Mutations of Arginine 222 in Pre-transmembrane Domain I of Mouse 5-HT_{3A} Receptor Abolish 20(*R*)- But Not 20(*S*)-Ginsenoside Rg_3 Inhibition of 5-HT-Mediated Ion Currents

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Ginsenosides, active ingredients of *Panax* ginseng, exist as stereoisomers depending on the position of the hydroxyl group on carbon-20; *i.e.* 20(*R*)-ginsenoside and 20(*S*)-ginsenoside are epimers. We previously investigated the structure–activity relationship of the ginsenoside Rg_3 stereoisomers, 20-*R*-protopanaxatriol-3-[*O*-β-D-glucopyranosyl (1→2)-β-glucopyranoside], (20(*R*)-Rg_3) and 20-*S*-protopanaxatriol-3-[*O*-β-D-glucopyranosyl (1→2)-β-glucopyranoside], (20(*S*)-Rg_3) in regulating 5-HT_{3A} receptor-mediated ion currents ($I_{5\text{-HT}}$) expressed in *Xenopus* oocytes and found that 20(*S*)-Rg_3 rather than 20(*R*)-Rg_3 was more stronger inhibitor of $I_{5\text{-HT}}$. In the present study, we further investigated the effects of 20(*R*)-Rg_3 and 20(*S*)-Rg_3 on mouse 5-HT_{3A} receptor channel activity after site-directed mutations of 5-HT_{3A} receptor facilitation site, which is located at pre-transmembrane domain I (pre-TM1). 5-HT_{3A} receptor was expressed in *Xenopus* oocytes, and $I_{5\text{-HT}}$ was measured using two-electrode voltage clamp technique. In wild-type, both 20(*R*)-Rg_3 and 20(*S*)-Rg_3 inhibited $I_{5\text{-HT}}$ with concentration-dependent and reversible manner. Induction of 5-HT_{3A} receptor facilitation by point mutations of pre-TM1 amino acid residue R222 to R222A, R222D, R222E or R222T not only decreased EC_{50} values for $I_{5\text{-HT}}$ compared to wild-type but also abolished 20(*R*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$. Those mutations also shifted the IC_{50} values by 20(*S*)-Rg_3 into right direction by 2- to 4-folds compared with wild-type. These results indicate that 5-HT_{3A} receptor facilitation differentially affects 20(*R*)-Rg_3- and 20(*S*)-Rg_3-mediated 5-HT_{3A} receptor channel regulation.

Key words ginseng; ginsenoside stereoisomer; 5-HT_{3A} receptor; ligand-gated ion channel; *Xenopus* oocyte

Ginseng, the root of *Panax ginseng* C. A. Meyer, is well known as natural medicine with restorative and promoting health. In traditional herbal medicine, ginseng alone has been used for the alleviation of symptoms such as anorexia, dyspepsia, vomiting and pain.[1] The main molecular components responsible for the actions of ginseng are ginsenosides, which are also known as ginseng saponins. Ginsenosides have a four-ring, steroid-like structure with sugar moieties attached, and about 30 different forms have been isolated and identified from the root of *Panax* ginseng. Each also has at least three side chains on carbon-3, -6, and -20, which are free, or bound to monomeric, dimeric, or trimeric sugars. They also exist as stereoisomers; 20(*R*)-ginsenoside and 20(*S*)-ginsenoside are epimers of each other depending on the position of the hydroxyl group on carbon-20 (Fig. 1).[2,3]

Recent reports show that the mixture of 20(*R*)- and 20(*S*)-ginsenosides regulates voltage-dependent Ca^{2+} channel activity and several types of ligand-gated ion channels. They inhibit high-threshold voltage-gated Ca^{2+} channels in chromaffin cells[4,5] and sensory neurons.[6—8] In cells expressing nicotinic acetylcholine (nACh) receptors, such as bovine chromaffin cells, they inhibit acetylcholine-stimulated Na^+ influx.[9,10] More directly, Choi *et al.*[11,12] and Sala *et al.*[13] showed that ginsenosides inhibit ACh- and 5-HT-induced inward currents in *Xenopus* oocytes expressing several subtypes of neuronal and $\alpha\beta\delta\varepsilon$ muscle-type nACh and 5-HT_{3A} receptors, respectively. Ginsenosides also inhibit NMDA receptor-mediated intracellular Ca^{2+} increases and NMDA-induced currents in cultured rat hippocampal neurons.[14] The effects of ginsenosides on voltage-dependent and ligand-gated ion channels suggest that they may affect synaptic transmission by controlling neurotransmitter release.[9,10]

Relatively, very little is known about the structure–activity relationships of ginsenosides in biological systems. In partic-

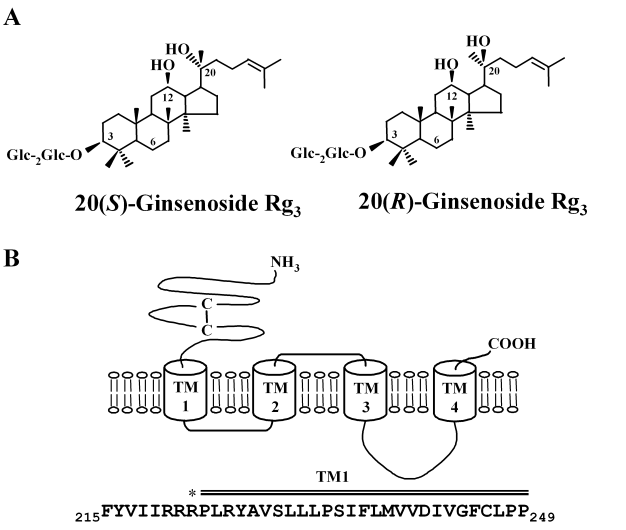

Fig. 1. Structures of 20(*S*)- and 20(*R*)-Ginsenosides Rg_3 and the Primary Amino Acid Sequence of the Pre-TM1 of the Mouse 5-HT_{3A} Receptor

(A) Structures of 20(*S*)- and 20(*R*)-ginsenosides Rg_3. Glc, glucopyranoside. Subscripts indicate the carbon in the glucose ring that links the two carbohydrates. (B) Partial amino acid sequence of the pre-TM1 region of the cloned mouse 5-HT_{3A} receptor. The (*) indicates amino acid residues that were mutated in the present study.

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ular, it is not known whether ion channel regulation by ginsenosides is stereospecific. To answer this question, we previously compared the effects of ginsenoside Rg_3 (20-*R*-protopanaxatriol-3-[*O*-β-D-glucopyranosyl (1→2)-β-glucopyranoside]), (20(*R*)-Rg_3) and ginsenoside Rg_3 (20-*S*-protopanaxatriol-3-[*O*-β-D-glucopyranosyl (1→2)-β-glucopyranoside]), (20(*S*)-Rg_3) in ion channels and receptors and found that 20(*S*)-Rg_3 but not 20(*R*)-Rg_3 is a main regulator of voltage-dependent Ca^{2+}, K^+, or Na^+ channels. We also found that both stereoisomers inhibit 5-HT_{3A} and $\alpha3\beta4$ nACh receptor channel activity but that 20(*S*)-Rg_3 rather than 20(*R*)-Rg_3 more potently inhibit 5-HT_3 receptor-mediated currents ($I_{5\text{-HT}}$).[15] In the present study, we further examined the changes of the inhibitory effects of 20(*R*)-Rg_3 and 20(*S*)-Rg_3 on $I_{5\text{-HT}}$ after mutations of 5-HT_{3A} receptor facilitation site, which is located at pre-transmembrane domain I (pre-TM1). We found that 5-HT_{3A} receptor facilitation abolished 20(*R*)-Rg_3- but not 20(*S*)-Rg_3-mediated inhibition of $I_{5\text{-HT}}$ and shifted the IC_{50} values by 20(*S*)-Rg_3 into right direction by 2- to 4-folds compared with wild-type. We report here that facilitation of 5-HT_{3A} receptor differentially affect Rg_3 stereoisomer-mediated 5-HT_{3A} receptor channel regulation.

MATERIALS AND METHODS

Materials Figure 1A shows the structure of 20(*R*)-Rg_3 and 20(*S*)-Rg_3. 20(*R*)-Rg_3 and 20(*S*)-Rg_3 used in this study were dissolved in dimethyl sulfoxide (DMSO) as previous report[15] and were diluted with bath medium before use. Final DMSO concentration was less than 0.01%. Other chemical agents were obtained from Sigma (St. Louis, MO, U.S.A.).

Oocyte Preparation *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 were 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 $CaCl_2$-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 units penicillin/ml, and 100 μg 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 $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) supplemented with 0.5 mM theophylline and 50 μg gentamycin/ml. All solutions were changed every day. All experiments were performed within 2—4 d following isolation of the oocytes.[12]

Oocyte Recording A single oocyte was placed in a small Plexiglas net chamber (0.5 ml) and was constantly superfused with ND96 medium in the absence or presence of 5-HT or quercetin during recording. The microelectrodes were filled with 3 M KCl and had a resistance of 0.2—0.7 MΩ. Two-electrode voltage-clamp recordings were performed at room temperature with Oocyte Clamp (OC-725C, Warner Instrument) with Digidata 1200A. For most of the electrophysiological experiments, the oocytes were clamped at a holding potential of −80 mV. For current–voltage relationship, voltage ramps were applied from −100 to +60 mV for 300-ms.

cRNA Preparation of 5-HT_{3A} Receptor and Microinjection Recombinant plasmid containing mouse 5-HT_{3A} cDNA insert was linearized by digestion with appropriate restriction enzymes. The cRNAs from linearized templates were obtained by using an *in vitro* transcription kit (mMessage mMachine; Ambion, Austin, TX, U.S.A.) with a T3 polymerase. The RNA was dissolved in RNase-free water at 1 μg/μl, divided into aliquots, and stored at −80 °C until used. Oocytes were injected with H_2O or mouse 5-HT_{3A} 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 μm-OD.[12]

Site-Directed Mutagenesis of 5-HT_{3A} Receptor and *in Vitro* Transcription of 5-HT_{3A} Receptor The substitution mutation of single or three amino acids was performed by *Pfu* DNA polymerase (QuikChange™ XL Site-Directed Mutagenesis Kit, STRATAGENE) and mutated sense and antisense primers. The overlap extension at the target domain by sequential polymerase chain reaction was performed in accordance with the supplier instruction manual with some modifications. Final PCR products were transformed to *E. coli* DH5α strain, and screened by PCR method. The mutations were confirmed by DNA sequencing analysis on target region. The mutant DNA constructs were linearized at the 3′ end by *Sal* I digestion, and run-off transcripts were prepared using methylated cap analog $m^7G(5')ppp(5')G$ and cRNAs were prepared using T3 RNA polymerase included in the mMessage mMachine transcription kit (Ambion, Austin, TX, U.S.A.). The final cRNA products were resuspended with RNase-free water at concentration of 1 μg/μl and stored at −80 °C.

Data Analysis To obtain the concentration–response curve for 5-HT-induced current in the presence of quercetin, 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 20(*R*)-Rg_3 and 20(*S*)-Rg_3, y_{max}, % of maximal inhibition, IC_{50} is the concentration of 20(*R*)-Rg_3 and 20(*S*)-Rg_3 producing half-maximum inhibition of the control response to 5-HT, and [A] is the concentration of 20(*R*)-Rg_3 and 20(*S*)-Rg_3. n is the interaction coefficient. All values are presented as means±S.E.M. The differences between means of control and quercetin treatment data were analyzed using unpaired Student's t test and one-way ANOVA test. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

As shown in previous report,[12,16,17] the addition of 5-HT to bathing solution induced a large inward current ($I_{5\text{-HT}}$) in oocytes injected with wild-type 5-HT_{3A} receptor cRNA. $I_{5\text{-HT}}$ was blocked by selective 5-HT_{3A} receptor antagonist 0.5 μM MDL-72222 and in H_2O-injected control oocyte, treatment of 5-HT did not induce any inward current as shown in previous report (data not shown).[12,16,17] Both 20(*R*)-Rg_3 and 20(*S*)-Rg_3 (100 μM each) itself had no effect in oocytes expressing 5-HT_{3A} receptor at a holding potential of −80 mV (data not shown). But co-treatment of 20(*R*)-Rg_3 or 20(*S*)-Rg_3 (100 μM) with 5-HT (1 μM) for 30 s inhibited $I_{5\text{-HT}}$ in

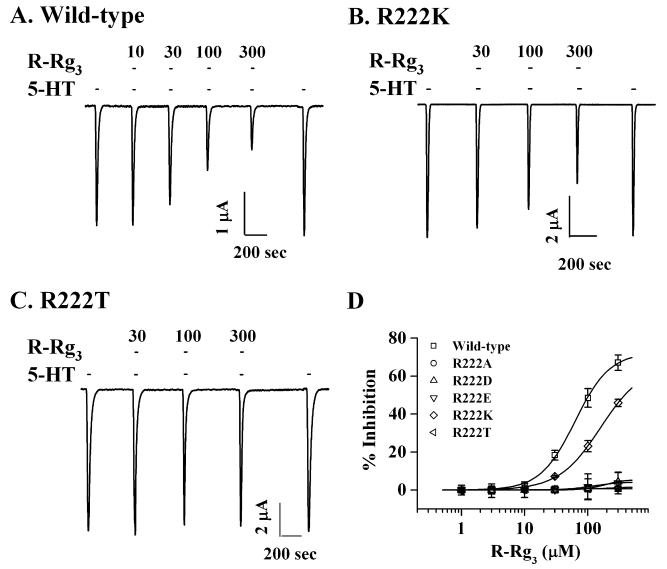

Fig. 2. Concentration-Dependent Effects of 20(*R*)-Rg_3 on $I_{5\text{-HT}}$ in Wild-Type and Mutant Receptors

$I_{5\text{-HT}}$ in oocytes expressing wild-type, R222A, R222D, R222E, R222K, and R222T mutant receptors was elicited at a holding potential of −80 mV for the indicated time in the presence of 1 μM 5-HT, and then the indicated concentrations of 20(*R*)-Rg_3 were co-applied with 5-HT. (A—C) Traces are representative of nine separate oocytes from three different frogs. (D) Concentration–response curves for the effect of 20(*R*)-Rg_3 on oocytes expressing the wild-type and various mutant receptors. 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 2 (mean±S.E.M.; n=9—10 oocytes for each point).

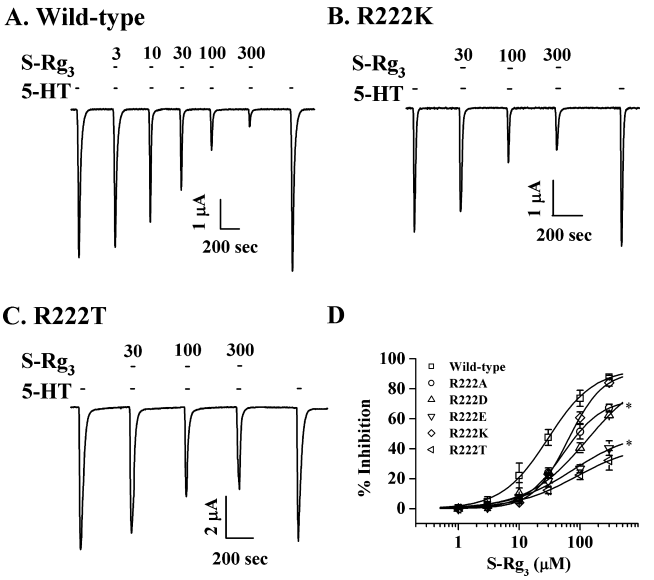

Fig. 3. Concentration-Dependent Effects of 20(*S*)-Rg_3 on $I_{5\text{-HT}}$ in Wild-Type and Mutant Receptors

$I_{5\text{-HT}}$ in oocytes expressing wild-type, R222A, R222D, R222E, R222K, and R222T mutant receptors was elicited at a holding potential of −80 mV for the indicated time in the presence of 1 μM 5-HT, and then the indicated concentrations of 20(*S*)-Rg_3 were co-applied with 5-HT. (A—C) Traces are representative of nine separate oocytes from three different frogs. (D) Concentration–response curves for the effect of 20(*S*)-Rg_3 on oocytes expressing the wild-type and mutant receptors. 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 3 (mean±S.E.M.; n=8—10 oocytes for each point).

oocytes expressing wild-type 5-HT_{3A} receptor (Figs. 2 and 3, n=10—12 from three different frogs). Thus, co-treatment of 20(*R*)-Rg_3 or 20(*S*)-Rg_3 with 5-HT induced the inhibition of $I_{5\text{-HT}}$ by 48.5±4.9 and 73.6±5.4%, respectively (Fig. 4, $*p<0.05$, $**p<0.005$ compared with 20(*R*)-Rg_3 or 20(*S*)-Rg_3 untreated control oocytes). In concentration-dependent experiments with 20(*R*)-Rg_3 or 20(*S*)-Rg_3, co-treatment with 20(*R*)-Rg_3 or 20(*S*)-Rg_3 for 30 s inhibited $I_{5\text{-HT}}$ in a concentration-dependent manner in oocytes expressing wild-type 5-HT_{3A} receptors (Figs. 2A, 3A). Interestingly, the low concentrations of 20(*R*)-Rg_3 or 20(*S*)-Rg_3 showed a slight inhibition of $I_{5\text{-HT}}$. Thus, 20(*R*)-Rg_3 inhibited $I_{5\text{-HT}}$ by 0.1±1.0, 0.2±3.5, 2.0±1.5, 18.4±2.6, 48.5±4.9 and 67.1±4.1% at 1, 3, 10, 30, 100, and 300 μM in oocytes expressing wild-type 5-HT_{3A} receptors, respectively. 20(*S*)-Rg_3 inhibited $I_{5\text{-HT}}$ by 0.5±1.0, 4.5±3.4, 22.1±8.3, 47.5±5.3, 73.6±5.4 and 92±1.1% at 1, 3, 10, 30, 100, and 300 μM in oocytes expressing 5-HT_{3A} receptors, respectively. The IC_{50}s of $I_{5\text{-HT}}$ were 62.5±4.9 and 34.3±4.9 μM, respectively, for by 20(*R*)-Rg_3 and 20(*S*)-Rg_3 in oocytes expressing 5-HT_{3A} receptor (n=10—12, from three different frogs for each point) (Figs. 2D, 3D).

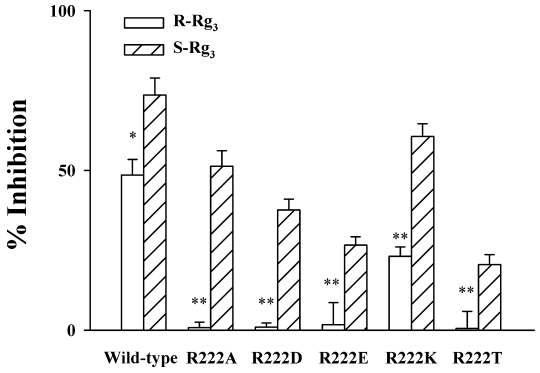

Fig. 4. Summary Histograms on Comparisons between 100 μM 20(*R*)-Rg_3- and 20(*S*)-Rg_3-Induced Inhibition of $I_{5\text{-HT}}$ (1 μM 5-HT) in Wild-Type and Mutant Receptors

$*p<0.05$, $**p<0.005$ compared with 20(*S*)-Rg_3 (mean±S.E.M.; n=8 oocytes for each point).

The fact that 20(*S*)-Rg_3 is more active than 20(*R*)-Rg_3 in the inhibition of $I_{5\text{-HT}}$ indicates that a slight difference in its hydroxyl group at carbon 20 may contribute for better geometrical alignment with the hydroxyl acceptor group in the 5-HT_{3A} receptor than that of 20(*R*)-Rg_3. If facilitation on 5-HT_{3A} receptor activation achieved through site-directed mutations,[19] it might be possible to observe some changes in 20(*R*)-Rg_3- or 20(*S*)-Rg_3-induced regulation of 5-HT_{3A} receptor channel activity. To test this possibility, we next constructed five different kinds of 5-HT_{3A} mutant receptors in pre-TM1 of N-terminal domain by replacing amino acid residues R222 to alanine (R222A), threonine (R222T), aspartate (R222D), glutamate (R222E), and lysine (R222K) (Fig. 1B).[18] We choose these sites for site-directed mutations, since mutation of pre-TM1 of N-terminal domain facilitates 5-HT_{3A}-mediated $I_{5\text{-HT}}$ and attenuated receptor antagonist-induced inhibition of $I_{5\text{-HT}}$.[18] Table 1 shows a summary of EC_{50} values after receptor facilitation in wild-type and mutant channels. Thus, mutation of R222 to other amino acids such as alanine, aspartate, glutamate and threonine decreased EC_{50} values by 18.4, 13.5, 12.3, 2.9-fold compared with wild-type, respectively. Interestingly, we could not observe a change of EC_{50} value in R222K mutant (Table 1). These results are well consistent with previous reports.[19] Next, we examined the effect of 20(*R*)-Rg_3 or 20(*S*)-Rg_3 on mutant receptors. Figures 2 and 3 show the representative traces in the absence or

Table 1. Summary of Concentration Response on Wild-Type and Mutant 5-HT_{3A} Receptors Expressed in *Xenopus* Oocytes

	EC_{50}	*n*H
Wild-type	4.05±0.42	1.7±0.2
R222A	0.22±0.01*	2.1±0.1
R222D	0.30±0.09*	1.0±0.2
R222E	0.33±0.05*	1.3±0.2
R222K	4.78±0.80	1.5±0.3
R222T	1.42±0.29*	1.1±0.2

Currents were elicited at a holding potential of −80 mV. EC_{50} (μM) and Hill coefficient values were determined as described in Materials and Methods. Values represent the mean±S.E.M. (n=9—12/group). *p<0.005 compared with wild-type 5-HT_{3A} receptors.

Table 2. Summary on Inhibition of 20(*R*)-Rg_3 on Wild-Type and Mutant 5-HT_{3A} Receptors Expressed in *Xenopus* Oocytes

	IC_{50}	V_{max}	*n*H
Wild-type	62.5±4.9	72.4±2.7	1.6±0.1
R222A	ND	ND	ND
R222D	ND	5.6±0.1**	ND
R222E	ND	4.3±0.1**	ND
R222K	159.3±20.9**	65.9±4.7*	1.3±0.1
R222T	ND	ND	ND

Currents were elicited at a holding potential of −80 mV. IC_{50} (μM), V_{max}, and Hill coefficient values were determined as described in Materials and Methods. Values represent the mean±S.E.M. (n=9—10/group). ND, not determined. *p<0.05, **p<0.005 compared with wild-type 5-HT_{3A} receptors.

Table 3. Summary of Inhibition of 20(*S*)-Rg_3 on Wild-Type and Mutant 5-HT_{3A} Receptors Expressed in *Xenopus* Oocytes

	IC_{50}	V_{max}	*n*H
Wild-type	29.5±2.7	93.4±2.6	1.1±0.2
R222A	53.0±3.3	73.5±2.0*	1.4±0.1
R222D	131.7±36.8**	92.4±12.8	0.9±0.3
R222E	77.3±24.9*	52.0±12.8*	0.9±0.3
R222K	63.7±2.8	91.9±1.9	1.5±0.1
R222T	86.2±20.0**	42.8±3.6**	0.9±0.1

Currents were elicited at a holding potential of −80 mV. IC_{50} (μM), V_{max}, and Hill coefficient values were determined as described in Materials and Methods. Values represent the mean±S.E.M. (n=8—10/group). *p<0.05, **p<0.005 compared with wild-type 5-HT_{3A} receptors.

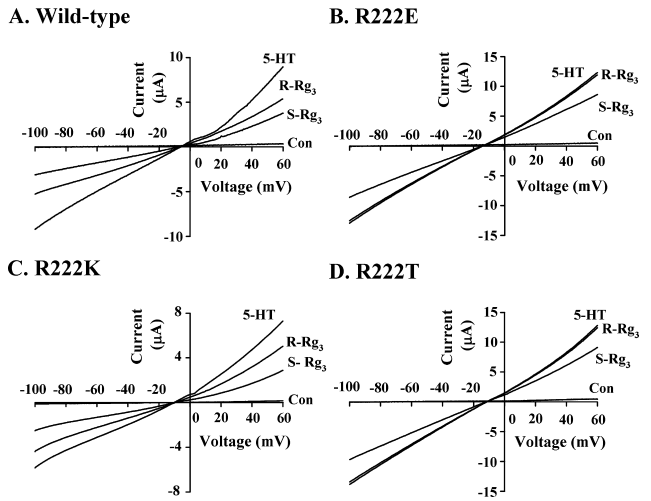

Fig. 5. Current–Voltage Relationships on $I_{5\text{-HT}}$ in Oocytes Expressing 5-HT_{3A} Receptor by 20(*R*)-Rg_3 and 20(*S*)-Rg_3 in Wild-Type and Mutants

The representative current–voltage relationship was obtained using voltage ramps from −100 and +60 mV with 300-ms duration at the holding potential −80 mV (A—D). Voltage steps were applied before and after application of 1 μM 5-HT in the absence or presence of 100 μM 20(*R*)-Rg_3 and 20(*S*)-Rg_3.

presence of 20(*R*)-Rg_3 or 20(*S*)-Rg_3 in each mutant of pre-TM1 of N-terminal domain sites. Interestingly, mutations of R222 to R222A, R222D, R222E and R222T but not R222K completely abolished 20(*R*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$ (Fig. 2), whereas although the degree of inhibition of $I_{5\text{-HT}}$ by 20(*S*)-Rg_3 on those mutant receptor was significantly attenuated, the inhibitory effects of 20(*S*)-Rg_3 was still maintained on those mutant receptor (Fig. 3). Figures 2D and 3D also show the concentration–response relationship for $I_{5\text{-HT}}$ inhibition by 20(*R*)-Rg_3 or 20(*S*)-Rg_3 in wild-type and various mutants at amino acid residue R222 and the smooth lines represent the best fits of the data using the Hill equation with the parameters of the fits shown in Figs. 2 and 3. Thus, mutations of R222 to R222A, R222D, R222E, R222T and R222K shifted the IC_{50} values by 20(*S*)-Rg_3 into right direction by 2- to 4-folds compared with wild-type (Table 3). Figure 4 shows a summary on comparison of 20(*R*)-Rg_3- and 20(*S*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$ in wild-type and various mutant receptors. Interestingly, we could observe that the degree of attenuation of 20(*S*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$ was less profound in mutation of R222 to R222K than other mutant receptors and furthermore, 20(*R*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$ was not abolished in R222K mutant receptor.

In experiments of the current–voltage relationship, the membrane potential was held at −80 mV and a voltage ramp was applied from −100 to +60 mV during 300 ms. In the absence of 5-HT, the inward current at −100 mV was <0.01 μA and the outward current at +60 mV was near 0.1 μA. The addition of 5-HT to the bathing medium induced mainly an inward current at negative voltages and outward current at positive voltages. Co-treatment of 20(*R*)-Rg_3 or 20(*S*)-Rg_3 with 5-HT decreased both inward and outward currents in wild-type (Fig. 5A). The reversal potential was near 0 mV in both 5-HT alone and 5-HT plus 20(*R*)-Rg_3 or 20(*S*)-Rg_3. This indicates that 5-HT induces the cation current.[20] Also, co-treatment of 20(*R*)-Rg_3 or 20(*S*)-Rg_3 with 5-HT did not affect the 5-HT receptor channel property because 20(*R*)-Rg_3 or 20(*S*)-Rg_3 did not change reversal potential of 5-HT_{3A} receptor (Fig. 5). In mutant 5-HT_{3A} receptors such as R222E and R222T but not R222K the inhibitory effect of 20(*S*)-Rg_3 on inward and outward $I_{5\text{-HT}}$ was greatly attenuated, whereas in mutant 5-HT_{3A} receptors such as R222E and R222T but not R222K the inhibitory effect of 20(*R*)-Rg_3 on inward and outward $I_{5\text{-HT}}$ was abolished (Fig. 5) (n=7—8, from three different frogs).

Ginsenosides consist of aglycone, carbohydrates portions, and alkene side chain. Aglycone is their main backbone with a hydrophobic four-ring steroid-like structure with hydroxyl group at carbon-20. The hydroxyl group at carbon-20 of the backbone structure generates stereoisomers, the (*R*)- and (*S*)-epimers (Fig. 1).[3] We have previously demonstrated that the slight difference in chemical structure between the two epimers greatly affects their ability to inhibit Ca^{2+}, K^+ and Na^+ channel activities: only (*S*)-Rg_3 inhibited the Ca^{2+}, K^+ and Na^+ channel currents. Our observations indicate that the hydroxyl group of 20(*S*)-Rg_3 may be better aligned than that of 20(*R*)-Rg_3 with the hydroxyl acceptor group of the Ca^{2+},

K^+ and Na^+ channel proteins.[21] In addition, we also showed in the present and previous studies that both 20(*R*)-Rg_3 and 20(*S*)-Rg_3 inhibited $I_{5\text{-HT}}$, although 20(*S*)-Rg_3 gave a greater maximal inhibition of this current than 20(*R*)-Rg_3. It is not known why the Rg_3 stereoisomers show much less stereospecificity for the ligand-gated ion channels than for the voltage-dependent ion channels.

In the present study, we further demonstrated that (1) mutations of N-terminal of pre-TM1 amino acid residue R222 to R222A, R222D, R222E or R222T shifted the 5-HT concentration–response curves into left direction by decreasing the EC_{50} values as previous reports[19] and (2) in site-directed mutagenesis experiments we found that mutations of N-terminal of pre-TM1 abolished 20(*R*)-Rg_3- but not 20(*S*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$. These results indicate that the position of hydroxyl group at carbon-20 of the backbone structure of ginsenosides is closely related with wild-type and mutant 5-HT_{3A} receptor regulations.

Interestingly, R222 that is located at just pre-transmembrane (TM1) domain of mouse 5-HT_{3A} receptor exhibits two characteristics (Fig. 1). First, this residue of the mouse 5-HT_{3A} receptor is conserved in all of 5-HT_{3A} receptors and α7 nicotinic acetylcholine receptors that have been cloned from various species.[18] Second, some mutations at R222 can alter the sensitivity of 5-HT_{3A} receptors to agonists and produce channels that open spontaneously.[19] Thus, it seems that R222 is not only involved in signal transduction that couples the agonist binding to channel opening in 5-HT_{3A} receptors but also facilitates the linking agonist binding to channel gating by decreasing the EC_{50} of 5-HT concentration–response curve.[18]

In the present study, we could also observe that mutations of R222 to R222A, R222D, R222E and R222T decreased the EC_{50} values of the 5-HT concentration–response curves by shifting left direction. These present results are well consistent with previous reports.[19] Interestingly, as mentioned above, 20(*S*)-Rg_3 gave a greater inhibition of this current than 20(*R*)-Rg_3: 20(*S*)-Rg_3 inhibited $I_{5\text{-HT}}$ by 73.6±5.4%, whereas 20(*R*)-Rg_3 inhibited $I_{5\text{-HT}}$ by 43.5±4.9% (Fig. 4). However, when we induce a facilitation on 5-HT receptor activation by mutation R222 to R222A, R222D, R222E and R222T but not R222K, we could observe a marked difference in 20(*R*)-Rg_3- or 20(*S*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$. Thus, the inhibitory effect of 20(*R*)-Rg_3 on $I_{5\text{-HT}}$ was abolished and the degree of 20(*S*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$ was attenuated as much as that of 20(*R*)-Rg_3 observed in wild-type receptor. Interestingly, we could observe a relationship between 5-HT_{3A} receptor facilitation and attenuation or abolishment of 20(*R*)-Rg_3- or 20(*S*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$, since mutation of R222 to R222K did not exhibit receptor facilitation and had no significant effects on 20(*R*)-Rg_3- and 20(*S*)-Rg_3 actions.

It needs to be speculated what the possible explanations underlying differential Rg_3 stereoisomers-induced regulations in wild-type and mutant 5-HT_{3A} receptor channel activities. Lee *et al.*[17] demonstrated that 20(*S*)-Rg_3 interacts several amino acids in pore region of TM2, since mutation of amino acid F292 to F292A abolished 20(*S*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$ and also showed that in addition, 20(*S*)-Rg_3 acts as open channel blocker of 5-HT_{3A} receptor.[17] Based on these results, it is possible to speculate that the 20(*S*)-Rg_3 interaction with amino acids in pore region of TM2 might not only induce a blockage of channel gating and but also allosterically induce a change of conformation of 5-HT_{3A} receptors, which might further interfere agonist binding to channel protein. This notion was supported by the results from our present pre-TM1 and previous TM2 pore region mutation experiments. First, mutations of TM2 pore region but not pre-TM1 region abolished 20(*S*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$, indicating that 20(*S*)-Rg_3 might more interact with TM2 pore region rather than pre-TM1 region. Second, induction of 5-HT_{3A} receptor facilitation by mutation of pre-TM1 abolished the less efficient 20(*R*)-Rg_3- but not 20(*S*)-Rg_3-induced inhibition of $I_{5\text{-HT}}$. These results show a possibility that receptor facilitation by mutation of pre-TM1 could overcome the inhibitory effect of 20(*R*)-Rg_3- but not 20(*S*)-Rg_3. Future studies will be necessary to determine the exact roles of 20(*R*)-Rg_3 and 20(*S*)-Rg_3 for the regulations of 5-HT_{3A} receptor channel activity.

In conclusion, we found that 20(*S*)-Rg_3 rather than 20(*R*)-Rg_3 is an efficient regulator of 5-HT_{3A} receptor channel activity through site-directed mutagenesis and these results, further, showed that the regulations of wild-type and mutant 5-HT_{3A} receptor channel activity by 20(*R*)-Rg_3 and 20(*S*)-Rg_3 might be closely related with the position of hydroxyl group at carbon-20 of the backbone structure.

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