

## Differential Regulations of Quercetin and Its Glycosides on Ligand-Gated Ion Channels

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**Quercetin, one of the flavonoids, is a compound of low molecular weight found in various plants and shows a wide range of diverse neuropharmacological actions. In fruits and vegetables, quercetin exists as monomer-(quercetin-3-*O*-rhamnoside) (Rham1), dimer- (Rutin), or trimer-glycosides [quercetin-3-(2<sup>G</sup>-rhamnosylrutinoside)] (Rham2) at carbon-3. In the previous studies, we demonstrated that quercetin inhibits both glycine and 5-hydroxytryptamine type 3, (5-HT<sub>3A</sub>) receptor channel activities expressed in *Xenopus* oocytes. However, the effects of quercetin glycosides on glycine and 5-HT<sub>3A</sub> receptor channel activities are not well known. In the present study, we investigated the effects of quercetin glycosides on the human glycine  $\alpha$ 1 receptor and mouse 5-HT<sub>3A</sub> receptor channel activities expressed in *Xenopus* oocytes using a two-electrode voltage clamp technique. In oocytes expressing glycine or 5-HT<sub>3A</sub> receptors, quercetin- or its glycosides-induced inhibitions on glycine- ( $I_{Gly}$ ) and 5-HT-induced current ( $I_{5-HT}$ ) were dose-dependent and reversible. Applications of quercetin and its glycosides inhibited  $I_{Gly}$  in order of quercetin > Rutin  $\geq$  Rham1 > Rham2. Applications of quercetin and its glycosides inhibited  $I_{5-HT}$  in order of Rham2  $\geq$  quercetin > Rutin = Rham1. The inhibitions of  $I_{Gly}$  by quercetin glycosides were non-competitive and voltage-sensitive, whereas the inhibitions of  $I_{5-HT}$  by quercetin glycosides were competitive and voltage-insensitive manners. These results also indicate that quercetin glycosides might regulate the human glycine  $\alpha$ 1 and mouse 5-HT<sub>3A</sub> receptors with differential manners.**

**Key words** flavonoid; quercetin glycoside; ligand-gated ion channel; *Xenopus* oocyte

The glycine and 5-hydroxytryptamine type 3 (5-HT<sub>3</sub>) receptors are the superfamily of ligand-gated ion channel receptors with cysteine loop and share a structural similarity with nicotinic acetylcholine and  $\gamma$ -aminobutyric acid A (GABA<sub>A</sub>) receptors.<sup>1</sup> The glycine receptor is predominantly expressed in spinal cord, brain stem and other regions of the central nervous system.<sup>2</sup> The activations of glycine receptor in these regions mediate post-synaptic inhibitions for reflex responses, voluntary motor control, and the processing of sensory signals,<sup>2</sup> since the glycine receptor forms a chloride-permeable and -selective transmembrane channel. Thus, the glycine receptor is responsible for fast inhibitory synaptic transmissions.<sup>3</sup>

On the other hand, 5-HT<sub>3</sub> receptor mediates rapid and transient membrane depolarizing effects of 5-HT in the central and peripheral nervous system like nicotinic acetylcholine receptors.<sup>1</sup> 5-HT<sub>3A</sub> receptor composes of a N-terminal domain, four transmembrane domains (TM1 to TM4), and an intracellular domain and finally homomeric pentamers of each subunit consist of 5-HT<sub>3</sub> receptor as glycine receptor does.<sup>4</sup> The physiological and pathological roles of 5-HT<sub>3</sub> receptor involve pain transmission, analgesia, vomiting, and mood disorders and drug abuse.<sup>5</sup>

Recently, we have reported that quercetin regulates glycine and 5-HT<sub>3A</sub> receptor channel activities through interaction with amino acid residues existing in pore region of transmembrane domain 2 and N-terminal of pre-transmembrane domain 1, respectively.<sup>6,7</sup> Interestingly, in fruits and vegetables quercetin naturally exists as monomer- (quercetin-3-*O*-rhamnoside), (Rham1) dimer- (Rutin), or trimer-glycosides

[quercetin-3-(2<sup>G</sup>-rhamnosylrutinoside)] (Rham2) at carbon-3 and other glycosidic forms.<sup>8,9</sup> When animal or human intakes fruits or vegetables, the dietary quercetin glycosides are metabolized to quercetin or conjugated with glucose or sulfate. A line of evidence showed that quercetin glycosides in addition to quercetin also exert their physiological or pharmacological effects.<sup>8,9</sup> However, until now little is known on how quercetin glycosides affect on the regulations of glycine and 5-HT<sub>3A</sub> receptor channel activities.

In the present study, we examined the effects of quercetin glycosides on the human glycine  $\alpha$ 1 receptor and mouse 5-HT<sub>3A</sub> receptor channel activities expressed in *Xenopus* oocytes using a two-electrode voltage clamp technique. We found that applications of quercetin and its glycosides reversibly inhibited glycine-induced current ( $I_{Gly}$ ) in order of quercetin > Rutin  $\geq$  Rham1 > Rham2. In oocytes expressing 5-HT<sub>3A</sub> receptor, applications of quercetin and its glycosides also reversibly inhibited 5-HT-induced current ( $I_{5-HT}$ ) in order of Rham2  $\geq$  quercetin > Rutin = Rham1. The inhibitions of  $I_{Gly}$  by quercetin glycosides were not competitive and voltage-sensitive, whereas the inhibitions of  $I_{5-HT}$  by quercetin glycosides were competitive and voltage-insensitive manners. These results indicate that quercetin glycosides might regulate the human glycine  $\alpha$ 1 and mouse 5-HT<sub>3A</sub> receptors with differential manners.

### MATERIALS AND METHODS

**Materials** Figure 1 shows the structure of quercetin and its glycosides used in this study were dissolved in dimethyl

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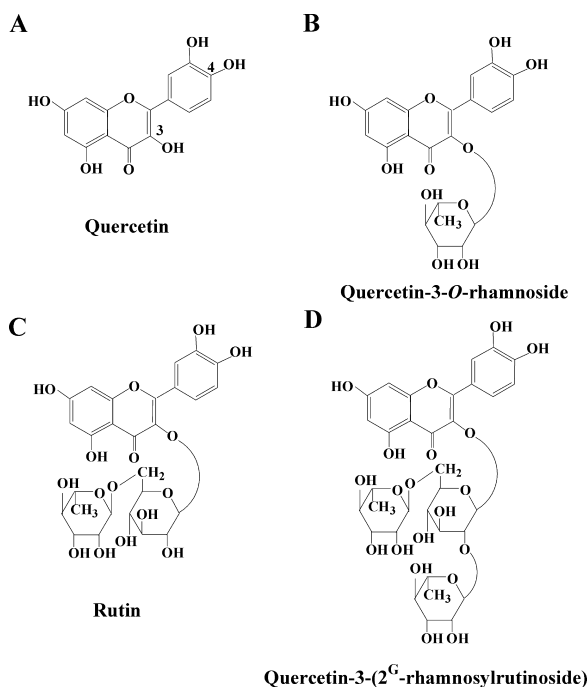


Fig. 1. Chemical Structures of Quercetin and Its Glycosides

(A) Quercetin, (B) quercetin-3-O-rhamnoside (Rham1), (C) rutin and (D) quercetin-3-(2'-O-rhamnosylrutinoside) (Rham2).

sulfoxide (DMSO) and were diluted with bath medium before use. cDNAs containing the human glycine  $\alpha 1$  and mouse 5-HT<sub>3A</sub> receptors were kindly provided by Dr. H. Betz (Max-Planck-Institute, Germany) and Dr. D. Julius (UCSF, U.S.A.), respectively. Final DMSO concentration was less than 0.01%. Quercetin and other chemical agents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Quercetin glycosides were purified from *Magnolia obovata*.<sup>10)</sup>

**Oocyte Preparation** *Xenopus laevis* frogs were purchased from *Xenopus* I (Ann Arbor, MI, U.S.A.). *Xenopus laevis* care and handling were in accordance with the guide for the *Care and Use of Laboratory Animals* published by NIH, U.S.A. Frogs underwent surgery only twice, separated by at least 3 weeks. To isolate oocytes, frogs were anesthetized with an aerated solution of 3-amino benzoic acid ethyl ester. Oocytes were separated by treatment with collagenase, by gentle shaking for 2 h in CaCl<sub>2</sub>-free medium containing 82.5 mM, NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 units penicillin/ml, and 100  $\mu$ g streptomycin/ml. Only stage 5 or 6 oocytes were collected and maintained at 18 °C with continuous gentle shaking in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, and 5 mM HEPES, pH 7.5) supplemented with 0.5 mM theophylline and 50  $\mu$ g gentamycin/ml. All solutions were changed every day. All experiments were performed within 2–4 d following isolation of the oocytes.

**Oocyte Recording** A single oocyte was placed in a small Plexiglas net chamber (0.5 ml) and was constantly superfused with ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, and 5 mM HEPES, pH 7.5). The microelectrodes were filled with 3 M KCl and had a resistance of 0.2–0.7 M $\Omega$ . Two-electrode voltage-clamp recordings were performed at room temperature with Oocyte Clamp (OC-725C, Warner Instrument, Hamden, CT, U.S.A.) with

Digidata 1200A (Axon Instruments, Union City, CA, U.S.A.). For most of the electrophysiological experiments, the oocytes were clamped at a holding potential of  $-80$  mV and 300-ms voltage steps for current–voltage relationship were applied from  $-100$  to  $+40$  mV and  $+60$  mV for glycine  $\alpha 1$  receptor and 5-HT<sub>3A</sub> receptor, respectively.<sup>6,7)</sup>

**cRNA Preparation of the Glycine  $\alpha 1$  or 5-HT<sub>3A</sub> Receptor and Microinjection** The cDNAs encoding the human glycine  $\alpha 1$  receptor and mouse 5-HT<sub>3A</sub> receptor was linearized by digestion with appropriate restriction enzymes. The cRNAs were transcribed from linearized templates with *in vitro* transcription kit (mMessage mMachine; Ambion, Austin, TX, U.S.A.) using a T3 polymerase. The cRNA was dissolved in RNase-free water at a final concentration of approximately 1  $\mu$ g/ $\mu$ l and stored at  $-70$  °C until used. Oocytes were injected with H<sub>2</sub>O, human glycine receptor, or mouse 5-HT<sub>3A</sub> receptor cRNAs (5–10 ng) by using a Nanoject Automatic Oocyte Injector (Drummond Scientific, Broomall, PA, U.S.A.). The injection pipette was pulled from glass capillary tubing used for recording electrodes and the tip was broken to *ca.* 20- $\mu$ m-OD.

**Data Analysis** To obtain the concentration–response curve for glycine or 5-HT-induced current in the presence of quercetin or its glycosides, the observed peak amplitudes were normalized and plotted and then fitted to the Hill equation below using Origin software (Northampton, MA, U.S.A.).  $y/y_{\max} = [A]^n / ([A]^n + [IC_{50}]^n)$ , where  $y$ , % inhibition at given concentration of quercetin or its glycosides,  $y_{\max}$ , % of maximal inhibition,  $IC_{50}$  is the concentration of quercetin producing half-maximum inhibition of the control response to glycine or 5-HT, and  $[A]$  is the concentration of quercetin or its glycosides.  $n$  is the interaction coefficient. All values are presented as means  $\pm$  S.E.M. The differences between means of control and quercetin or its glycosides treatment data were analyzed using unpaired Student's  $t$  test and one-way ANOVA with *post-hoc* Tukey test. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

### Effects of Quercetin and Its Glycosides on Human Glycine $\alpha 1$ and Mouse 5-HT<sub>3A</sub> Receptor-Mediated Currents

As previous reports, we could observe that application of glycine (100  $\mu$ M) or 5-HT (1  $\mu$ M) to bathing medium induced a large inward current ( $I_{\text{Gly}}$  or  $I_{5\text{-HT}}$ ) in oocytes injected with cRNAs coding glycine or 5-HT<sub>3A</sub> receptor (Fig. 2). Strychnine (0.5  $\mu$ M) and MDL72222 (0.5  $\mu$ M), of which the respective glycine receptor and 5-HT<sub>3A</sub> receptor antagonist blocked  $I_{\text{Gly}}$  and  $I_{5\text{-HT}}$  (data not shown), indicating that the glycine and 5-HT<sub>3A</sub> receptor were functionally expressed in this system as previous reports.<sup>6,7)</sup> In the present study, we examined the effects of quercetin glycosides on  $I_{\text{Gly}}$  or  $I_{5\text{-HT}}$ . Quercetin and its glycosides (each 100  $\mu$ M) themselves had no effect in oocytes expressing the glycine or 5-HT<sub>3A</sub> receptor at a holding potential of  $-80$  mV. However, co-application of quercetin (Que) or its glycosides with glycine or 5-HT inhibited  $I_{\text{Gly}}$  or  $I_{5\text{-HT}}$  in a reversible manner (Fig. 2A,  $n = 15$  from three different frogs) (Figs. 2A, B). The percent inhibitions were  $88.7 \pm 2.0$ ,  $53.3 \pm 5.7$ ,  $58.3 \pm 5.1$ ,  $35.6 \pm 8.8\%$  for  $I_{\text{Gly}}$  and  $74.2 \pm 3.0$ ,  $52.4 \pm 4.9$ ,  $58.6 \pm 2.1$ ,  $83.6 \pm 1.6\%$  for  $I_{5\text{-HT}}$  by quercetin, Rham1, Rutin, and Rham2, respectively

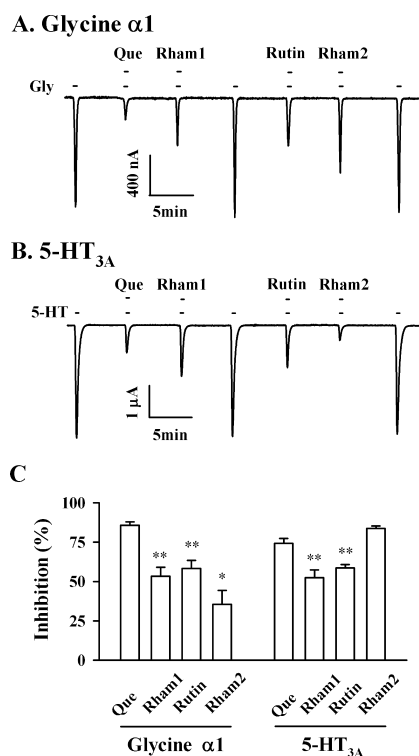


Fig. 2. Effects of Quercetin and Its Glycosides on the Human Glycine  $\alpha 1$  and 5-HT<sub>3A</sub> Receptor Channel in *Xenopus* Oocytes after Glycine  $\alpha 1$  and 5-HT<sub>3A</sub> Receptor cRNA Injection

The inward currents were recorded at a holding potential of  $-80$  mV. (A) Glycine (Gly;  $100 \mu\text{M}$ ) induced a large inward current in oocytes expressing the glycine  $\alpha 1$  receptor. Co-treatment of  $100 \mu\text{M}$  of quercetin, quercetin-3-O-rhamnoside (Rham1), rutin (Rutin) and quercetin-3-(2'-rhamnosylrutinoside) (Rham2) with glycine inhibited  $I_{\text{Gly}}$  in the representative trace. Oocytes were exposed to glycine alone, glycine+quercetin, or quercetin+its glycosides treatment for 30 s. (B) 5-HT ( $1 \mu\text{M}$ ) induced a large inward current in oocytes expressing the 5-HT<sub>3A</sub> receptor. Co-treatment of  $100 \mu\text{M}$  of quercetin, Rham1, Rutin and Rham2 with 5-HT inhibited  $I_{5\text{-HT}}$  in the representative trace. The representative trace shows both quercetin and its glycosides inhibit  $I_{\text{Gly}}$  and  $I_{5\text{-HT}}$ . Oocytes were exposed to 5-HT alone or 5-HT+quercetin and+its glycosides treatment for 30 s. (C) Co-treatment of quercetin and its glycosides with glycine or 5-HT exhibits differential inhibitory effects in summary histograms. \* $p < 0.05$ , \*\* $p < 0.005$  compared quercetin with its glycosides treatment by one-way ANOVA with *post-hoc* Tukey test. Each point represents the mean  $\pm$  S.E.M. ( $n = 9-12$  oocytes/group).

(Fig. 2C). Thus, the inhibitory potency for  $I_{\text{Gly}}$  was in order of quercetin > Rutin  $\approx$  Rham1 > Rham2 and the inhibitory potency for  $I_{5\text{-HT}}$  was in order of Rham2  $\approx$  quercetin > Rutin = Rham1. Glycosylations of quercetin affect on glycine but not 5-HT<sub>3A</sub> receptor channel current inhibitions. In addition, since quercetin more strongly inhibited  $I_{5\text{-HT}}$  or  $I_{\text{Gly}}$  than other quercetin glycosides tested, we tested using quercetin whether pre-application of quercetin caused further inhibition on  $I_{5\text{-HT}}$  or  $I_{\text{Gly}}$  compared to its co-application with glycine or 5-HT. As shown in Figs. 3A and B, pre-application of quercetin did not induce further inhibition of  $I_{5\text{-HT}}$  or  $I_{\text{Gly}}$  compared to co-application of quercetin with glycine or 5-HT. Thus, the percent inhibitions in co-application of quercetin were  $52.3 \pm 6.4\%$  for  $I_{\text{Gly}}$  and  $49.5 \pm 5.0\%$  for  $I_{5\text{-HT}}$ . The percent inhibitions in pre-application of quercetin were  $54.3 \pm 3.4\%$  for  $I_{\text{Gly}}$  and  $52.1 \pm 3.4\%$  for  $I_{5\text{-HT}}$  (Fig. 3C), indicating that either co- or pre-application causes the same extent of inhibitions on  $I_{5\text{-HT}}$  and  $I_{\text{Gly}}$ .

**Concentration-Dependent Effect of Quercetin and Its Glycosides on  $I_{\text{Gly}}$  and  $I_{5\text{-HT}}$**  Quercetin and its glycosides inhibited both  $I_{\text{Gly}}$  and  $I_{5\text{-HT}}$  with concentration-dependent

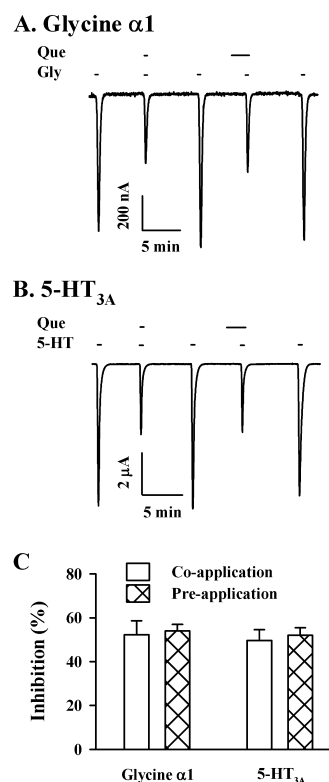


Fig. 3. Pre-application of Quercetin Does Not Induce Further Inhibition of  $I_{5\text{-HT}}$  or  $I_{\text{Gly}}$  Compared to Co-application of Quercetin with Glycine or 5-HT

(A) Glycine (Gly;  $100 \mu\text{M}$ ) induced a large inward current in oocytes expressing the glycine  $\alpha 1$  receptor. The representative trace shows that co-treatment or pre-treatment of quercetin ( $30 \mu\text{M}$ ) with glycine exhibited the same extent inhibition of  $I_{\text{Gly}}$ . Oocytes were exposed to glycine alone or glycine+quercetin for 30 s, or pretreatment of quercetin for 2 min and co-treatment of quercetin with glycine for 30 s. (B) 5-HT ( $5 \mu\text{M}$ ;  $1 \mu\text{M}$ ) induced a large inward current in oocytes expressing the 5-HT<sub>3A</sub> receptor. The representative trace shows that co-treatment or pre-treatment of quercetin ( $30 \mu\text{M}$ ) with 5-HT exhibited the same extent of inhibition of  $I_{5\text{-HT}}$ . Oocytes were exposed to 5-HT alone or 5-HT+quercetin for 30 s, or pretreatment of quercetin for 2 min and co-treatment of quercetin with 5-HT for 30 s. (C) The summary histograms show the same extent inhibitions of  $I_{\text{Gly}}$  and  $I_{5\text{-HT}}$  either co- or pre-treatment of quercetin ( $n = 10-12$  oocytes/group).

manners (Figs. 4A, B). The  $\text{IC}_{50}$ s for  $I_{\text{Gly}}$  were  $23.8 \pm 0.5$ ,  $62.5 \pm 7.4$ ,  $66.4 \pm 2.8$ ,  $97.4 \pm 0.9 \mu\text{M}$  by quercetin, Rham1, Rutin, and Rham2, respectively. The  $\text{IC}_{50}$ s for  $I_{5\text{-HT}}$  were  $18.8 \pm 2.4$ ,  $61.4 \pm 3.9$ ,  $25.5 \pm 3.3$ ,  $23.6 \pm 0.1 \mu\text{M}$  by quercetin, Rham1, Rutin, and Rham2, respectively ( $n = 8-12$  from three different frogs for each point) (Figs. 4A, B, Table 1). The  $\text{IC}_{50}$  values of quercetin glycosides on glycine receptor were significantly higher than that of quercetin. Especially, it appears that the increase in  $\text{IC}_{50}$  value was related with number of quercetin glycosidation. Finally, quercetin's glycosylation caused dose-response curves to rightward shift. These results show a possibility that quercetin rather than quercetin glycosides is the main regulator of glycine receptor channel activity. In 5-HT<sub>3A</sub> receptors, the  $\text{IC}_{50}$  values were not significantly changed between quercetin and its glycosides, except for Rham1 (Fig. 4, Table 1), indicating that glycosylations of quercetin did not much affect on 5-HT<sub>3A</sub> receptor channel regulations.

**Quercetin Glycosides Inhibit  $I_{\text{Gly}}$  with Non-competitive, Whereas They Inhibit  $I_{5\text{-HT}}$  with Competitive Manner** To study further the mechanism by which quercetin and its glycosides inhibit  $I_{\text{Gly}}$  and  $I_{5\text{-HT}}$ , we first analyzed the effects of quercetin and its glycosides on  $I_{\text{Gly}}$  evoked by different

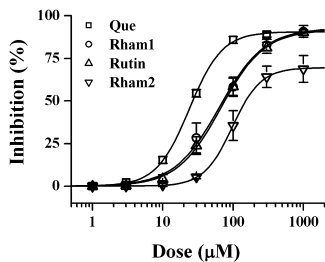
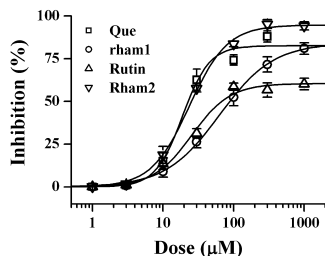
A. Glycine  $\alpha 1$ B. 5-HT<sub>3A</sub>

Fig. 4. Concentration-Dependent Effects of Quercetin and Its Glycosides on the Human Glycine  $\alpha 1$  and 5-HT<sub>3A</sub> Receptor Channel

(A)  $I_{Gly}$  in oocytes expressing glycine receptor was elicited at a holding potential of  $-80$  mV for 30 s in the presence of  $100 \mu\text{M}$  glycine, and then the indicated concentrations of quercetin and its glycosides were co-applied with glycine. (B)  $I_{5-HT}$  in oocytes expressing 5-HT<sub>3A</sub> receptor was elicited at a holding potential of  $-80$  mV for 30 s in the presence of  $1 \mu\text{M}$  5-HT, and then the indicated concentrations of quercetin and its glycosides were co-applied with 5-HT. The solid lines were fit by the Hill equation. Additional  $IC_{50}$ , Hill coefficient, and  $V_{max}$  values for the various mutants are presented in Table 1 (mean  $\pm$  S.E.M.;  $n=9-12$  oocytes for each point).

Table 1. Inhibitory Effects of Quercetin and Its Glycosides on Glycine  $\alpha 1$  and 5-HT<sub>3A</sub> Receptors-Mediated Currents Expressed in *Xenopus* Oocytes

Receptors	Drug	$V_{max}$	$IC_{50}$	$nH$
Glycine $\alpha 1$	Quercetin	$90.6 \pm 1.4$	$23.8 \pm 0.5$	$1.9 \pm 0.1$
	Rham1	$92.6 \pm 3.6$	$62.5 \pm 7.4^*$	$1.4 \pm 0.2$
	Rutin	$91.6 \pm 1.4$	$66.4 \pm 2.8^*$	$1.4 \pm 0.1$
	Rham2	$70.2 \pm 0.3^*$	$97.4 \pm 0.9^{**}$	$2.1 \pm 0.1$
5-HT <sub>3A</sub>	Quercetin	$82.6 \pm 4.0$	$18.8 \pm 2.4$	$2.3 \pm 0.5$
	Rham1	$84.1 \pm 1.6$	$61.4 \pm 3.9^{**}$	$1.2 \pm 0.1^*$
	Rutin	$60.5 \pm 2.4^*$	$25.5 \pm 3.3$	$1.6 \pm 0.3$
	Rham2	$94.7 \pm 1.5^*$	$23.6 \pm 1.2$	$1.6 \pm 0.1$

Values represent the mean  $\pm$  S.E.M. ( $n=10-12$ /group). Currents were elicited at a holding potential of  $-80$  mV.  $IC_{50}$  ( $\mu\text{M}$ ),  $V_{max}$ , and Hill coefficient values were determined as described in Materials and Methods.  $*p < 0.05$ ,  $**p < 0.005$  compared with quercetin by one-way ANOVA.

glycine concentrations in oocytes expressing the glycine receptors (Fig. 5A). Co-application of quercetin or its glycosides for 30 s with different concentrations of glycine did not significantly shift the dose-response curve of glycine. The  $EC_{50}$  values were  $168.9 \pm 0.9$ ,  $167.4 \pm 2.0$ ,  $173.9 \pm 0.3$ ,  $193.5 \pm 0.1$  and  $156.5 \pm 0.4 \mu\text{M}$  for glycine alone, glycine+quercetin, glycine+Rham1, glycine+Rutin, and glycine+Rham2, respectively and the Hill coefficients were 2.6, 3.4, 2.9, 2.5 and 2.8, respectively. Thus, quercetin, Rham1, Rutin, and Rham2 significantly inhibited the  $I_{Gly}$  elicited by 100, 300 and 1000  $\mu\text{M}$  of glycine, which are independent on the glycine concentrations ( $n=9-12$  from three different frogs) (Fig. 5A, Table 2). These results indicate that quercetin and its glycosides inhibit the  $I_{Gly}$  in a non-competitive manner.

Next, we also analyzed the effects of quercetin and its gly-

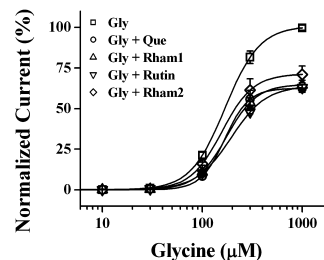
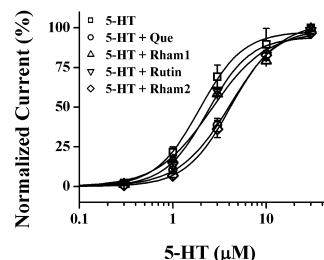
A. Glycine  $\alpha 1$ B. 5-HT<sub>3A</sub>

Fig. 5. (A) Dose-Response Relationship for Glycine and Glycine +Quercetin and Its Glycosides in Oocytes Expressing the Human Glycine  $\alpha 1$  Receptor

$I_{Gly}$  in oocytes expressing the glycine receptor was measured with the indicated concentration of glycine alone ( $\square$ ) and glycine +  $25 \mu\text{M}$  quercetin ( $\circ$ ), +  $60 \mu\text{M}$  Rham1 ( $\Delta$ ), +  $65 \mu\text{M}$  Rutin ( $\nabla$ ) and +  $100 \mu\text{M}$  Rham2 ( $\diamond$ ). Oocytes were voltage-clamped at a holding potential of  $-80$  mV. Oocytes were exposed to glycine alone or glycine+quercetin, +Rham1, +Rutin and +Rham2 treatment for 30 s.

(B) Dose-Response Relationship for 5-HT and 5-HT+Quercetin and Its Glycosides in Oocytes Expressing the Mouse 5-HT Receptor

$I_{5-HT}$  in oocytes expressing the 5-HT<sub>3A</sub> receptor was measured with the indicated concentration of 5-HT alone ( $\square$ ) and 5-HT+  $20 \mu\text{M}$  quercetin ( $\circ$ ), +  $60 \mu\text{M}$  Rham1 ( $\Delta$ ), +  $25 \mu\text{M}$  Rutin ( $\nabla$ ) and +  $25 \mu\text{M}$  Rham2 ( $\diamond$ ). Oocytes were voltage-clamped at a holding potential of  $-80$  mV. Oocytes were exposed to 5-HT alone or 5-HT+quercetin, +Rham1, +Rutin and +Rham2 treatment for 30 s. Each point represents the mean  $\pm$  S.E.M. ( $n=9-12$  oocytes/group).

Table 2. Competitive and Non-competitive Effects of Quercetin and Its Glycosides on Glycine  $\alpha 1$  and 5-HT<sub>3A</sub> Receptors-Mediated Currents Expressed in *Xenopus* Oocytes

Receptors	Drug	$EC_{50}$	$nH$
Glycine $\alpha 1$	Glycine	$168.9 \pm 0.9$	$2.6 \pm 0.1$
	Glycine+Quercetin	$167.4 \pm 2.0$	$3.4 \pm 0.1$
	Glycine+Rham1	$173.9 \pm 0.3$	$2.9 \pm 0.1$
	Glycine+Rutin	$193.5 \pm 0.1$	$2.5 \pm 0.0$
	Glycine+Rham2	$156.5 \pm 0.4$	$2.8 \pm 0.1$
5-HT <sub>3A</sub>	5-HT	$1.9 \pm 0.2$	$1.9 \pm 0.3$
	5-HT+Quercetin	$4.1 \pm 0.1^{**}$	$1.6 \pm 0.1$
	5-HT+Rham1	$2.6 \pm 0.6^*$	$1.5 \pm 0.4$
	5-HT+Rutin	$2.3 \pm 0.4$	$1.9 \pm 0.5$
	5-HT+Rham2	$4.1 \pm 0.1^{**}$	$1.8 \pm 0.1$

Values represent the mean  $\pm$  S.E.M. ( $n=10-12$ /group). Currents were elicited at a holding potential of  $-80$  mV.  $EC_{50}$  ( $\mu\text{M}$ ), and Hill coefficient values were determined as described in Materials and Methods.  $*p < 0.05$ ,  $**p < 0.005$  compared with 5-HT or glycine alone by one-way ANOVA.

cosides on  $I_{5-HT}$  evoked by different 5-HT concentrations in oocytes expressing 5-HT<sub>3A</sub> receptors (Fig. 5B). Co-application of quercetin and its glycosides for 30 s with different concentrations of 5-HT significantly shifted the dose-response curve of 5-HT to the rightward. The  $EC_{50}$  values were  $1.9 \pm 0.2$ ,  $4.1 \pm 0.1$ ,  $2.6 \pm 0.6$ ,  $2.3 \pm 0.4$  and  $4.1 \pm 0.1 \mu\text{M}$  for 5-HT alone, 5-HT+quercetin, 5-HT+Rham1, 5-HT+Rutin, and 5-HT+Rham2, respectively ( $*p < 0.05$ ;  $**p <$

0.005, compared with 5-HT alone) and the Hill coefficients were 1.9, 1.6, 1.5, 1.9 and 1.8, respectively (Table 2). Thus, these results indicate that quercetin and its glycosides inhibit the  $I_{5-HT}$  in a competitive manner. Altogether, these results indicate that quercetin and its glycosides regulate glycine and 5-HT<sub>3A</sub> receptor channel activities with differential manners.

**Current–Voltage Relationships in Quercetin and Its Glycosides-Mediated Human Glycine  $\alpha 1$  and 5-HT<sub>3A</sub> Receptor Regulations** In experiments of the current–voltage relationship, the membrane potential was held at  $-80$  mV and a voltage ramps for 300 ms were applied from  $-100$  to  $+40$  mV for glycine receptor. In the absence of glycine, the inward current at  $-100$  mV was  $<0.1 \mu A$  and the outward current at  $+40$  mV was  $0.1$ – $0.4 \mu A$  in the glycine receptor (Fig. 6A). The addition of glycine to the bathing medium resulted in an increase of the inward current at a potential more negative than approximately  $-20$  mV. In contrast, at a potential more positive than approximately  $-20$  mV, glycine led to a large increase in outward current. Co-application of quercetin and its glycosides with glycine inhibited both the inward and outward currents more than those induced by glycine treatment alone. The reversal potential was also near  $-20$  mV in both glycine alone and glycine+quercetin and its glycosides. The inhibitory effects of quercetin and its glycosides on  $I_{Gly}$  in oocytes expressing the glycine receptor were voltage-sensitive (Fig. 6A). Thus, quercetin, Rham1, Rutin, and Rham2 inhibited  $I_{Gly}$  by  $47.3 \pm 2.5\%$ ,  $45.7 \pm 2.4\%$ ,  $40.6 \pm 3.1$  and  $36.4 \pm 2.1\%$ , respectively, at  $-100$  mV; and by  $66.8 \pm 3.1\%$ ,  $58.9 \pm 2.1\%$ ,  $56.7 \pm 3.7$  and  $52.8 \pm 2.2\%$ , respectively, at  $+40$  mV ( $n=10$ – $12$ , from three different frogs). These results indicate that quercetin and its glycosides inhibit  $I_{Gly}$  in a voltage-sensitive manner.

In experiments of the current–voltage relationship using 5-HT<sub>3A</sub> receptors, the membrane potential was also held at

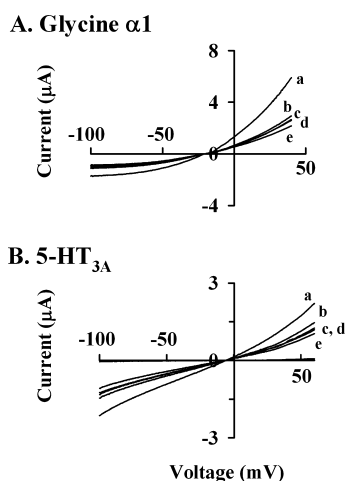


Fig. 6. Voltage-Dependent and -Independent Inhibition of  $I_{Gly}$  and  $I_{5-HT}$  in Oocytes Expressing the Human Glycine  $\alpha 1$  and Mouse 5-HT<sub>3A</sub> Receptor by Quercetin and Its Glycosides

(A) The representative current–voltage relationship was obtained using voltage ramps from  $-100$  and  $+40$  mV for a 300-ms duration. Voltage steps were applied before and after application of  $100 \mu M$  glycine in the absence (a) or presence of  $100 \mu M$  Rham2 (b),  $65 \mu M$  Rutin (c),  $60 \mu M$  Rham1 (d) and  $25 \mu M$  quercetin (e). (B) The representative current–voltage relationship was obtained using voltage ramps from  $-100$  and  $+60$  mV for a 300-ms duration. Voltage steps were applied before and after application of  $1 \mu M$  5-HT in the absence (a) or presence of  $25 \mu M$  Rutin (b),  $60 \mu M$  Rham1 (c),  $20 \mu M$  quercetin (d) and  $25 \mu M$  Rham2 (e). Each point represents the means  $\pm$  S.E.M. ( $n=10$ – $12$  oocytes in three different frogs).

$-80$  mV and a voltage ramps for 300 ms were applied from  $-100$  to  $+60$  mV for 5-HT<sub>3A</sub> receptors, respectively. In the absence of 5-HT, the inward current at  $-100$  mV was  $<0.1 \mu A$  and the outward current at  $+60$  mV was  $0.1 \mu A$  in the 5-HT<sub>3A</sub> receptor (Fig. 6B). The addition of 5-HT to the bathing medium resulted in an increase of the inward current at negative voltages, and an outward current at positive voltages. The reversal potential was near  $0$  mV with 5-HT alone as well as 5-HT+quercetin and its glycosides. Co-application of quercetin and its glycosides with 5-HT inhibited both the inward and outward currents more than those induced by 5-HT treatment alone. The inhibitory effects of quercetin and its glycosides on  $I_{5-HT}$  in oocytes expressing 5-HT<sub>3A</sub> receptor were voltage-insensitive (Fig. 6B). Thus, quercetin, Rham1, Rutin and Rham2 inhibited  $I_{5-HT}$  by  $43.1 \pm 2.4\%$ ,  $40.1 \pm 2.2\%$ ,  $33.0 \pm 3.0$  and  $50.7 \pm 2.7\%$  at  $-100$  mV, respectively; and by  $45.8 \pm 3.0\%$ ,  $44.1 \pm 2.0\%$ ,  $32.9 \pm 3.7$  and  $54.2 \pm 2.1\%$  at  $+60$  mV, respectively ( $n=10$ – $12$ , from three different frogs). These results indicate that quercetin inhibits  $I_{Gly}$  in a voltage-insensitive manner.

## DISCUSSION

Quercetin, one of the flavonoids, and its glycosides are substances of low molecular weight mainly found in apple, tomato, ginkgo and other red fruits (Fig. 1A).<sup>12</sup> Quercetin shows a wide range of biological activities,<sup>13–15</sup> with neuropharmacological actions such as analgesia, motility and sleep,<sup>16,17</sup> neuronal oxidative modulations,<sup>18</sup> proconvulsant, anticonvulsant, sedative and anxiolytic effects.<sup>19–21</sup> The cellular mechanisms underlying quercetin, especially on synaptic transmissions are not well studied. In the previous studies, we could demonstrate that quercetin regulates glycine and 5-HT<sub>3A</sub> receptor channel activities.<sup>6,7</sup> However, although most of quercetin in fruits and vegetables exist as quercetin glycosides, the pharmacological and physiological roles of those quercetin glycosides are relatively unknown in nervous systems.

In the present study, we demonstrated that (1) quercetin was more potent for the inhibition of  $I_{Gly}$  than other quercetin glycosides, whereas quercetin and its glycosides except Rham1 and rutin were equally potent for the inhibition of  $I_{5-HT}$ ; (2) quercetin glycosides inhibited both  $I_{Gly}$  and  $I_{5-HT}$  in a concentration-dependent and reversible manner in oocytes expressing the human glycine  $\alpha 1$  or mouse 5-HT<sub>3A</sub> receptors; (3) quercetin glycosides-induced inhibitions of  $I_{Gly}$  were non-competitive and voltage-sensitive, whereas quercetin glycosides-induced inhibitions of  $I_{5-HT}$  were competitive and voltage-insensitive, indicating that quercetin glycosides regulate glycine and 5-HT<sub>3A</sub> receptor with differential manners.

It has been known that dietary quercetin and its glycosides are metabolized into three ways. First is to remain in intact quercetin or conjugated with glucoses or sulfates to produce quercetin glucuronides or quercetin sulfates in blood stream; second way is to deglycosylate quercetin to quercetin aglycone; third way is to remain with quercetin glycosides without further metabolisms.<sup>8,9</sup> Interestingly, quercetin glucuronides or quercetin sulfates maintain their biological activities as antioxidants if they are conjugated with carbon-3 but not carbon-4. These results indicate that the antioxidant effects of quercetin and its metabolites are due to catechol

ring of quercetin backbone structure (Fig. 1).<sup>8)</sup> However, it is unknown whether catechol ring of quercetin is also involved in ligand-gated ion channel activity regulations. In future, further investigations using flavonoids, which are conjugated at carbon-4, will be required to evaluate whether intact catechol ring of flavonoids is also necessary for the regulation of ligand-gated ion channel activity.

In the present study, we found the position of glycosylation to quercetin plays important roles in glycine but not 5-HT<sub>3A</sub> receptor channel activity. As shown in Fig. 4 and Table 1, the IC<sub>50</sub> values of quercetin with monomer or dimer of carbohydrate at carbon-3 were about 2.5-fold higher than that of quercetin. Moreover, the IC<sub>50</sub> value of quercetin with trimer of carbohydrate was about 3-fold higher than that of quercetin (Fig. 4A). Thus, glycosylations of quercetin at carbon-3 attenuate quercetin action in quercetin-induced glycine receptor regulations. These results show a possibility that if quercetin glycosides are metabolized into quercetin aglycone, their regulatory effects on glycine receptor channel activity will be recovered as much as quercetin. Interestingly, in case of 5-HT<sub>3A</sub> receptor, the degree of quercetin glycosylations did not much affect quercetin-mediated 5-HT<sub>3A</sub> receptor channel activity except Rham1, since the IC<sub>50</sub> values of quercetin with monomer or trimer of carbohydrate were not significantly higher than that of quercetin. In addition, it appeared that the maximum inhibition of Rutin on 5-HT<sub>3A</sub> receptor channel activity was about 60% compared to quercetin and other quercetin glycosides shown Fig. 4B and Table 1, even though its IC<sub>50</sub> was similar to others except Rham1. It seems that Rutin might have a low efficiency in the inhibition of 5-HT<sub>3A</sub> receptor channel currents compared with quercetin and other quercetin glycosides tested.

From the results of quercetin glycosides' effects on glycine and 5-HT<sub>3A</sub> receptor channel activities, we could observe that the possible interaction site(s) of quercetin glycosides with glycine receptor might be different from that of 5-HT<sub>3A</sub> receptor, since quercetin glycosides-induced inhibitions of  $I_{Gly}$  were non-competitive and voltage-sensitive, whereas quercetin glycosides-induced inhibitions of  $I_{5-HT}$  were competitive and voltage-insensitive manners. It has been known that open channel blockers of ligand-gated ion channels are usually non-competitive and voltage-sensitive, due to the charge that they carry in the transmembrane electrical field of channel pore.<sup>26–28)</sup> This notion is again supported by previous report that site-directed mutations of S267 to S267Y at transmembrane domain 2 (TM2), which is forming channel pore region, almost abolished quercetin-induced inhibition of  $I_{Gly}$ .<sup>7)</sup> The present and previous results show a possibility that quercetin and its glycosides might play as open channel blockers of glycine receptor.

On the other hand, we also found that quercetin- and its glycosides-induced inhibitions of  $I_{5-HT}$  were competitive and voltage-insensitive manners. It is also known that the competitive inhibitors of 5-HT<sub>3A</sub> receptor usually regulate channel activity by inhibiting 5-HT binding to its binding site(s) in N-terminal regions of 5-HT<sub>3A</sub> receptor.<sup>29)</sup> In competition experiments, we could observe that the presence of quercetin- and its glycosides shifted the concentration of 5-HT in oocytes expressed with 5-HT<sub>3A</sub> receptor without significant changes of Hill coefficient (Fig. 5B). Thus, the competitive modulation of 5-HT<sub>3A</sub> receptor channel activity by

quercetin and its glycosides shows a possibility that quercetin and its glycosides might have interaction site(s) in N-terminal of 5-HT<sub>3A</sub> receptor. In previous report, we have shown that site-directed mutations of N-terminal region of pre-transmembrane domain 1 (pre-TM1) such as R246T and R246A but not R246D, R246E and R246K abolished quercetin-induced inhibition of  $I_{5-HT}$ , supporting that quercetin regulates 5-HT<sub>3A</sub> receptor channel activities through interaction with N-terminal region of 5-HT<sub>3A</sub> receptor.<sup>6)</sup> However, identification of the exact interaction sites of quercetin and its glycosides involved in the regulation of glycine or 5-HT<sub>3A</sub> receptor channel activity might require further investigations in future.

Interestingly, it is well known that activations of glycine receptor at postsynaptic sites exhibit fast inhibitory postsynaptic potentials, whereas activations of 5-HT<sub>3A</sub> at postsynaptic sites exhibit fast excitatory postsynaptic potentials.<sup>22,23)</sup> Therefore, it will be questioned what are the physiological or pharmacological roles of quercetin and its glycosides following the inhibitions of excitatory or inhibitory ligand-gated ion channel activities. Glycine receptors are rich in spinal cord and brain stem, whereas 5-HT<sub>3</sub> receptors exist peripheral nervous systems such as intestines and brain stem area related with emesis in central nervous system.<sup>24,25)</sup> Although we could not clearly answer the roles of quercetin and its glycosides that inhibit both ligand-gated ion channel activities in nervous systems, it seems that quercetin and its glycosides might exert their effects with differential manners in different regions of nervous systems.

In summary, we found that quercetin glycosides inhibit  $I_{Gly}$  and  $I_{5-HT}$  in oocytes expressing the human glycine  $\alpha 1$  or mouse 5-HT<sub>3A</sub> receptors. The glycosylation of quercetin exhibited differential effects on  $I_{Gly}$  and  $I_{5-HT}$ , in that quercetin was more potent for the inhibition of  $I_{Gly}$  than other quercetin glycosides, whereas quercetin and its glycosides except Rham1 were equally potent for the inhibition of  $I_{5-HT}$ .

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