29 cells (3) indicated that EGCG and PolyE inhibit activation of the EGFR. We therefore investigated whether EGCG also affect EGFR dimerization. Serum-starved HT29 cells were stimulated with EGF (100 ng/mL) for 15 min after pretreatment with the solvent (0.1% DMSO), EGCG, EC, or PolyE, and then the intact cells were exposed to the membrane-impermeable cross-linker BS<sub>3</sub> (32). Cell extracts were then prepared and examined by Western blot analysis with an anti-EGFR antibody (Fig. 1C). As expected, treatment with EGF enhanced EGFR dimer formation (Fig. 1C, compare lanes 1 and 2). In addition, the EGFR dimer was found in the Triton X-100-insoluble fraction (data not shown), which is consistent with the results obtained in Fig. 1B. On the other hand, EGCG and PolyE (Fig. 1C, lanes 3 and 5), but not EC (Fig. 1C, lane 4), inhibited dimerization of the EGFR. These results provide further evidence that EGCG and PolyE suppress ligand-dependent activation of EGFR.

**EGCG and PolyE, but not EC, inhibit EGF binding.** It was previously reported that EGCG inhibited the binding of <sup>125</sup>I-EGF to intact A431 cells (28). To further investigate how EGCG inhibits activation of EGFR, we examined effects of EGCG, EC, and PolyE on EGF binding to intact HT29 cells using Alexa Fluor 488-labeled EGF and fluorescence microscopy (Fig. 2). First, we confirmed that Alexa-EGF stimulated phosphorylation of the EGFR (Fig. 2A). When HT29 cells were exposed to Alexa-EGF for 1 h at 4°C, the cell membranes displayed strong fluorescence (Fig. 2B, 2). This binding of Alexa-EGF to the cells was completely blocked by an excess of unlabeled EGF (data not shown). Pretreatment of the cells with 20 µg/mL EGCG at 37°C for 10 min before the addition of Alexa-EGF (Fig. 2B, 3) markedly inhibited, whereas pretreatment with 20 µg/mL EGCG or PolyE for 30 min almost completely inhibited, the binding of Alexa-EGF to these cells (Fig. 2B, 4, 7, and 9). When EGCG and Alexa-EGF were added simultaneously to the cells at 4°C, EGCG did not reduce the binding of Alexa-EGF to the cells (data not shown), which suggests that the inhibition of Alexa-EGF binding to the cells is not simply due to physical competition for binding sites. It is of interest that previous studies showing that EGCG inhibits <sup>125</sup>I-EGF binding to A431 cells were also done at 37°C (28). Pretreatment of the cells with 20 µg/mL EC had no detectable effect on Alexa-EGF binding to the cells (Fig. 2B, 8). These data provide evidence that the ability of EGCG and PolyE to inhibit phosphorylation (Fig. 1B) and dimerization of the EGFR (Fig. 1C) are a consequence of the ability of EGCG to inhibit binding of EGF to the EGFR. We also found that when the cells were treated with EGCG for 30 min at 37°C and then washed and incubated at 37°C with fresh medium lacking EGCG, Alexa-EGF binding was restored to control levels within 4 h. Therefore, this effect of EGCG is reversible (data not shown).

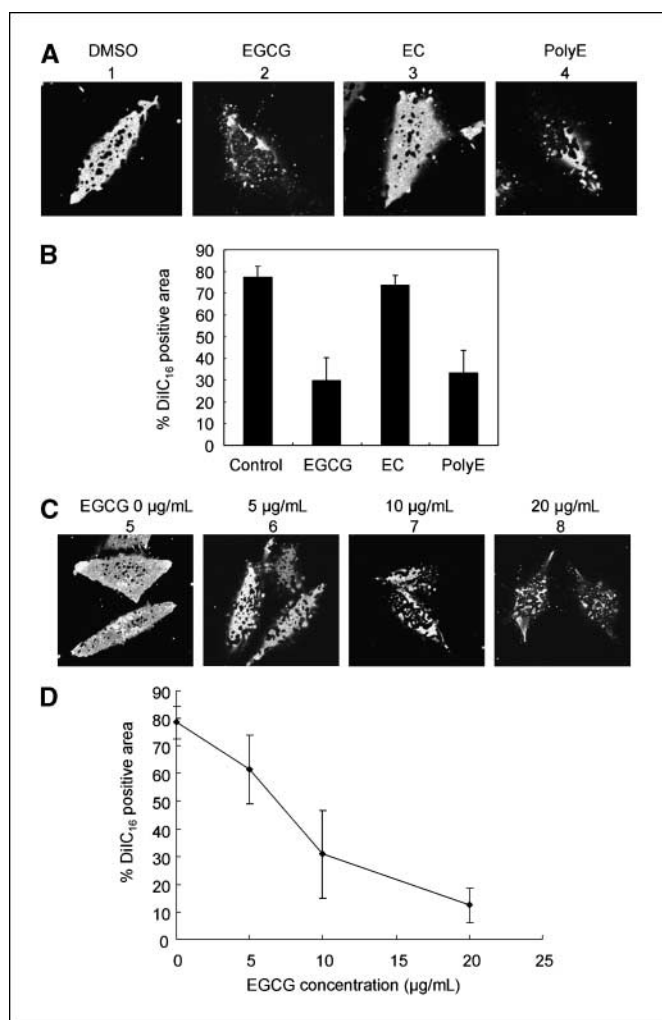
**Treatment of HT29 cells with EGCG causes a reduction in Triton X-100 resistance of the plasma membrane.** As mentioned in Introduction, there is evidence that lipid organization can affect the activation and functions of EGFR (10–13, 24, 25) and other membrane-associated receptors (14, 15). Indeed, we found that the activated form of the EGFR is specifically associated with the Triton X-100-insoluble fraction of HT29 cells (see Fig. 1), which is enriched in ordered lipids. Therefore, we carried out a series of studies to determine possible effects of EGCG on lipid order in cell membranes. We used an assay in which the cells are first fluorescently stained with the compound DiI<sub>16</sub>, a lipid-mimetic dialkylindocarbocyanine that preferentially incorporates into

detergent-resistant plasma membrane lipid domains (20). The cells were then exposed to cold Triton X-100, which preferentially extracts the disordered domains. Thus, after this extraction, DiI<sub>16</sub> labels the detergent-resistant cell membranes, and the extent of these detergent-resistant membranes can be quantified by fluorescent confocal microscopy (18).

Figure 3A (1) shows HT29 cells that were labeled with DiI<sub>16</sub> for 3 min and then exposed to the DMSO-containing solvent (control) for 30 min at 37°C before extraction with cold Triton X-100. As has been reported for other cells (18), a large fraction of the plasma membrane lipids remain unextracted by cold Triton X-100, and the remaining plasma membrane forms a large sheet with many small



**Figure 2.** The effects of EGCG, EC, and PolyE on EGF binding to HT29 cells. **A**, colocalization of Alexa-EGF and p-EGFR in HT29 cells. The cells were treated with Alexa-EGF at 37°C for 15 min and fixed with 2% paraformaldehyde followed by immunoblotting with an anti-p-EGFR antibody to confirm that Alexa-EGF stimulates p-EGFR. **B** and **C**, the cells were treated at 37°C with the indicated compound (20 µg/mL) for the indicated time. After this pretreatment, the cells were exposed to Alexa-EGF (100 ng/mL) for 1 h at 4°C and then washed with cold CSB (see Materials and Methods). The cells were then fixed with 2% paraformaldehyde and examined by confocal microscopy (see *top panels*). *Bottom*, the same cells were imaged by light transmission. 1 and 5, no exposure to Alexa-EGF; 2 to 4 and 6 to 9, exposed to Alexa-EGF (100 ng/mL) for 1 h at 4°C. The cells were pretreated with 20 µg/mL EGCG for 10 min (3) or 30 min (4 and 7), and with 20 µg/mL EC (8) or 20 µg/mL PolyE (9). **C**, quantification of cell surface-bound Alexa-EGF. Aliquots of the same cells shown in panels 5 to 9 in (B) were collected at 4°C, fixed with 2% paraformaldehyde, and examined by flow cytometry to quantify cell surface Alexa-EGF (see Materials and Methods).



**Figure 3.** The effects of EGCG, EC, and PolyE on lipid rafts using the DiIC<sub>16</sub> staining and cold Triton X-100 solubility assay. Although attached to the culture plates, HT29 cells were first stained with DiIC<sub>16</sub> for 3 min at 37°C and then exposed to solvent (DMSO) or 20 μg/mL of EGCG, EC, or PolyE for 30 min at 37°C. After washing with cold CSB, the attached cells were extracted with 0.5% cold Triton X-100 for 30 min at 4°C (on ice). They were then fixed with 2% paraformaldehyde for 15 min and examined by confocal microscopy. *A*, the representative images presented are single confocal sections near the bottom adherent surfaces of the cells. *B*, quantitative analysis of the results presented in (*A*), expressed as the percentage of the total cell area that was positive for DiIC<sub>16</sub> staining. Bars, SD. See Materials and Methods. *C*, in a dose-dependent study, the cells were first stained with DiIC<sub>16</sub> and then exposed at 37°C to increasing concentrations of EGCG for 30 min, and then extracted with cold Triton X-100, fixed, stained, and examined by confocal microscopy, as described in (*A*). *D*, quantitative analysis of the results presented in (*C*). The results are shown as percentage of the total cell area that is DiIC<sub>16</sub> positive. Bars, SD.

holes (Fig. 3*A*, 1 and *C*, 5). Similar results were obtained when the DiIC<sub>16</sub>-stained cells were treated with 20 μg/mL EC for 30 min at 37°C (Fig. 3*A*, 3). Treatment with 20 μg/mL EGCG or PolyE led to extensive loss of DiIC<sub>16</sub>-labeled membrane after cold Triton X-100 extraction (Fig. 3*A*, 2 and 4). The percentage of unextracted cell area is quantified in Fig. 3*B*. As shown in Fig. 3*C*, when the cells were treated with 5, 10, or 20 μg/mL EGCG, there was a dose-dependent decrease in the amount of unextracted DiIC<sub>16</sub>-labeled membrane. These results are quantified in Fig. 3*D*. As little as 5 μg/mL EGCG and a 30-min exposure was sufficient to reduce the content of detergent-resistant membrane in HT29 cells. Indeed, we

found that only 5-min exposure of the cells to EGCG was sufficient to cause a detectable decrease in the ordered domains (data not shown). This effect of EGCG is not confined to this cell type because we have seen similar effects with the SW480 colon cancer cell line (data not shown). In the studies shown in Fig. 3, the cells were treated with EGCG in serum-free medium. However, loss of detergent-resistant membrane was also seen when the cells were treated with EGCG in the presence of 10% serum, although the effect was diminished by ~30% (data not shown). We also found that when the cells were treated with EGCG for 30 min at 37°C and then washed and incubated at 37°C with fresh medium lacking EGCG, DiIC<sub>16</sub> staining of plasma membranes was restored to control levels within 4 h. Therefore, this effect of EGCG on disruption of lipid order is also reversible (data not shown).

In view of these results, it was of interest to also examine in the above assay system other catechins and the plant-derived polyphenolic compounds curcumin, resveratrol, cyanidin, and delphinidin because they have also been reported to have anticancer activity in various experimental systems (37). The results are summarized in Table 1. The green tea-derived catechin ECG was active with a potency similar to that of EGCG, whereas the catechin EGC, like EC, was inactive. Theaflavin-3, which is present in black tea, had an activity similar to that of EGCG (Table 1). Curcumin displayed weak activity at 50 μmol/L, but resveratrol, cyanidin, and delphinidin were inactive even when

**Table 1.** Effects of some phytochemicals on extractability of DiIC<sub>16</sub>-positive area

Compound (μmol/L)	Score
EGCG	
25	+
50	+
ECG	
25	+
50	+
EC	
50	-
EGC	
50	-
Theaflavin-3	
25	+
50	+
Curcumin	
25	-
50	±
Resveratrol	
50	-
Cyanidin	
50	-
Delphinidin	
50	-

NOTE: HT29 cells were prelabeled with DiIC<sub>16</sub> and then treated with the indicated compound followed by extraction with cold Triton X-100 and examined by Confocal microscopy for residual DiIC<sub>16</sub> staining as described for EGCG, EC and PolyE in Fig. 3. The effect of each compound was scored as follows: -, no decrease (<30%); ±, 30% to 50% decrease; +, >50% decrease of DiIC<sub>16</sub>-positive area of cell membranes.

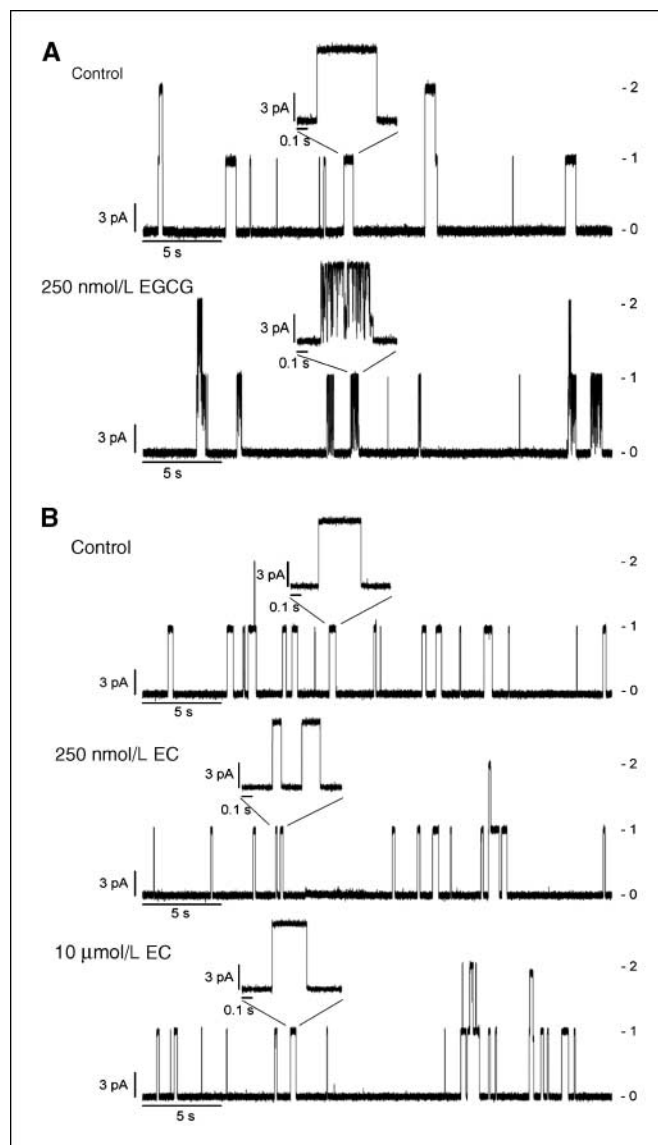
tested at 50  $\mu\text{mol/L}$ . The relative activities of the green tea catechins EGCG, ECG, EGC, and EC in this assay reflected their relative potencies for growth inhibition of HT29 cells because in the latter assays, their  $\text{IC}_{50}$  values in HT29 cells were 50, 80, 180, and  $>200$   $\mu\text{mol/L}$ , respectively (data not shown). In addition, theaflavin-3 is relatively potent in inhibiting the growth of some cancer cells (38, 39).

**EGCG also exerts effects on gramicidin channels imbedded in synthetic lipid bilayers.** To explore whether the EGCG-induced alteration of membrane domain organization could be due to EGCG-bilayer interactions, we examined whether EGCG altered the function of bilayer-spanning gramicidin channels. Gramicidin channels are single-molecule probes of lipid bilayer properties (40) and thus can be used to monitor how small molecules alter bilayer-protein interactions. Gramicidin channels are formed by the transmembrane dimerization of two nonconducting subunits residing in each bilayer leaflet. Because the hydrophobic length of the channels is less than the hydrophobic thickness of the bilayer, channel formation is associated with a local bilayer thinning meaning that the bilayer exerts a disjoining force on the channels. Therefore, drug-induced changes in bilayer properties would be expected to alter the function of gramicidin channels. In the simplest case, this would occur by altering the energetic of the bilayer deformation associated with changes in the gramicidin monomer  $\leftrightarrow$  dimer equilibrium. This feature makes gramicidin an excellent probe for measuring changes in lipid bilayer mechanical properties (40).

Figure 4A shows that EGCG is a potent modifier of AgA channel function in synthetic lipid bilayers. In the absence of EGCG, the AgA channels appear as stepwise transitions between three well-defined current levels corresponding to the presence of 0, 1, and 2 channels in the membrane. In the presence of only 250 nmol/L EGCG, the channel appearances changed qualitatively, with numerous downward transitions (flickers) from the conducting state, indicating that EGCG causes brief interruptions in the flow of ions through the channels. In some cases, the flickers were to the nonconducting current level; in other cases, the flickers were to some intermediate current level. Similar results were observed with the enantiomeric  $\text{AgA}^-$  channels (data not shown), indicating that the flickery channel activity is unlikely to result from direct interaction between EGCG and the AgA channels because this would be expected to vary with the channel chirality (right-handed versus left-handed helix sense). In contrast to the results with EGCG, EC had no detectable effect on AgA channel function up to 10  $\mu\text{mol/L}$  (Fig. 4B). EC did not cause the appearance of flickers in the conducting state; furthermore, it did not alter the channel appearance rate and lifetime.

## Discussion

The present study provides the first evidence that EGCG, a catechin present in green tea that has anticancer activity in several experimental systems, directly alters the organization of the plasma membrane in cancer cells. We found that treatment of HT29 colon cancer cells with EGCG at concentrations as low as 5  $\mu\text{g/mL}$  (10.9  $\mu\text{mol/L}$ ) caused within 30 min a reduction in ordered lipid domains in the plasma membrane of these cells (Fig. 3). Similar effects on disruption of detergent-resistant membranes were seen with ECG, PolyE (a mixture of green tea catechins), and theaflavin-3, whereas EC had no detectable effect on detergent resistance of plasma membrane lipids (Table 1). This correlates well with evidence that



**Figure 4.** Effects of EGCG and EC on gramicidin A channels in planar phospholipid bilayers. **A**, single-channel current traces recorded before and after addition of 250 nmol/L EGCG to the aqueous solution on both sides of an AgA-doped bilayer. *Numbers (right)*, number of conducting channels at the different current levels. The expanded current traces show a single channel at higher time resolution. **B**, single-channel current traces were recorded in the absence and presence of 0.25 and 10  $\mu\text{mol/L}$  EC added to both sides of an AgA-doped bilayer. The expanded traces show a single channel at higher time resolution to show that EC does not induce flickering channel activity.

the first three compounds inhibit the *in vitro* growth of cancer cells (3, 39, 41). Previous studies indicate that auto-oxidation may play a role in some of the cell culture effects of EGCG (42). However, we found that when our assays were done in the presence of superoxide dismutase (SOD), the ability of EGCG to induce a loss of detergent-resistant lipids in HT29 cells was actually enhanced (data not shown), thus providing evidence that the change in membrane organization induced by EGCG is exerted by the parent compound itself. Because EGCG caused marked inhibition of cellular levels of p-EGFR even in the presence of 15 units/mL SOD (data not shown), it is unlikely that in our cell system, the inhibitory effect of EGCG on activation of the EGFR is due to auto-oxidation of EGCG.

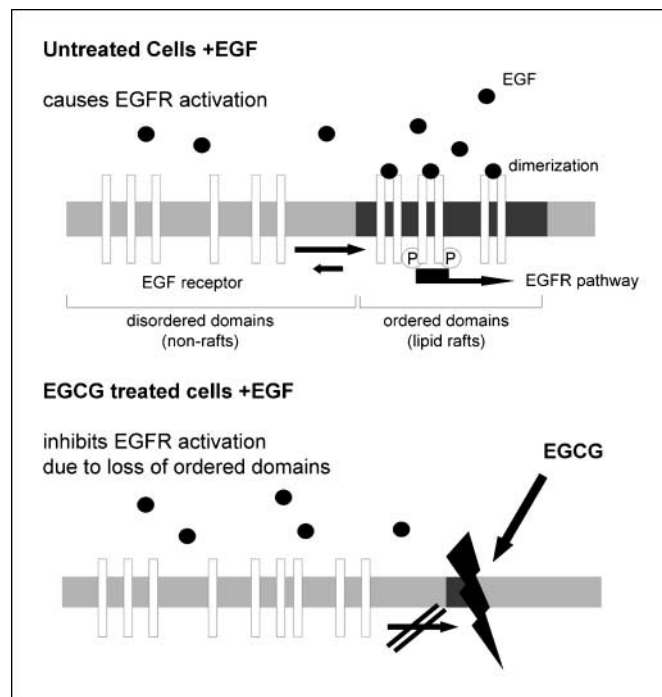
Detergent-resistant membranes are enriched in cholesterol and sphingomyelin (16, 17, 43), and previous studies indicated that EGCG can deplete cholesterol from synthetic lipid micelles (44). However, using filipin staining to quantify membrane cholesterol content, we did not observe an appreciable loss of cholesterol in EGCG-treated HT29 cells. In addition, cholesterol repletion studies indicated that although this restored detergent resistance to the lipids in cells previously treated with the cholesterol-depleting agent, M $\beta$ CD, cholesterol repletion did not restore detergent resistance in EGCG treated cells.<sup>5</sup> Furthermore, EGCG altered the function of gramicidin channels in a synthetic lipid bilayer system that lacks cholesterol (Fig. 4). Therefore, cholesterol does not seem to be the critical target for EGCG in the plasma membrane of cells. Further studies are required to determine at the molecular level how EGCG inserts into membrane structures and thereby disrupts lipid organization.

In previous studies, we and other investigators found that EGCG inhibits activation of the EGFR, and downstream signaling pathways, in several types of cancer cells (2, 3, 5, 26), but the precise mechanism has not been elucidated. Furthermore, there is evidence that the EGFR is associated with detergent-resistant domains in the plasma membrane of cells (10–13, 24, 25). Therefore, our finding that EGCG decreases the amount of the plasma membrane that is detergent-resistant domains may explain, at least in part, the inhibitory effects of EGCG on EGFR function. Indeed, we found that under the same conditions in which EGCG alters lipid organization in HT29 cells, it also inhibited binding of Alexa-labeled EGF to the surface of these cells and inhibited dimerization and autophosphorylation of the EGFR (Figs. 1B and C and 2). Furthermore, we found that when HT29 cells were treated with EGF to activate the EGFR and the cells were then extracted with cold Triton X-100, most of the total cellular EGFR protein was present in the cold Triton X-100-soluble fraction, but the phosphorylated and activated form of EGFR was present in the Triton X-100-insoluble fraction. In addition, pretreatment with EGCG or PolyE, but not EC, markedly decreased the level of phosphorylation of EGFR in the Triton X-100-insoluble fraction (Fig. 1B). Although the Triton X-100 fractionation procedure may not be precise, these results provide evidence that most of the total cellular EGFR resides mainly in disordered domains, whereas the activated form of EGFR resides mainly in ordered domains in the plasma membrane of these cells.

Figure 5 presents a hypothetical model that may explain these results. We postulate that in HT29 cells grown in the absence of EGF, most of the total cellular EGFR resides in the disordered domains (non-lipid raft fraction) of the plasma membrane and that during activation by EGF or TGF $\alpha$  EGFR is translocated to ordered domains or that ordered domains are formed in association with the process of EGFR activation. This would explain why we found most of the total cellular EGFR in the Triton X-100-soluble fraction and p-EGFR in the Triton X-100-insoluble fraction (Fig. 1B). Thus, the ability of EGCG to disrupt ordered domains in these cells (Fig. 3; Table 1) might explain the ability of EGCG to inhibit activation of the EGFR. Consistent with this model, EGCG, ECG, PolyE, and theaflavin-3 disrupt lipid ordered domains in HT29 cells and also inhibit activation of the EGFR, whereas EC and EGC exert neither of these functions (Fig. 1B and C; Table 1).<sup>5</sup> We should, however,

emphasize that our findings do not exclude the possibility that the inhibition of activation of the EGFR by EGCG occurs independent of the effects of EGCG on ordered lipid domains, and further studies are required to clarify this association. Nevertheless, the model presented in Fig. 5 is consistent with recent studies on the IgE receptor Fc $\epsilon$ RI in human basophilic cells (45). The latter study provided evidence that before activation uncross-linked Fc $\epsilon$ RI receptors reside largely outside lipid rafts and that the cross-linking of these receptors induced by specific antigen enhances their association with lipid rafts and the activation of these receptors by the Lyn kinase. Furthermore, other investigators found that in this cell system, EGCG is associated with lipid rafts and can suppress expression of Fc $\epsilon$ RI-activated receptors (46). Further studies are also required to determine how EGCG disrupts lipid organization in the plasma membrane and whether this also perturbs the subsequent internalization and recycling of the EGFR.

Our finding that relatively low concentrations of EGCG (0.25–3  $\mu$ mol/L), but not EC, alter gramicidin channel function in synthetic lipid bilayers (Fig. 4) provides evidence that EGCG can directly modify the structure of lipid bilayers. It is unlikely that this is due to direct interaction between EGCG and the gramicidin channel because EGCG had similar effects on channels formed by the enantiomeric pair of gramicidin analogues AgA and AgA<sup>-</sup> channels. At higher concentrations (>3  $\mu$ mol/L), EGCG also increased the average channel lifetime (data not shown), indicating that EGCG reduces the bilayer stiffness (the disjoining force the bilayer imposes on the bilayer-spanning gramicidin channels). Taken together with previous studies on the effects of genistein (47) and curcumin (48) on gramicidin channel function in synthetic lipid bilayers, these results suggest that the disruption of ordered



**Figure 5.** A hypothetical model of how EGCG inhibits activation of the EGFR in HT29 cells. According to this model, most of the total cellular EGFR resides mainly in disordered domains (non-lipid rafts) in the plasma membrane. Binding of the ligand, EGF, or TGF $\alpha$  to EGFRs and the subsequent activation (autophosphorylation) and dimerization of these molecules occurs mainly in lipid rafts. Therefore, the disruption of lipid rafts by EGCG inhibits ligand binding to the EGFR, autophosphorylation, and dimerization of EGFRs.

<sup>5</sup> Unpublished data.

lipid domains in the plasma membrane of cells by EGCG may not involve direct binding of EGCG to cholesterol, sphingomyelin, or specific membrane-associated proteins.

On the other hand, EGCG can bind with high affinity to the 67-kDa laminin receptor protein, which, curiously, is associated with lipid rafts, and there is evidence that this binding plays an important role in mediating the Fc $\epsilon$ RI-suppressive action of EGCG in basophilic KU812 cells (29, 49). In addition, there is evidence that EGCG can directly inhibit activation of the EGFR (28) and that it binds directly to vimentin (50). Thus, EGCG might exert biological effects in cancer cells by targeting multiple components in cell membranes, thereby disrupting several aspects of membrane organization and function.

In summary, the present study provides evidence that the ability of EGCG and related naturally occurring compounds to inhibit activation of the EGFR and related downstream signaling pathways in cancer cells is due, at least in part, to disruption of ordered domains in the plasma membrane of these cells. These effects on membrane domain organization may also explain the ability of EGCG to inhibit activation of other membrane-associated RTKs,

such as IGF-IR and ErbB2, because these receptors also seem to be associated with lipid rafts (14, 15). Our studies suggest a novel mechanism by which EGCG, and perhaps several other naturally occurring polyphenolic compounds, exert anticancer effects in various experimental systems. Further studies are required to determine whether these effects of EGCG are preferential for cancer cells, perhaps because of alterations in the structure of their plasma membrane, and whether they also occur *in vivo*.

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