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Effect of 3-*O*-Octanoyl-(+)-catechin on the Responses of $GABA_A$ Receptors and Na^+/Glucose Cotransporters Expressed in *Xenopus* Oocytes and on the Oocyte Membrane Potential

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Recently, 3-*O*-octanoyl-(+)-catechin (OC) was synthesized from (+)-catechin (C) by incorporation of an octanoyl chain into C in the light of (−)-epicatechin gallate (ECg) and (−)-epigallocatechin gallate (EGCg), which are the major polyphenols found in green tea and have strong physiological activities. OC was found to inhibit the response of ionotropic γ-aminobutyric acid (GABA) receptors ($GABA_A$ receptors) and Na^+/glucose cotransporters expressed in *Xenopus* oocytes in a noncompetitive manner more strongly than does C. OC also induced a nonspecific membrane current and decreased the membrane potential of the oocyte, and thus death of the oocyte occurred even at lower concentrations than that induced by C or EGCg. Although EGCg produced H_2O_2 in aqueous solution, OC did not. This newly synthesized catechin derivative OC possibly binds to the lipid membrane more strongly than does C, Ecg, or EGCg and as a result perturbs the membrane structure.

KEYWORDS: Catechin; cell death; $GABA_A$, receptor; Na^+/glucose cotransporter; Xenopus oocyte

INTRODUCTION

Catechin derivatives, which are polyphenol compounds found in abundance in green tea, possess various physiological effects including antioxidative, antitumor, and antibacterial activities (*1*, *2*). Green tea contains four main catechins, (−)-epicatechin (EC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCg) (*3*), which exhibit variations in their biological activities. For example, the strength of their bactericidal activity occurs in the order ECg > EGCg > EGC > EC (*4*). This suggests the importance of a galloyl residue in catechin derivatives for their activity.

Recently, 3-*O*-acyl-catechins were synthesized to examine their antitumor-promoting and antibacterial activities (*5*, *6*) since the incorporation of fatty acid hydrocarbon chains into compounds can increase their propensity to interact with lipid bilayers (*7*). 3-*O*-Acylation of catechin derivatives increased the inhibitory effect of the activation of Epstein−Barr virus early antigen as a function of the acyl carbon chain length (*5*) and also increased the anti-staphylococcal activity as a function of the acyl carbon chain length, thereby resulting in membrane damage (*6*).

Hydrogen peroxide (H_2O_2) is produced by polyphenols and polyphenol-rich beverages under quasi-physiological conditions (*8*). In addition, the bactericidal effect of EGCg is dependent on hydrogen peroxide levels produced by EGCg and is reduced by treatment with catalase (*9*), indicating the involvement of H_2O_2 in the bactericidal action of EGCg. Thus, it is important to determine whether the action of polyphenols in cells occurs through production of H_2O_2 or membrane damage.

In previous studies the inhibition of the electrical responses of $GABA_A$ receptors (*10*) and Na^+/glucose cotransporters (*11*) expressed in *Xenopus* oocytes was measured in the presence of catechin derivatives, and it was found that ECg and EGCg caused nonspecific membrane currents when their concentrations were high (*11*).

In this study we examined the effect of 3-*O*-octanoyl-(+)-catechin (OC) on the responses of $GABA_A$ receptors and Na^+/glucose cotransporters expressed in *Xenopus* oocytes in comparison with C. We also investigated the effects of C, OC, and EGCg on the membrane potential of the oocyte and measured the production of H_2O_2 by various polyphenols by ferrous ion oxidation-xylenol orange (FOX) assay (*12*).

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MATERIALS AND METHODS

Materials. 3-*O*-Octanoyl-(+)-catechin (OC) was synthesized as previously reported (*5*), and (+)-catechin (C) and tannic acid were purchased from Nacalai Tesque Co., Ltd., Kyoto, Japan. (−)-Epigallocatechin gallate (EGCg) was acquired from Sigma Chemical Co., St. Louis, MO, and saponin from tea seeds and catalase (11 500 units/mg; 1 unit of catalase decomposes 1 mmol/min H_2O_2 at 25 °C and pH 7.0 in the presence of 0.2% H_2O_2) from bovine liver were purchased from Wako Pure Chemical Industries, Osaka, Japan. All chemicals were of guaranteed reagent quality.

Preparation of cRNA and *Xenopus* Oocytes. The cRNAs of the α_1 and β_1 subunits of the bovine $GABA_A$ receptors were synthesized from cloned cDNAs of bovine brain receptors with RNA polymerase (Promega, Madison, WI) according to standard procedures. The cloned cDNAs were a gift from Prof. Eric A. Barnard of the MRC Center, London, U.K. The cRNA of Na^+/glucose cotransporters was prepared as previously described (*13*).

Adult female frogs (*Xenopus laevis*) were purchased from Hamamatsu Seibutsu Kyozai Co., Hamamatsu, Japan. The oocytes were dissected from adult frog ovaries that had been kept in ice flakes for 1 h. They were first manually detached from the inner ovarian epithelium and follicular envelope after incubation in collagenase (type I, 1 mg/mL; Sigma) solution for 1 h according to the procedure of Kusano et al. (*14*). Then the oocytes were microinjected with cRNAs in sterilized water and incubated in modified Barth solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$ and 0.41 mM $CaCl_2$ in 5 mM Tris at pH 7.6) containing 25 mg/L penicillin and 50 mg/L streptomycin at 17.5 °C for 2−7 days before electrophysiological measurements were made (*15*).

Measurement of Electrical Responses. The membrane current of the receptors evoked by GABA or glucose was measured by the voltage clamping method with a voltage clamp amplifier (CEZ-1100; Nihon Kohden Kogyo, Tokyo, Japan) according to a previously described procedure (*15*). To examine the effect of OC on the $GABA_A$ receptors (*10, 15*), a mixture of OC and GABA dissolved in frog normal Ringer solution (115 mM NaCl, 1 mM KCl and 1.8 mM $CaCl_2$ in 5 mM Tris at pH 7.2) was applied to oocytes that expressed the $GABA_A$ receptors, and the induced electrical response was compared with the response caused by 1 μM GABA solution without the compound. To examine the effect of OC on the Na^+/glucose cotransporter (*11, 13*), a mixture of OC and glucose dissolved in frog normal Ringer solution was applied to oocytes that expressed the Na^+/glucose cotransporters, and the induced electrical response was compared with the response caused by 0.5 mM glucose solution without the compound. One or the other solution was selected by switching a valve in the flow system (*16*). Measurements were repeated several times using the same oocyte, and control values were measured every two or three measurements. The data were usually expressed as the means of four experiments, and a Student's *t* test was used to evaluate the significance of differences between the mean values of the mixture and those of the control.

Measurement of the Membrane Potential. Oocytes were incubated in 20 mL of modified Barth solution with and without a catechin derivative and/or catalase (575 units/mL after dilution) and stored at 17.5 °C. The membrane potential was measured by inserting a microelectrode filled with 3 M KCl using a voltage clamp amplifier (TEV-200A, Dagan Co., Minneapolis, MN). During measurement the oocytes were perfused with frog normal Ringer solution and the value of the membrane potential was recorded after it stabilized, usually within a few minutes. The data were usually expressed as the means of five experiments.

Measurement of Hydrogen Peroxide by the FOX Assay. The concentration of H_2O_2 was measured as described by Akagawa et al. (*9*). Briefly, each polyphenol was incubated in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C for 24 h. FOX reagent was prepared by adding one volume of Reagent 1 to nine volumes of Reagent 2, where Reagent 1 was 4.4 mM 2,6-di-*tert*-butyl-4-methylphenol (BHT) in methanol and Reagent 2 was 1 mM xylenol orange plus 2.56 mM ammonium ferrous sulfate in 250 mM H_2SO_4. The polyphenol sample (100 μL) was added to the FOX reagent (3 mL) and vortexed for 5 s. After incubation for 30 min at room temperature, the absorbance at 560 nm was measured using a spectrometer (Hitachi U-2000A). The FOX assay was calibrated using a standard H_2O_2 solution, whose concentration was estimated by using a molar extinction coefficient of 43 M^{-1} cm^{-1} at the 240 nm absorbance wavelength of H_2O_2.

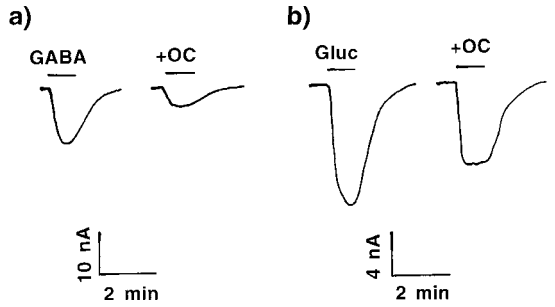

Figure 1. Effect of OC on the response of the $GABA_A$ receptors (a) and Na^+/glucose cotransporters (b). $GABA_A$ receptors and Na^+/glucose cotransporters were expressed in *Xenopus* oocytes by injecting their respective cRNA. All traces were obtained using a voltage clamp at −40 mV. An inward current is shown as a downward curve, and the upper bars show when the compound(s) was applied. The responses for a given panel were obtained from the same cRNA-injected oocyte, but the responses shown in a and b were obtained from different oocytes. The concentrations of OC, GABA, and D-glucose (Gluc) were 0.1 mM, 10 μM, and 0.5 mM, respectively.

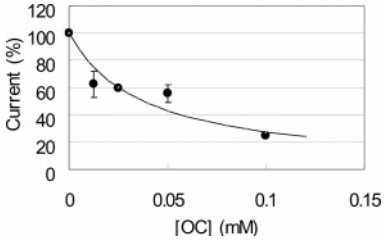

Figure 2. Dose–response relationship of the inhibition induced by OC on the response of the $GABA_A$ receptors expressed in *Xenopus* oocytes. The effect was measured in the presence of 1 μM GABA, and a theoretical curve was drawn using a noncompetitive inhibition constant of 0.038 mM. The data are shown as means ± SD (bars) for four experiments. $*p < 0.01$ as determined by Student's *t* test for the values between the control and OC.

RESULTS

$GABA_A$ receptors and Na^+/glucose cotransporters were expressed in *Xenopus* oocytes by injecting cRNAs synthesized from their respective cloned cDNAs. **Figure 1** shows typical currents of the receptors and cotransporters and their inhibition by 0.1 mM OC, and **Figures 2** and **3** show the dose-inhibition relationship of the responses of the $GABA_A$ receptors and Na^+/glucose cotransporters to OC. Since the inhibition by OC was almost independent of the GABA or glucose concentration (data not shown), OC inhibited the responses in a noncompetitive manner. Unfortunately, induction of a nonspecific current in the oocyte prevented measurement of the effect of OC at high concentrations (>0.1 mM) on both the receptors and the cotransporters. The inhibition constant of OC was estimated to be 0.038 mM for the $GABA_A$ receptor response and 0.28 mM for the Na^+/glucose cotransporter response.

Since OC induced a nonspecific current even at low concentrations, we examined the effect of OC on the membrane potential of the noninjected oocytes when they were incubated with various concentrations of OC overnight. **Figure 4** shows the dose dependence of OC, C, and EGCg on the membrane potential after overnight incubation in modified Barth solution.

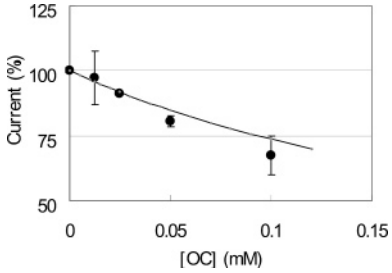

Figure 3. Dose–response relationship of the inhibition caused by OC on the response of the Na^+/glucose cotransporters expressed in *Xenopus* oocytes. The effect of OC was measured in the presence of 0.5 mM glucose, and a theoretical curve was drawn using a noncompetitive inhibition constant of 0.28 mM. The data are shown as means ± SD (bars) for four experiments. *$p < 0.01$ as determined by Student's *t* test for the values between the control and OC except the value at 0.0125 mM OC.

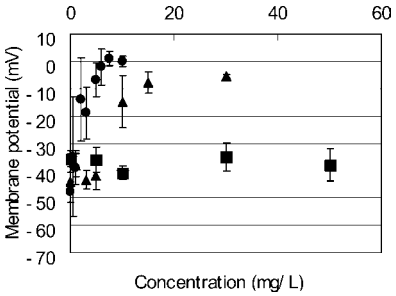

Figure 4. Dose-dependence relationship of C, OC, and EGCg on the membrane potential of oocytes after overnight incubation at 17.5 °C. The membrane potential of the oocytes incubated in various concentrations of C (■), OC (●), and EGCg (▲) were measured by inserting a microelectrode filled with 3 M KCl using a voltage clamp amplifier. The data are shown as means ± SD (bars) for five experiments.

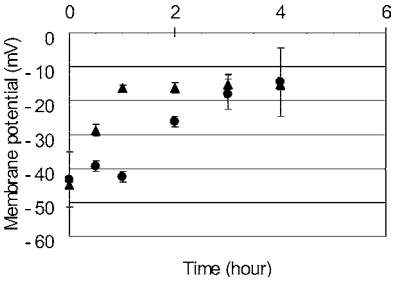

Figure 5. Time-dependence relationship of the membrane potential change in oocytes when OC and EGCg were applied. The oocytes were incubated in 10 OC (●) or 30 mg/L EGCg (▲) in frog Barth solution at 17.5 °C, and the membrane potential was measured at various times. The data are shown as means ± SD (bars) for two experiments.

OC and EGCg decreased the membrane potential of the oocytes with increasing concentration, while C caused no effect up to at least 50 mg/L. **Figure 5** shows the time dependence of the membrane potential of the oocytes when OC and EGCg were applied. The resulting decrease in the membrane potential was irreversible (**Table 1**), leading to death of the oocytes.

Since the production of H_2O_2 by polyphenols might promote death in bacteria (*9*), we measured the production of H_2O_2 by FOX assay. **Figure 6** shows that EGCg induced the production of H_2O_2 while OC and C had almost no effect. Next, we examined the effect of catalase on the decrease in the oocyte membrane potential induced by OC and EGCg (**Figure 7**). It protected, in part, the decrease in the membrane potential caused by EGCg but did not have any effect on that induced by OC.

Table 1. Irreversible Decrease in the Membrane Potential (mV) of Oocytes Induced by OC and EGCg[a]

original	incubation with OC for 30 min	washing in Barth solution for 1 h
–52.1	–19.7	–14.0
–38.4	–16.3	–9.6
–50.8	–13.1	–9.5
–54.4	–16.5	–7.2
–55.0	–18.4	–8.6

original	incubation with EGCg for 30 min	washing in Barth solution for 1 h
–44.6	–24.7	–20.6
–46.7	–23.2	–17.6
–39.2	–32.0	–26.0
–48.3	–13.0	–10.6
–46.6	–12.6	–8.3

[a] After the membrane potential of the oocyte was measured, it was incubated in 50 mg/L OC or 40 mg/L EGCg dissolved in modified Barth solution for 30 min and the membrane potential of the oocyte was measured. Then it was transferred and incubated in modified Barth solution without the compound for 1 h, and its membrane potential was again measured. Five different oocytes were used for the experiment, and they were incubated at 17.5 °C.

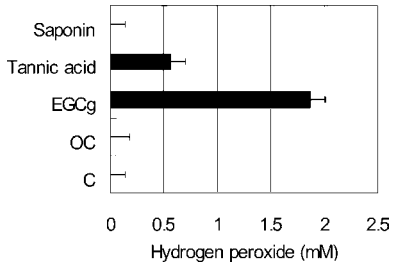

Figure 6. Production of H_2O_2 induced by various polyphenols. Each polyphenol (250 μM) was incubated in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C for 24 h, and then the formation of H_2O_2 was measured by FOX assay as described in the Materials and Methods section. However, the concentration of saponin used was 0.1 g/L, because its molecular weight was unknown. The data are shown as means ± SD (bars) for three experiments. *$p < 0.05$ as determined by Student's *t* test for tannic acid and OC.

DISCUSSION

Catechin derivatives in tea possess several useful physiological properties such as antioxidative-, antibacterial-, and antitumor-promoting activities (*2*). It would be worthwhile to develop new catechin derivatives and characterize them in the search for useful compounds. From this stand point, 3-*O*-acyl-(+)-catechins and 3-*O*-acyl-(−)-epigallocatechins were synthesized from C (*5*, *6*) and EG (*17*, *18*) by incorporation of fatty acid hydrocarbon chains and were found to have stronger antibacterial- and antitumor-promoting activities than those of C and EG. This increase in activity may be due to the increase in their interaction with lipid bilayer and the resultant membrane damage (*6*).

Since we observed the inhibition of $GABA_A$ receptors (*10*) and Na^+/glucose cotransporters (*11*) expressed in *Xenopus* oocytes by catechin derivatives, we measured the effect of OC on their electrical responses. Since C is cheaper than EG and catechin derivatives with a C-3 acyl chain of C_8−C_{11} carbon atoms show promise in their inhibitory effects on Epstein−Barr virus activation and anti-*Staphylococcus aureus* activity, we used OC for the comparison with C. OC noncompetitively inhibited the response of the $GABA_A$ receptors and Na^+/glucose cotransporters with an inhibition constant of 0.038 and 0.28 mM, respectively (*10*, *11*). These constants were less than those of

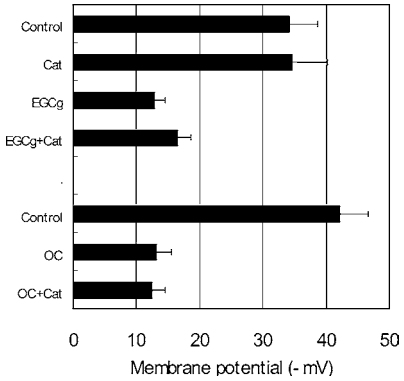

Figure 7. Effect of catalase (Cat) on the decrease in the membrane potential of the oocyte caused by EGCg and OC. Oocytes were incubated in Barth solution containing EGCg (50 mg/L) or OC (20 mg/L) with and without catalase (0.05 mg/L, 575 units/mL) at 17.5 °C. The membrane potentials of seven oocytes were then measured after incubation for 4 (EGCg) or 3 h (OC). The oocytes from different frogs were used for the EGCg and OC experiments, but the oocytes from the same frog were used for the comparison. $*p < 0.005$ as determined by Student's *t* test between the values of EGCg and those of EGCg+Cat.

C (1.7 and 2.3 mM), indicating an increase in its binding affinity to the receptors and cotransporters.

Although the incorporation of a C-3 acyl chain to C increased its inhibition of the receptors and cotransporters, it induced a nonspecific membrane current in the oocytes (*11*). Overnight application of OC to the oocytes induced a decrease in the membrane potential in a dose-dependent manner ($EC_{50} = 2$ mg/L), while the application of C did not up to 50 mg/L. The catechin derivative EGCg also decreased the membrane potential at concentrations between 5 and 10 mg/L. As previously reported (*8*, *9*), EGCg produced H_2O_2 during incubation in aqueous solution (*8*) while OC and C did not (**Figure 6**). Since the addition of catalase to EGCg solution protected, in part, a decrease in the membrane potential of the oocytes induced by EGCg (**Figure 7**) and H_2O_2 also induced a decrease in the membrane potential of the oocytes (data not shown), EGCg possibly caused a decrease in the membrane potential of the oocytes through not only membrane damage but also production of H_2O_2. On the other hand, since OC did not produce detectable amounts of H_2O_2 and the addition of catalase to OC solution did not protect against the decrease in the membrane potential caused by OC, OC must directly bind to the membrane of the oocyte, thereby perturbing the membrane and causing membrane structure damage (*6*, *19*). As suggested by Akagawa et al. (*8*), EGCg and tannic acid, which are phenolic compounds OH substituted at the 2 and 4 positions of phenol, produced H_2O_2 while C and OC and saponin did not (**Figure 6**). Although most these compounds have some cell toxicity, the mechanism of action of these compounds may vary.

The *Xenopus* oocyte expression system combined with electrophysiological measurements are useful for examining the effect of various compounds on receptors or transporters because *Xenopus* oocytes, which are globular with a diameter >1 mm, are more stable, larger, and simpler than neuronal or epitherial cells. Therefore, electrophysiological measurement of their responses can be made easily and repetitively performed over a long period (*20*). *Xenopus* oocytes are also useful for examining membrane damage induced by compounds which cause death of the oocyte.

In conclusion, incorporation of C-3 octanoyl chain into C increases the inhibition of $GABA_A$ receptors and Na^+/glucose cotransporters. The inhibition of Na^+/glucose cotransporters by OC may be helpful for reducing the blood glucose level in diabetic patients. However, care must be exercised if it is given as a dietary supplement due to the cell toxicity induced by OC through membrane damage (*21*). OC may also be useful as an antitumor or antibacterial compound if it is determined to have specificity toward cancer cells (*22*) or bacteria, since it is very toxic toward the oocyte cells.

ABBREVIATIONS USED

C, (+)-catechin; EC, (−)-epicatechin; ECg, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCg, (−)-epigallocatechin gallate; FOX, ferrous ion oxidation-xylenol orange; GABA, γ-aminobutyric acid.

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