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Identification of ginsenoside interaction sites in 5-HT_{3A} receptors

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Abstract

We previously demonstrated that 20(*S*)-ginsenoside Rg_3 (Rg_3), one of the active components of *Panax ginseng*, non-competitively inhibits 5-HT_{3A} receptor channel activity on extracellular side of the cell. Here, we sought to elucidate the molecular mechanisms underlying Rg_3-induced 5-HT_{3A} receptor regulation. We used the two-microelectrode voltage-clamp technique to investigate the effect of Rg_3 on 5-HT-mediated ion currents ($I_{5\text{-HT}}$) in *Xenopus* oocytes expressing wild-type or 5-HT_{3A} receptors harboring mutations in the gating pore region of transmembrane domain 2 (TM2). In oocytes expressing wild-type 5-HT_{3A} receptors, Rg_3 dose-dependently inhibited peak $I_{5\text{-HT}}$ with an IC_{50} of 27.6 ± 4.3 μM. Mutations V291A, F292A, and I295A in TM2 greatly attenuated or abolished the Rg_3-induced inhibition of peak $I_{5\text{-HT}}$. Mutation V291A but not F292A and I295A induced constitutively active ion currents with decrease of current decay rate. Rg_3 accelerated the rate of current decay with dose-dependent manner in the presence of 5-HT. Rg_3 and TMB-8, an open channel blocker, dose-dependently inhibited constitutively active ion currents. The IC_{50} values of constitutively active ion currents in V291A mutant receptor were 72.4 ± 23.1 and 6.5 ± 0.7 μM for Rg_3 and TMB-8, respectively. Diltiazem did not prevent Rg_3-induced inhibition of constitutively active ion currents in occlusion experiments. These results indicate that Rg_3 inhibits 5-HT_{3A} receptor channel activity through interactions with residues V291, F292, and I295 in the channel gating region of TM2 and further demonstrate that Rg_3 regulates 5-HT_{3A} receptor channel activity in the open state at different site(s) from those of TMB-8 and diltiazem.

Keywords: Ginsenosides; 5-HT_{3A} receptor; Site-directed mutagenesis; *Xenopus* oocytes

1. Introduction

The 5-hydroxytryptamine type 3 (5-HT_3) receptors are cationic ion channels that may be activated by serotonin or its agonists. Various agents exert their pharmacological effects by targeting 5-HT_3 receptors (Dang et al., 2000; Lopreato et al., 2003; Zhang et al., 2002; Hu et al., 2003), which share structural similarity with other ligand-gated ion channels such as $GABA_A$, glycine, and nicotinic acetylcholine receptors (Keramidas et al., 2004; Lummis, 2004). The 5-HT_3 receptor consists of a homomeric pentamer; each subunit includes

Abbreviations: Rg_3, ginsenoside Rg_3; 5-HT, 5-hydroxytryptamine (serotonin); 5-HT_{3A} receptor, serotonin receptor 3A subunit; MES, 4-Morpholineethanesulfonic acid; NMDG, *N*-methyl-D-glucamine; TM2, transmembrane domain 2; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester.

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a large N-terminal extracellular domain, four hydrophobic transmembrane domains (TM1–TM4) and an intracellular C-terminal domain (Fig. 1B). Studies have suggested that the agonist-binding sites are located in the N-terminal domain at the subunit–subunit interfaces, while the TM2 domains of the five subunits combine to form the channel pore (Keramidas et al., 2004; Lummis, 2004). Recently, site-directed mutagenesis has been used to characterize the detailed actions and binding sites of various drugs and toxins that regulate 5-HT_3 receptors (Zhang et al., 2002; Hu et al., 2003). Similarly, the substituted cysteine accessibility method has been used to infer the channel gating regions in this receptor. In particular, Reeves et al. (2001) and Panicker et al. (2002, 2004) revealed that amino acids from D274 to D298 in TM2 form the putative pore-lining face, and that residues D274, G276, E277, S280, T284, L285, L287, S290, V291, F292, L293, I295, and V296 appear to form a gating region that is susceptible to the sulfhydryl reagent, MTSET. This putative gating region in the 5-HT_{3A} receptor is similar to that previously identified for the nicotinic acetylcholine receptor (Le Novere and Changeux, 1999). However, the precise mechanisms by which the binding of serotonin or agonist leads to channel opening and gating are still poorly understood at the molecular level (Keramidas et al., 2004).

Ginseng, the root of *Panax ginseng* C.A. Meyer, is well known in herbal medicine as a tonic and restorative agent. The main molecular ingredients responsible for the actions of ginseng are the ginsenosides (also called ginseng saponins). These amphiphatic molecules comprise a hydrophobic backbone of aglycone (a hydrophobic four-ring steroid-like structure) linked to hydrophilic carbohydrate side chains consisting of monomers, dimers or tetramers. The ginsenosides may be classified as either protopanaxadiols or protopanaxatriols, according to the positions of the carbohydrate moieties at carbons-3, -6 and -20, which can be either free or connected to sugar rings (Nah, 1997). Recent studies have shown that ginsenosides exert their physiological and pharmacological effects by regulating ligand-gated ion channel activity. In cells expressing nicotinic acetylcholine receptors, such as bovine chromaffin cells, ginsenosides were shown to inhibit acetylcholine-stimulated Na^+ influxes (Kudo et al., 1998; Tachikawa et al., 1995). More directly, ginsenosides and a related metabolite were shown to inhibit acetylcholine- and 5-HT-induced inward currents in *Xenopus* oocytes expressing several subtypes of neuronal and αβδε muscle-type nicotinic acetylcholine receptors (Choi et al., 2002; Sala et al., 2002), or 5-HT_{3A} receptors (Choi et al., 2003; Lee et al., 2004). Furthermore, Lee et al. (2004) and Jeong et al. (2004) demonstrated that ginsenoside Rg_3 (Rg_3) (Fig. 1A), one of the active ginsenosides, non-competitively inhibits 5-HT_{3A} receptor channel activity by acting outside the cell. However, no previous works have specifically examined the underlying interaction site(s) responsible for Rg_3-mediated regulation of 5-HT_{3A} receptor channel activity.

Here, we sought to identify the possible interaction site(s) of Rg_3 with the 5-HT_{3A} receptor and further tried characterizations on Rg_3-induced 5-HT_{3A} receptor regulation, using

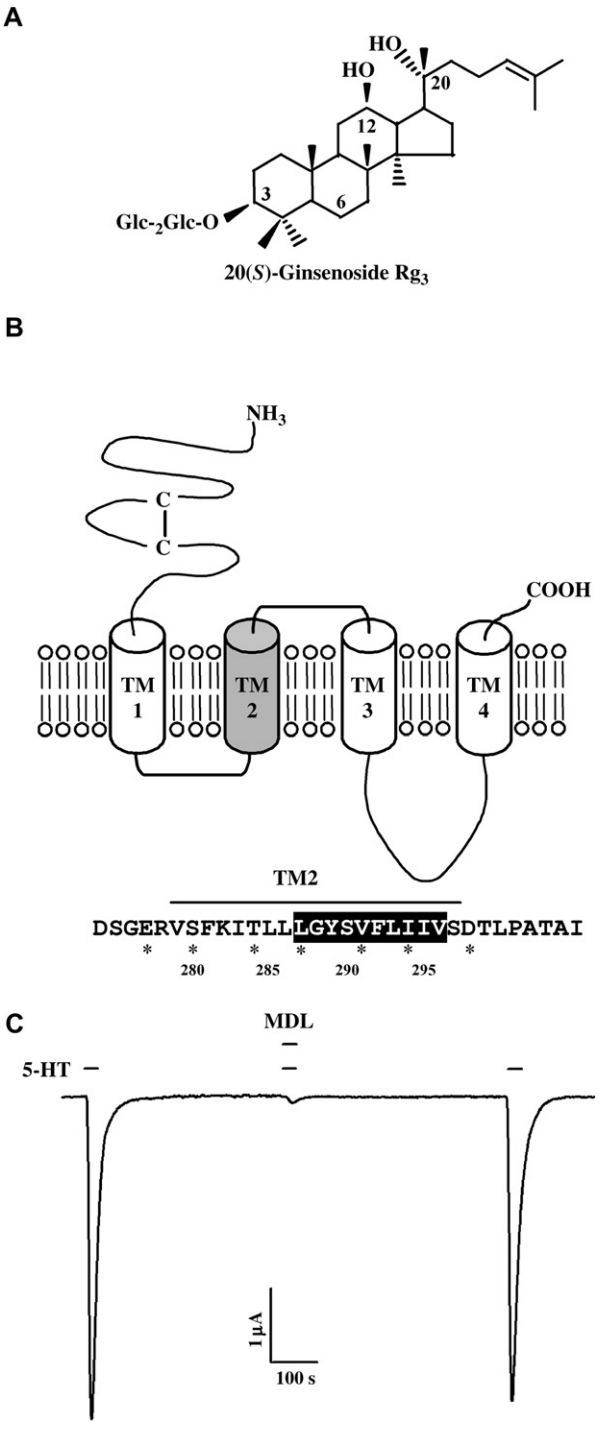

Fig. 1. Structure of 20(*S*)-ginsenoside Rg_3 (Rg_3), the primary amino acid sequence of the channel-lining regions (TM2) of the mouse 5-HT_{3A} receptor, and the effect of 5-HT and an antagonist on oocytes expressing the wild-type 5-HT_3 receptor. (A) Structure of 20(*S*)-ginsenoside Rg_3. Glc, glucopyranoside. Subscripts indicate the carbon in the glucose ring that links the two carbohydrates. (B) Partial amino acid sequence of the TM2 region of the cloned mouse 5-HT_{3A} receptor. The (*) corresponds to amino acid residues exposed to the lumen of the channel, as identified with the substituted cysteine accessibility method (SCAM) (Panicker et al., 2002; Reeves et al., 2001). The box indicates amino acid residues that were mutated in the present study. (C) In oocytes expressing wild-type 5-HT_{3A} receptors, 5-HT (1 μM) induced the expected inward current ($I_{5\text{-}HT}$). Treatment of these oocytes with MDL-72222 (MDL) (0.5 μM), a selective 5-HT_3 receptor antagonist, blocked $I_{5\text{-}HT}$.

a *Xenopus* oocyte gene expression system. This model system has few endogenous ion channels (Dascal, 1987) and allows heterologous expression of ion channels for biochemical study (Choi et al., 2002; Sala et al., 2002; Lee et al., 2004). We expressed wild-type or various TM2-mutant 5-HT_{3A} receptors (Fig. 1B, box) by intraoocyte cRNA injection, and examined the 5-HT-elicited ion currents ($I_{5\text{-HT}}$) in these oocytes in the absence and presence of Rg_3. In wild-type receptors, Rg_3 inhibited the peak $I_{5\text{-HT}}$ with dose-dependent manner. Oocytes expressing receptors with mutation V291A showed a decrease of rate of current decay in the presence of 5-HT compared with wild-type. Mutation V291A also showed constitutively active ion currents in the absence of 5-HT. Rg_3 treatment accelerated the rate of current decay with slight inhibition of peak $I_{5\text{-HT}}$ and inhibited the constitutively active ion currents with dose-dependent manner. Oocytes expressing F292A mutant receptors showed no Rg_3-induced inhibition of peak $I_{5\text{-HT}}$. Finally, oocytes expressing the I295A mutant receptor showed a large attenuation of Rg_3-induced inhibition of $I_{5\text{-HT}}$. Collectively, these results indicate that Rg_3 regulates 5-HT_{3A} receptor channel activity when the channel is in the open state, through interactions with residues V291, F292, and I295 of the gating region in TM2.

2. Materials and methods

2.1. Materials

The 20(*S*)-ginsenoside Rg_3 (Rg_3) was kindly provided by the Korea Ginseng Cooperation (Daejon, Korea); its chemical structure is shown in Fig. 1A. The mouse 5-HT_{3A} receptor cDNA was kindly provided by Dr. D. Julius (University of California, San Francisco, CA, USA) (Maricq et al., 1991). TMB-8 and the other agents were purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of Xenopus oocytes and microinjection

Xenopus laevis frogs were purchased from *Xenopus* I (Ann Arbor, MI, USA). Their care and handling were in accordance with the highest standards of institutional guidelines. For isolation of oocytes, frogs were anesthetized with an aerated solution of 3-amino benzoic acid ethyl ester. Oocytes were surgically removed and separated by collagenase treatment followed by agitation for 2 h in a Ca^{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/ml penicillin, and 100 μg/ml streptomycin. Stage V–VI oocytes were collected and stored 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/ml gentamicin. This oocyte-containing solution was maintained at 18 °C with continuous gentle shaking and changed daily. Electrophysiological experiments with oocytes were performed within 5–6 days of their isolation. Chemicals were bath-applied. One day after harvest, 40 nl of cRNAs were injected into the animal or vegetal pole of each oocyte using a 10 μl VWR microdispenser (VWR Scientific, San Francisco, CA, USA) fitted with a tapered glass pipette tip that was 15–20 μm in diameter (Choi et al., 2002).

2.3. Site-directed mutagenesis of 5-HT_{3A} receptors and in vitro transcription of 5-HT_{3A} receptor cDNAs

The substitution mutation of one or two amino acids was performed using Pfu DNA polymerase (QuickChange™ Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA) and mutated sense and antisense primers. Sequential polymerase chain reaction (PCR) was used for overlap extension at the target domain, as described in the provided manual. Final PCR products were transformed to *Escherichia coli* strain DH5α, and screened by PCR. Mutations were confirmed by DNA sequence analysis of the target region, and sequencing was used to confirm that there were no changes throughout the 5-HT_{3A} receptor-encoding sequence, including the 5′- and 3′-UTRs. The mutant DNA constructs were linearized at the 3′ end by *Sal*I digestion, and run-off transcripts were prepared using the methylated cap analog, $m^7G(5')ppp(5')G$. For generation of wild-type cRNAs, recombinant plasmids containing the wild-type 5-HT_{3A} receptor cDNA were linearized by digestion with appropriate restriction enzymes. All cRNAs were prepared using T3 RNA polymerase and the mMessage mMachine Transcription Kit (Ambion, Austin, TX, USA). The final cRNA products were resuspended at 1 μg/μl with RNase-free water and stored at −80 °C until use (Choi et al., 2002). The absence of degraded RNA was confirmed by denaturing agarose gel electrophoresis followed by ethidium bromide staining.

2.4. Data recording

A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings. The chamber was constructed by milling two concentric wells into the chamber bottom (diameter/height: upper well, 8/3 mm; lower well, 6/5 mm) and gluing plastic mesh (~0.4 mm grid diameter) onto the bottom of the upper well. The perfusion inlet (~1 mm in diameter) was formed through the wall of the lower well, and a suction tube was placed on the edge of the upper well. The oocyte was placed on the net that separated the upper and lower wells, with the net grids serving to keep the oocyte in place during the electrophysiological recordings. Oocytes were impaled with two microelectrodes filled with 3 M KCl (0.2–0.7 MΩ). Recordings were performed in ND96 solution. In experiments using mutant 5-HT_{3A} receptors, ND96 solution containing 300 μM niflumic acid, a Ca^{2+}-activated Cl^- channel blocker (White and Aylwin, 1990), was used to exclude any contributions of Cl^- currents. In the indicated experiments, Na^+-free ND96 was used and the Na^+ was replaced with the organic cation *N*-methyl-D-glucamine (NMDG) (96 mM), which was impermeant. The electrophysiological experiments were performed at room temperature using an Oocyte Clamp (OC-725C; Warner Instruments, Hamden, CT, USA) and stimulation and data acquisition were controlled by pClamp 8 (Axon Instruments, Union City, CA, USA) (Choi et al., 2002). We also measured ionic currents through the functionally expressed 5-HT_{3A} receptor using giga-ohm sealed membrane patch-clamp method in excised outside-out configuration with an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA). Pipettes were fire-polished with a microforge to give the pipette resistance of 3–6 MΩ for macropatch recording. The amplified analog data were filtered at 1 kHz with 80 dB/decade low-pass Bessel filter, and digitized with a Digidata 1200 (Axon Instruments, Union City, CA, USA). Membrane currents were recorded at a holding potential of −60 mV and the rate of solution exchange at the patch membrane was less than 1 s. Internal solution contained 125 mM CsOH, 5 mM CsCl, 10 mM EGTA, 10 mM glucose, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2 mM K_2-ATP, and 1 mM HEPES. The composition of external solution was 130 mM NaOH, 10 mM NaCl, 2.8 mM KOH, 10 mM HEPES, and 300 μM niflumic acid. The pH of all recording solutions was adjusted to 7.4 with 4-morpholineethanesulfonic acid (MES).

2.5. Data analysis

To obtain concentration–response curves of the effect of Rg_3 on $I_{5\text{-HT}}$, the peak amplitudes at different concentrations of Rg_3 were plotted and then fitted to the following Hill's equation using the Origin Software (OriginLab Corp., Northampton, MA, USA):

$$\text{Response} = E_{\max} - E_{\min}/1 + (\text{IC}_{50}/[A]^{n_H}) + E_{\min}$$

where $E_{\max}$ and $E_{\min}$ are maximal and minimal responses, respectively. $[A]$ is concentration of Rg_3 and n_H is the Hill's coefficient. IC_{50} is the concentration of Rg_3 required to decrease the response by 50%. All values are presented as means ± S.E.M. The differences between means of control and treatment data were determined using the unpaired Student's *t*-test or one-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of Rg_3 on $I_{5\text{-}HT}$ in oocytes expressing wild-type mouse 5-HT_{3A} receptors

We first used the two-electrode voltage-clamp method to examine the effect of Rg_3 on the 5-HT-stimulated inward current ($I_{5\text{-}HT}$) mediated by wild-type mouse 5-HT_{3A} receptors expressed in *Xenopus* oocytes. Addition of 1 μM 5-HT to the bath solution of oocytes maintained at a holding potential of −80 mV elicited inward currents. MDL-72222, a selective 5-HT_{3A} receptor antagonist, blocked $I_{5\text{-}HT}$ (Fig. 1C) indicating that the 5-HT_{3A} receptors were functionally expressed in this system (Maricq et al., 1991; Choi et al., 2002; Lee et al., 2004; Jeong et al., 2004). Addition of Rg_3 to this system inhibited the peak $I_{5\text{-}HT}$ (Fig. 2A) in a dose-dependent manner, blocking $I_{5\text{-}HT}$ by 0.8 ± 0.5, 6.0 ± 2.6, 17.3 ± 3.7, 45.6 ± 3.9, 65.8 ± 2.4, and 79.6 ± 3.6% at 1, 3, 10, 30, 100, and 300 μM, respectively (Fig. 2F). The IC_{50} for Rg_3 was 27.6 ± 4.3 μM, and the Hill's coefficient was 1.1 ± 0.2 (Fig. 2F and Table 1).

3.2. Rg_3-induced inhibition of peak $I_{5\text{-}HT}$ is abolished or attenuated in mutations V291S, V291I, F292A, F292L, F292S, and I295A in TM2

Previous studies showed that Rg_3 inhibits 5-HT_{3A} receptor channel activity in a non-competitive manner (Choi et al., 2002; Jeong et al., 2004). Based on this, we hypothesized that Rg_3 might affect $I_{5\text{-}HT}$ by interacting with as yet unidentified site(s) of the 5-HT_{3A} receptor protein. Since TM2 forms the pore region of 5-HT_{3A} receptors, and mutations in this region affect the inhibition of $I_{5\text{-}HT}$ in response to anesthetics, cadmium, and the open channel blocker, TMB-8 (Zhang et al., 2002; Hu et al., 2003; Panicker et al., 2004), we investigated the effect of Rg_3 on $I_{5\text{-}HT}$ in oocytes expressing 5-HT_{3A} receptors containing site-directed mutations in TM2 residues ranging from L287 to V296 (Fig. 1B, box). The constructed mutants included replacement of residues L287, 288, Y289, S290, V291, L293, I294, I295, and V296 with alanine (generating L287A, G288A, Y289A, S290A, V291A, L293A, I294A, I295A, and V296A, respectively), replacing residue V291 with leucine (V291L), serine (V291S) or isoleucine (V291I), or replacing residue F292 with alanine (F292A), leucine (F292L), serine (F292S) or threonine (F292T). Among these mutants, oocytes expressing G288A, Y289A and F292T failed to show $I_{5\text{-}HT}$ following 5-HT stimulation.

We then examined the effects of Rg_3 on these currents. Fig. 2 shows representative traces of peak $I_{5\text{-}HT}$ in the absence or presence of Rg_3 in oocytes expressing the S290A, F292A, L293A, and I295A mutated receptors. At a holding potential of −80 mV, the inhibitory effect of Rg_3 on peak $I_{5\text{-}HT}$ was greatly attenuated or abolished in oocytes expressing the mutant versus wild-type receptors, with the blockade of Rg_3-induced inhibition decreasing in the order of F292A > V291A > I295A > S290A > L293A (Fig. 2F and Table 1). Representative concentration–response relationships for peak $I_{5\text{-}HT}$ inhibition by Rg_3 in five different mutant receptors were fitted using the Hill's equation (Fig. 2F, smooth lines). The Hill's coefficients differed substantially from the wild-type values in oocytes expressing V291A, I295A, and V296A, while the V_{max} values differed significantly from wild-type in oocytes expressing V291A, V291I, V291S, I295A, and V296A ($^*p < 0.05$, $^{**}p < 0.005$; Table 1). Most of these mutants including V291A, V291I, V291S, F292L, F292S, I294A, and I295A had significantly higher IC_{50} values than the wild-type channels ($^*p < 0.05$, $^{**}p < 0.005$; Table 1).

Since mutations V291A, and F292A attenuated or abolished Rg_3-induced inhibition of peak $I_{5\text{-}HT}$, we further substituted V291 or F292 with other amino acid residues, including leucine, serine, and isoleucine (generating V291L, V291S, V291I, F292L, and F292S), and examined the effect of Rg_3 on $I_{5\text{-}HT}$ in oocytes expressing these mutants. Our results revealed that mutation V291S almost completely abolished Rg_3-induced inhibition of $I_{5\text{-}HT}$ (Fig. 3A) and mutation V291I significantly attenuated Rg_3-induced inhibition of $I_{5\text{-}HT}$ in terms of the V_{max} and significantly increased the IC_{50} (Table 1), while mutation V291L had no effect on Rg_3-induced inhibition of $I_{5\text{-}HT}$. Mutations F292L, and F292S significantly attenuated Rg_3-induced inhibition of $I_{5\text{-}HT}$ and increased the IC_{50} values as compared to wild-type, but these effects were not as potent as those seen in oocytes expressing F292A (Fig. 3 and Table 1). These results indicate that substitutions of V291 or F292 with amino acid residues such as alanine, isoleucine, leucine, or serine alleviated the Rg_3-induced inhibition of $I_{5\text{-}HT}$ but it is unlikely that the volume of the substituted amino acids is associated with alleviation the Rg_3-induced inhibition of $I_{5\text{-}HT}$.

3.3. V291A mutant receptors slow the rate of current decay, whereas Rg_3 accelerates the rate of current decay of V291A mutant receptors

Interestingly, treatment of oocytes expressing V291A mutant receptors with 1 μM 5-HT slowed the rate of desensitization in $I_{5\text{-}HT}$, as compared to oocytes expressing the wild-type receptors. The half-time of current recovery ($T_{1/2}$) was 13.6 ± 0.7 and 90.9 ± 2.1 s for wild-type and V291A mutant, respectively ($^*p < 0.001$, compared with wild-type; means ± S.E.M; $n = 10$–12 oocytes from three different frogs) (Fig. 4A). Next, we examined the effect of Rg_3 on V291A mutant receptor channel activity in the presence of 5-HT. Although Rg_3 inhibited the peak $I_{5\text{-}HT}$ slightly even at high concentrations (16.1 ± 3.8% inhibition of peak $I_{5\text{-}HT}$ at 300 μM Rg_3) (Fig. 4D), the presence of Rg_3 significantly and concentration-dependently accelerated the rate of current decay (Fig. 4C and E). Thus, treatment of 300 μM Rg_3 accelerated $T_{1/2}$ by 73% in oocytes expressing V291A mutant receptors ($^*p < 0.05$, means ± S.E.M; $n = 10$–12 oocytes from three different frogs). These results are consistent with the notion that the V291 residue is involved in channel gating activity (Reeves et al., 2001; Panicker et al., 2002, 2004), and further show that Rg_3 could regulate channel gating activity of 5-HT_{3A}. Similarly, Hargreaves et al. (1994) and Gunthorpe and Lummis (1999) also demonstrated that diltiazem and other

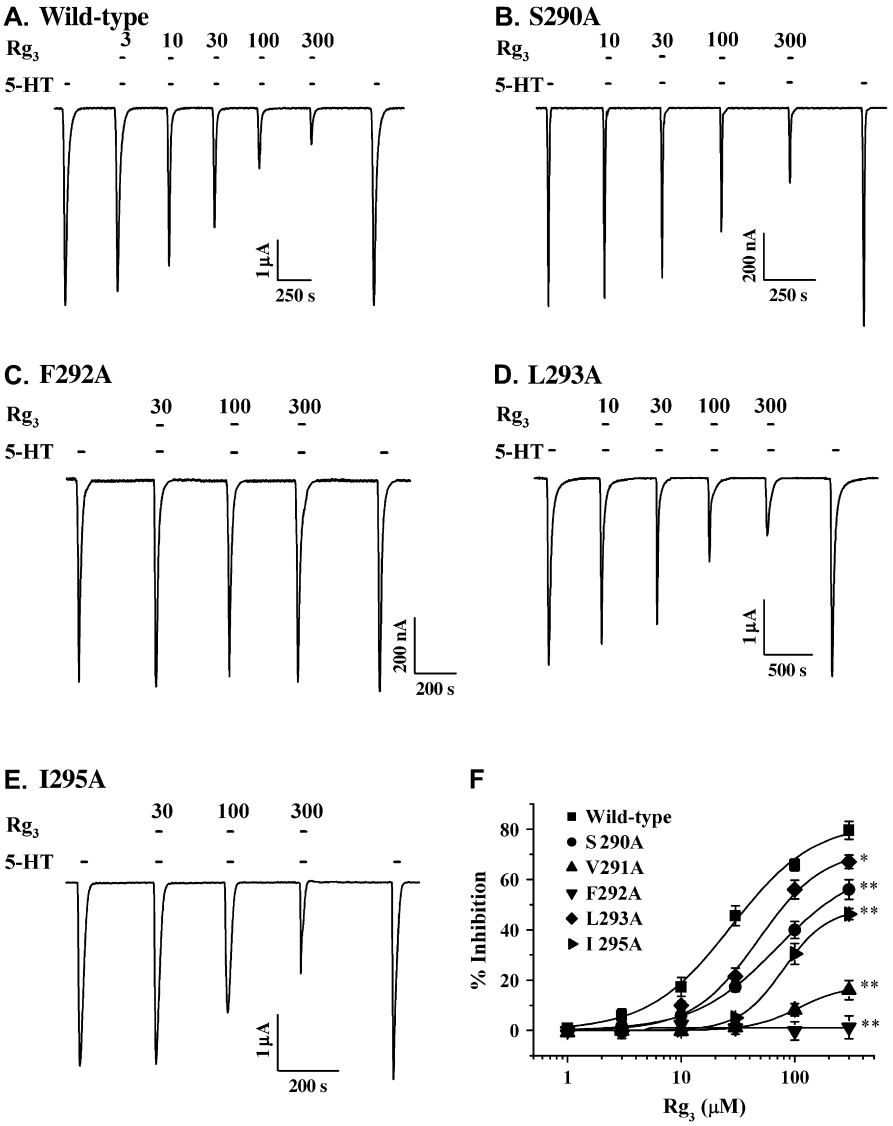

Fig. 2. Concentration-dependent effects of Rg_3 on $I_{5\text{-HT}}$ in wild-type and various mutant receptors. $I_{5\text{-HT}}$ in oocytes expressing wild-type, S290A, F292A, L293A, and I295A mutant receptors was elicited at a holding potential of −80 mV for the indicated time in the presence of 1 μM 5-HT, and then the indicated concentrations of Rg_3 were co-applied with 5-HT. (A–E) Traces are representative of nine separate oocytes from three different frogs. (F) Concentration–response curves for the effect of Rg_3 on oocytes expressing the wild-type and various mutant receptors. The solid lines were fit by the Hill's equation. $^*p < 0.05$, $^{**}p < 0.005$, compared with Rg_3-treated oocytes expressing wild-type receptors. Additional IC_{50}, Hill's coefficient, and V_{max} values for the various mutants are presented in Table 1 (means ± S.E.M; $n = 9$–12 oocytes for each point).

L-type Ca^{2+} channel antagonists inhibited the open state of receptors by increasing the receptor current decay without affecting peak $I_{5\text{-HT}}$.

3.4. Rg_3 inhibits constitutively active ion currents in mutation V291A

In addition to the changes in 5-HT_{3A} receptor channel activity in V291A mutant receptor compared to wild-type, we also found that mutation V291A induced constitutively active ion currents even in the absence of 5-HT. The average amplitude of these constitutively active ion currents was −1755 ± 179 nA ($n = 15$ from five different frogs) (Fig. 4B, *left panel*). To further determine the nature of the constitutive ion currents in oocytes expressing the V291A mutant receptor, we replaced the extracellular Na^+ with *N*-methyl-D-glucamine (NMDG), which is impermeant organic cation, and examined current flow. The replacement of extracellular Na^+ with NMDG abolished the constitutively active ion currents mediated by the V291A mutant receptor, indicating that the constitutively active ion currents observed in V291A mutant receptor are mainly cation currents traveling through the constitutively open channels (Fig. 4B, *right panel*). Since treatment of oocytes expressing V291 mutant channels with 100 μM Rg_3 alone inhibited the constitutively active ion currents (Fig. 4B, *left panel arrow*), we next examined the effects of Rg_3 and TMB-8 on the constitutively active V291A-mediated ion currents (Fig. 4F). Our results revealed

Table 1
Effect of Rg_3 on wild-type and various mutant 5-HT_{3A} receptors expressed in *Xenopus* oocytes

	IC_{50}	V_{max}	n_H
Wild-type	27.6 ± 4.3	83.7 ± 5.6	1.1 ± 0.2
L287A	40.3 ± 1.9	87.6 ± 1.8	1.1 ± 0.1
G288A	NC	NC	NC
Y289A	NC	NC	NC
S290A	70.3 ± 2.7*	66.0 ± 1.2	1.2 ± 0.1
V291A	112.8 ± 9.5*	19.0 ± 1.1*	1.8 ± 0.2*
V291L	36.1 ± 0.7	84.2 ± 0.7	1.1 ± 0.1
V291S	201.8 ± 50.5*	26.0 ± 3.7**	0.8 ± 0.1
V291I	70.5 ± 13.7*	56.8 ± 5.1*	1.2 ± 0.2
F292A	ND	ND	ND
F292L	92.5 ± 44.3*	86.0 ± 17.8	0.9 ± 0.2
F292S	124.4 ± 86.4*	81.0 ± 28.9	0.8 ± 0.2
L293A	47.8 ± 9.5	71.8 ± 6.4	1.5 ± 0.4
I294A	103.4 ± 55.1*	93.6 ± 23.4	1.0 ± 0.3
I295A	79.4 ± 0.7**	48.6 ± 0.3**	2.2 ± 0.1*
V296A	40.8 ± 1.5	62.9 ± 1.0*	2.7 ± 0.2*
V291A[a]	72.4 ± 23.1	40.6 ± 4.7	0.8 ± 0.1

Values represent the means ± S.E.M. ($n = 9–12$/group). Currents were elicited at a holding potential of −80 mV. IC_{50} (μM), V_{max}, and Hill's coefficient values were determined as described in Section 2.
NC, no current; ND, not determined.
*$p < 0.05$, **$p < 0.005$ compared with wild-type 5-HT_{3A} receptors.
[a] IC_{50} (μM), V_{max}, and Hill's coefficient values of the constitutively active ion currents in oocytes expressing V291A were determined in the absence of 5-HT.

that Rg_3 dose-dependently inhibited the constitutively active ion currents by 0 ± 0, 1.3 ± 0.3, 7.1 ± 3.7, 12.9 ± 1.7, 22.3 ± 3.7, and 30.9 ± 4.0% at 1, 3, 10, 30, 100, and 300 μM, respectively. Similarly, TMB-8 dose-dependently inhibited the constitutively active ion currents by 0.7 ± 0.3, 4.8 ± 1.2, 13.6 ± 1.1, 30.2 ± 2.3, 54.5 ± 3.3, 80.2 ± 4.9, 88.1 ± 3.6, and 91.4 ± 2.9% at 0.1, 0.3, 1, 3, 10, 30, 100, and 300 μM, respectively. The IC_{50} values of Rg_3 and TMB-8 were 72.4 ± 23.1 and 6.5 ± 0.7 μM, respectively. The IC_{50} value for Rg_3 was about 10-fold larger than that of TMB-8 in the inhibition of constitutively active ion currents mediated by V291A mutant receptors.

3.5. Effects of Rg_3 on wild-type and V291A mutant receptors in excised outside-out configuration

We further performed giga-ohm sealed membrane patch-clamp experiments in excised outside-out configuration using oocytes expressing wild-type and V291A mutant receptors. As shown in Fig. 5A, treatment of 5-HT (10 μM) to wild-type receptors induced rapid activation and desensitization in the continued presence of agonist, whereas in V291 mutant receptors treatment of 5-HT delayed the rate of current decay even in the presence of agonist. The half-time of current recovery ($T_{1/2}$) was 2.9 ± 0.4 and 58.9 ± 3.1 s for wild-type and V291A mutant, respectively (*$p < 0.001$, compared with wild-type; means ± S.E.M; $n = 5$ oocytes from three different frogs). Treatment of 100 μM Rg_3 inhibited peak $I_{5\text{-HT}}$ by 63.7 ± 5.7% in wild-type receptors (Fig. 5B and D, $n = 5$), which was also similar with results obtained from whole cell configurations (Fig. 5D). We did further test the effect of Rg_3 in V291A mutant receptors in excised outside-out configuration. Treatment of Rg_3 (100 μM) to V291A mutant receptors slightly inhibited on peak $I_{5\text{-HT}}$ by 10.9 ± 4.6% (Fig. 5D, *$p < 0.01$, compared with wild-type) but the presence of Rg_3 accelerated decay rate of currents as shown in whole cell configurations (Fig. 4C). Again, these results support data obtained from whole cell configurations and provide further evidences that Rg_3 regulates 5-HT_{3A} receptor channel activity through interaction with channel gating region.

3.6. Diltiazem and TMB-8 do not prevent Rg_3-induced inhibition of constitutively active ion currents in mutation V291A

We performed occlusion experiments using diltiazem and TMB-8 to determine whether Rg_3 shares a common binding site or pathway with diltiazem or TMB-8 in the inhibition of constitutively active ion currents (Gunthorpe and Lummis, 1999). As shown in Fig. 5, single application of Rg_3 (100 μM), diltiazem (Dil) (100 μM), and TMB-8 (3 μM)

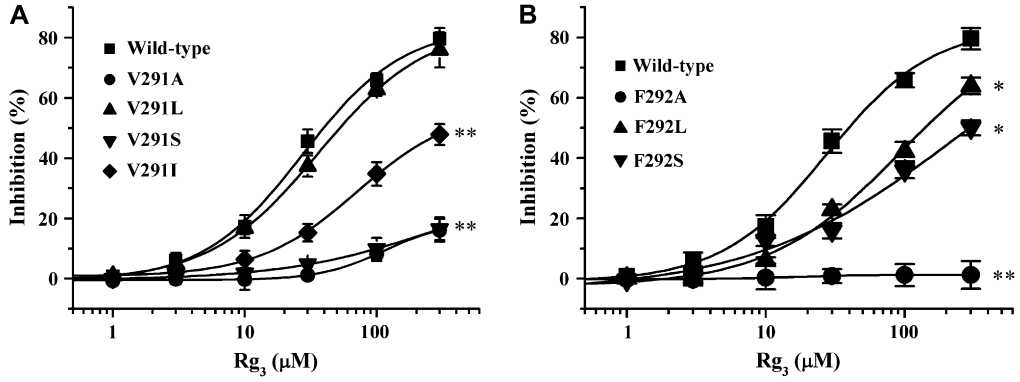

Fig. 3. Concentration-dependent effects of Rg_3 on $I_{5\text{-HT}}$ in additional mutants of V291 and F292. (A, and B) $I_{5\text{-HT}}$ was elicited in oocytes expressing wild-type or mutant receptors at a holding potential of −80 mV, and experiments were performed as described in Fig. 2. Concentration–response curves were generated for the effect of Rg_3 on the wild-type and mutant receptors. The solid lines were fit by the Hill's equation. *$p < 0.05$, **$p < 0.005$, compared with Rg_3-treated oocytes expressing the wild-type receptor. Additional IC_{50}, Hill's coefficient, and V_{max} values for the various mutants are presented in Table 1 (means ± S.E.M; $n = 9–12$ oocytes for each point).

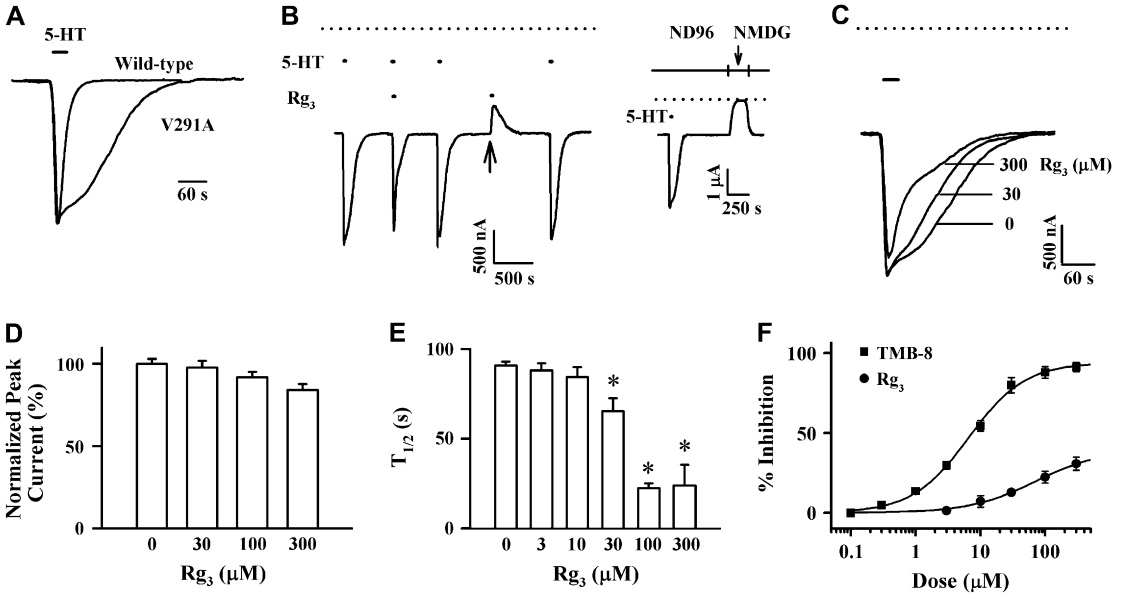

Fig. 4. Rg_3 accelerate the rate of current decay rather than peak $I_{5\text{-HT}}$ inhibition in V291A mutant receptor. (A) $I_{5\text{-HT}}$ in oocytes expressing wild-type or V291A mutant receptors was elicited at a holding potential of −80 mV for the indicated time in the presence of 1 μM 5-HT, and current decay was assessed. For comparison between the wild-type, and V291A mutant receptors, the traces were superposed after normalization. 5-HT treatment of oocytes expressing V291A mutant receptors decreased the rate of current decay and prolonged the channel opening compared to wild-type. The half-time of current recovery ($T_{1/2}$), determined as the time taken for the current to decay to half of its peak value in the absence of Rg_3, was 13.6 ± 0.7 and 90.9 ± 2.1 s for currents in wild-type and V291A channels, respectively (*$p < 0.001$, compared with wild-type; means ± S.E.M; $n = 10–12$ oocytes from three different frogs). (B) *Left panel*: the representative trace shows that mutation V291A induced constitutively active ion currents. In oocytes expressing this mutant, 5-HT treatment decreased the rate of receptor desensitization, but co-treatment with 5-HT and 100 μM Rg_3 accelerated the rate of current decay without significantly inhibiting peak $I_{5\text{-HT}}$. Rg_3 treatment alone for 30 s also inhibited the constitutively active ion currents in this oocyte (arrow). *Right panel*: the replacement of Na^+ with NMDG abolished the constitutively active ion currents in the presence of NFA. (C) Representative traces showing that co-treatment of oocytes expressing V291A mutant receptors with Rg_3 and 5-HT concentration-dependently accelerated the rate of current decay. For comparison, traces generated in the absence of Rg_3 (0) or presence of 30 or 300 μM Rg_3 and 1 μM 5-HT (bar) were superposed after normalization. The dotted line indicates the basal level. (D) Histograms show the concentration-dependent effects of Rg_3 on peak $I_{5\text{-HT}}$ (means ± S.E.M; $n = 10–12$ oocytes each). (E) Histograms showing that Rg_3 concentration-dependently accelerated the rate of current decay ($T_{1/2}$) (*$p < 0.05$, compared with Rg_3-free control; means ± S.E.M; $n = 10–12$ oocytes each). (F) Concentration–response curves for the effect of Rg_3 and TMB-8 on V291A mutant receptors (means ± S.E.M; $n = 12–15$ oocytes each).

inhibited the constitutively active currents by 31.4 ± 2.8, 39.3 ± 2.0, and $52.3 \pm 2.0\%$, respectively. Co-treatment of Rg_3 with diltiazem produced an additive inhibition of the constitutively active currents by $59.6 \pm 1.9\%$, whereas co-treatment of Rg_3 with or TMB-8 produced an additive inhibition of the constitutively active currents by $73.8 \pm 2.1\%$ (Fig. 6A and B). To further investigate how Rg_3 inhibits $I_{5\text{-HT}}$, we performed competition experiments with diltiazem. To simplify the comparison between the suppressive effects of Rg_3, diltiazem and of their mixture on $I_{5\text{-HT}}$, we assumed that if Rg_3 and diltiazem act by same interaction sites, the inhibitory effects produced by the mixture of Rg_3 and diltiazem should be less than the sum of their individual effects. On the contrary, if Rg_3 and diltiazem act by different interaction sites, the inhibitory effects produced by the mixture of Rg_3 and diltiazem should be equal or close to the sum of their individual effects. As shown in Fig. 6C, in series of experiments, 100 μM diltiazem and 30 μM Rg_3 inhibited $I_{5\text{-HT}}$ by $19.0 \pm 2.0\%$ and by $6.0 \pm 0.6\%$, respectively ($n = 7–9$). When 30 μM Rg_3, and 100 μM diltiazem were co-applied, $I_{5\text{-HT}}$ was suppressed by $23.3 \pm 1.3\%$ ($n = 7–9$), which was slightly smaller than $25.0 \pm 2.9\%$, the sum of their individual effects. Similar results were observed with other concentrations of Rg_3 and diltiazem. Rg_3 alone (100 μM) inhibited $I_{5\text{-HT}}$ by $16.5 \pm 0.8\%$. When 100 μM Rg_3, and 100 μM diltiazem were co-applied, $I_{5\text{-HT}}$ was inhibited by $32.3 \pm 3.3\%$ ($n = 6–7$), which was slightly smaller than $35.4 \pm 2.8\%$, the sum of their individual effects. Rg_3 alone (300 μM) suppressed $I_{5\text{-HT}}$ by $22.1 \pm 1.5\%$. When 300 μM Rg_3 and 100 μM diltiazem were co-applied, they suppressed $I_{5\text{-HT}}$ by $40.1 \pm 1.8\%$ ($n = 7$). This value was not much different from the sum of their individual effects, $41.0 \pm 2.8\%$. Altogether, these results suggest that Rg_3 regulates the constitutively active currents of V291A mutant receptor by acting on different site(s) from that of diltiazem.

3.7. Current–voltage relationships in wild-type and mutant 5-HT_{3A} receptors

To determine whether the V291A, and F292A mutations affected ion permeation through the receptor channel, we compared the mutant and wild-type current–voltage ($I–V$) relationships. We measured the amplitude of current activated by either 5-HT alone or 5-HT plus Rg_3 at holding potentials beginning at −80 mV and then ramping from −100 to +60 mV over a duration of 300 ms. In oocytes expressing wild-type receptors, untreated with 5-HT, the inward current

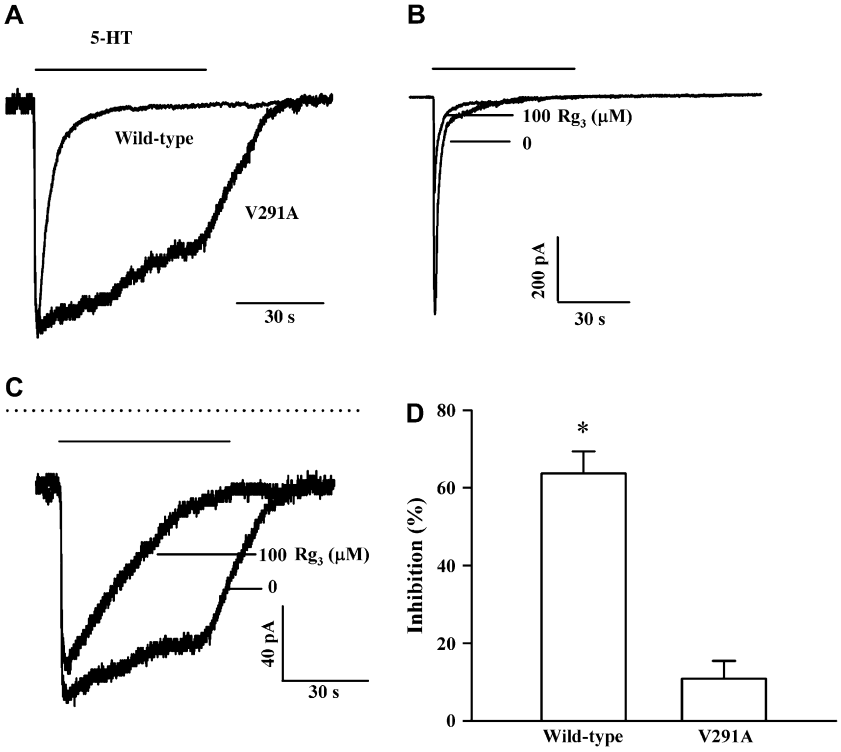

Fig. 5. Effects of Rg_3 on wild-type and V291A mutant receptors in excised outside-out configuration. (A) The representative traces on outside-out configuration of membrane patch in wild-type and V291A mutant. $I_{5\text{-HT}}$ in oocytes expressing wild-type or V291A mutant receptors was elicited at a holding potential of −60 mV for the indicated time in the presence of 10 μM 5-HT. For comparison between the wild-type and V291A mutant receptors, the representative traces from five different patches were superposed after normalization. Treatment of 5-HT to membrane patch expressing V291A mutant receptors decreased the rate of current decay in the continued presence of 5-HT compared to wild-type. (B) The representative traces on outside-out configuration of membrane patch show that treatment of Rg_3 to wild-type receptor inhibited peak $I_{5\text{-HT}}$. (C) Representative traces show that treatment of Rg_3 to membrane patch expressing V291A mutant receptors accelerated the rate of current decay. For comparison, traces generated in the absence of Rg_3 (0) or presence of 100 μM Rg_3 and 10 μM 5-HT (bar) were superposed after normalization. The dotted line indicates the basal level. (D) The histograms show the percent blockade of the peak $I_{5\text{-HT}}$ by 100 μM Rg_3 in wild-type and V291A mutant receptors. (*$p < 0.01$, compared with V291A mutant; means ± S.E.M; $n = 5$).

at −100 mV was <0.01 mA and the outward current at +60 mV was near 0.1 mA. Under these conditions, oocytes expressing V291A mutant channels showed significant increases in both the inward current at −100 mV and the outward current at +60 mV (Fig. 7). In oocytes expressing wild-type receptors treated with 5-HT, the current evoked by 5-HT reversed at about −5 mV, and as previously reported, the inward and outward currents were both inhibited by Rg_3 (Jeong et al., 2004). Oocytes expressing the V291A or F292A mutant channels showed no significant differences from wild-type with regards to the reversal potential for 5-HT in the absence or presence of Rg_3 (wild-type, -6.40 ± 0.6 mV; V291, -6.2 ± 0.7 mV; F292A, -5.0 ± 0.5 mV; $p < 0.5$) or the shape of the $I-V$ relationship. Co-treatment of oocytes expressing the V291A mutant channels with Rg_3 and 5-HT triggered a slight inhibition of both the inward and outward currents. In contrast, oocytes expressing the F292A mutant receptors showed no such effect (Fig. 7).

4. Discussion

Ginsenosides, the active components of *P. ginseng*, exhibit diverse effects on the central and peripheral nervous systems. Although the functional mechanisms of ginsenoside-based actions in the nervous system have not been fully elucidated, accumulating evidence suggests that these molecules may target ion channels involved in neuronal excitability (Jeong and Nah, 2005). Indeed, ginsenosides have been shown to affect several ion channels found at pre- and post-synaptic sites in the nervous system (Choi et al., 2002; Sala et al., 2002; Lee et al., 2004, 2005; Nah, 1997; Kim et al., 1998, 2002), and their effects are closely coupled to inhibition of neurotransmitter release (Kudo et al., 1998; Tachikawa et al., 1995). We recently demonstrated that Rg_3 non-competitively inhibits 5-HT_{3A} receptor-mediated ion currents, and that the inhibitory effect of Rg_3 on $I_{5\text{-HT}}$ is observed when it is applied extracellularly but not intracellularly (Lee et al., 2004). However, although these findings seem to indicate that Rg_3 probably interacts on the extracellular side of the receptor, very little is characterized on the Rg_3-induced 5-HT_{3A} receptor channel modulation at molecular levels.

In the present study, we utilized site-directed mutagenesis to identify possible interaction sites of Rg_3 with 5-HT_{3A} receptors. This well-known method allowed us to examine ligand-ion channel protein interaction site(s) by introducing specific site-directed point mutations of single amino acids into the wild-type ion channel protein. We then screened these mutants for losses or significant reductions of the expected action of the ligand on the ion currents. We generated 16 different

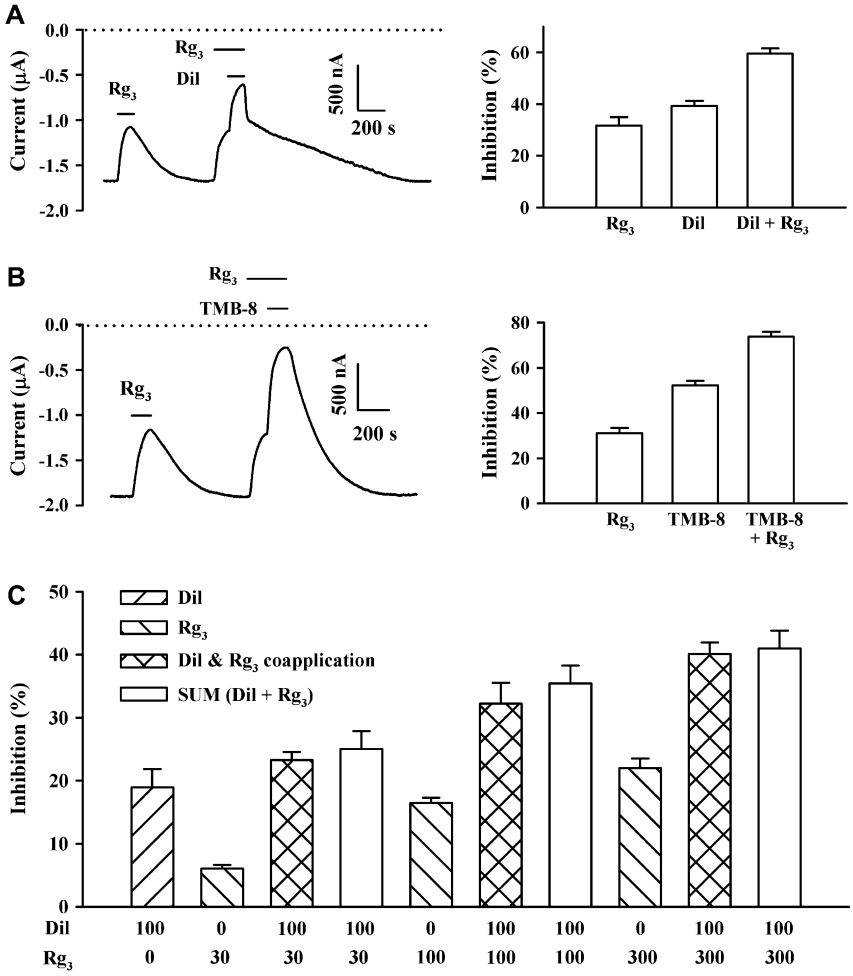

Fig. 6. Diltiazem and TMB-8 do not interfere with the action of Rg_3 in mutation V291A. (A) Rg_3 (100 μM) was first applied during the period indicated by the solid bars, and then diltiazem (100 μM) (Dil) was applied in the presence of Rg_3 as indicated by solid bars. The histograms show the percent blockade of the constitutively active ion currents by Rg_3, diltiazem, or Rg_3 + diltiazem (Dil). Data represent the means ± S.E.M. ($n = 8–10$/group). (B) Experiments were performed as above with TMB-8 (3 μM) used in place of diltiazem. Traces (A and B) are representatives of six separate oocytes from three different frogs. Data represent the means ± S.E.M. ($n = 10–12$/group). Oocytes were holding at −80 mV. (C) Competition experiments between Rg_3 and diltiazem (Dil). The suppressive effect of Rg_3 in indicated concentrations on $I_{5\text{-HT}}$ was not diminished by indicated concentration diltiazem. Rg_3 or/and diltiazem were treated for 30 s. The effects of co-applied Rg_3 with diltiazem on $I_{5\text{-HT}}$ were not much different from the sum of the suppressive effects when they were applied individually. Data represent the means ± S.E.M. ($n = 6–9$/group).

5-HT_{3A} receptor mutants spanning amino acids L287–V296, which are thought to line the pore region of TM2 (Table 1). Notably, we found that mutations at residues V291, F292, and I295 abolished or attenuated the inhibitory effect of Rg_3 on peak $I_{5\text{-HT}}$, suggesting that these residues likely form the main interaction sites for Rg_3 with the channel protein.

The ligand-gated ion channels, including the 5-HT_{3A} receptors, exist in three interconvertible states in channel gating activity: resting, open, and desensitized. Binding of 5-HT activates the receptor, induces a conformational change to the open state, and then enters the desensitized state (Lummis, 2004). Reeves et al. (2001) and Panicker et al. (2002, 2004) previously demonstrated that in the 5-HT_{3A} receptors, the amino acid sequence spanning D274–D298 in TM2 (which comprises the putative pore-lining face) include several amino acid residues that appear to be involved in channel gating activity (S290, V291, F292, L293, I295, and V296) (Fig. 1B). We found that mutations in V291, F292, and I295 but not L287, S290, L293, I294, and V296 abrogated or abolished the inhibitory effect of Rg_3 on $I_{5\text{-HT}}$. These findings provide evidence that Rg_3 might interact with specific amino acid residues in the channel gating pore region of the 5-HT_{3A} receptors. Thus, the V291, F292, and I295 residues in TM2 might play a key role in providing a favorable environment for Rg_3 to exhibit the inhibitory effect on 5-HT_{3A} receptor-mediated ion currents.

The previous findings suggest that residue V291 of the 5-HT_{3A} receptors is one of residues that might be directly involved in current decay, constitutive activation, and ion selectivity (Reeves et al., 2001; Panicker et al., 2002). Supporting this notion, we showed that V291A mutant receptor exhibits changes in 5-HT_{3A} receptor channel activity by slowing the rate of receptor desensitization. In the present study, we further showed that Rg_3 affects the channel gating activity

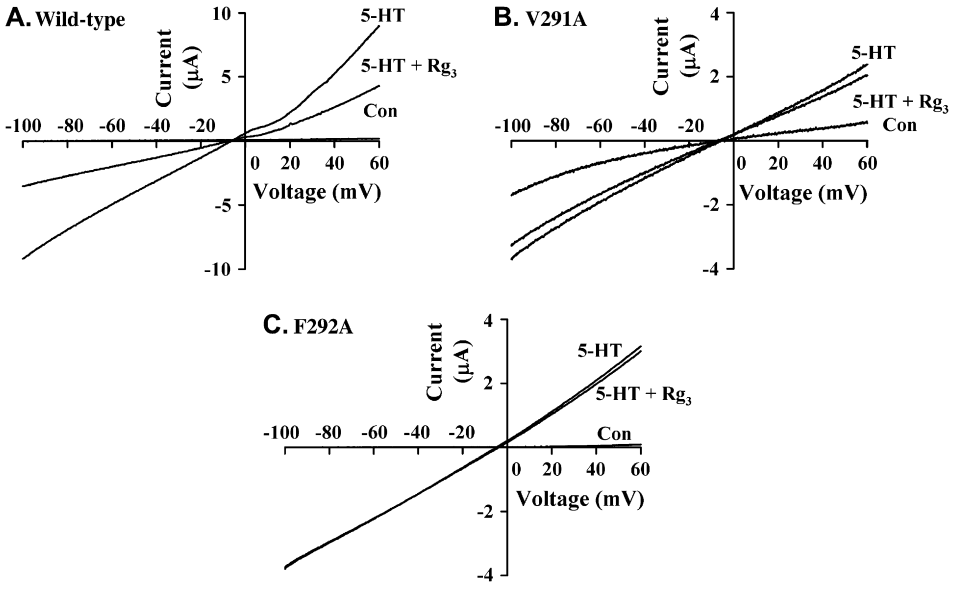

Fig. 7. Current–voltage relationships of $I_{5\text{-HT}}$ blockade by Rg_3 in wild-type V291A, and F292A mutant receptors. The representative current–voltage relationships were obtained using voltage ramps from −100 and +60 mV for 300 ms durations at a holding potential of −80 mV. Voltage steps were applied before and after application of 1 μM 5-HT in the absence or presence of 100 μM Rg_3. The reversal potential for the V291A, and F292A mutant receptors was not significantly different from that of the wild-type receptor in the absence or presence of Rg_3 (wild-type, -6.4 ± 0.6 mV; V291, -6.2 ± 0.7 mV; F292A, -5.0 ± 0.5 mV).

of V291A mutant receptor by accelerating the rate of current decay with a slight inhibition of peak $I_{5\text{-HT}}$ (Figs. 4 and 5). In addition to 5-HT_{3A} receptor, we had also investigated the effect of Rg_3 on rat brain Na^+ channel activation and inactivation process. In that study, we have shown that Rg_3 inhibits Na^+ currents with voltage-dependent manner and that Rg_3 treatment causes a depolarizing shift in the activation voltage compared with control oocytes but does not alter the steady-state inactivation voltage. However, single amino acid substitution (K859–K859Q) in the S4 voltage-sensor segment of domain II of Na^+ channels abolished the Rg_3-induced voltage shift of Na^+ channel activation (Lee et al., 2005). Thus, the previous and present results show that Rg_3 treatment might induce changes in channel gating activity of 5-HT_{3A} receptors as well as voltage-dependent Na^+ channels through interactions with specific amino acid residue of 5-HT_{3A} receptor and Na^+ channel, respectively.

We have shown that Rg_3 inhibits 5-HT_{3A} receptors (Jeong and Nah, 2005). However, we did not reveal which state of 5-HT_{3A} receptor channels are affected by Rg_3. Using V291A mutant receptor, we were also able to show that Rg_3 interacts with the open state of the receptor channel. Thus, we found that Rg_3 inhibited constitutively active currents of V291A mutant receptor, of which currents were also abolished by replacement of permeant Na^+ in the extracellular solution with impermeant NMDG (Fig. 4B, *right panel*). These results indicate that Rg_3 inhibits mainly inward cation currents through open channels. Reeves et al. (2001) and Panicker et al. (2002) reported that MTSES modification of V291C also produced constitutively active ion currents, and that these currents could be inhibited by diltiazem (Fig. 6). In previous study, we also demonstrated that Rg_3 inhibits open Na^+ channels. Thus, Rg_3 inhibited Na^+ currents with use-dependent manner in wild-type and inactivation gate deficient mutant (IFMQ3) Na^+ channels. The inhibitory effect of Rg_3 on Na^+ current was also not dependent on membrane holding potentials (Lee et al., 2005). These present findings in conjunction with the previous work strongly suggest that Rg_3 specifically regulates 5-HT_{3A} receptor channel activity in the open state through an interaction with TM2. But Rg_3 acts on different site(s) from those of diltiazem and TMB-8, since diltiazem and TMB-8 do not prevent Rg_3-induced inhibition of constitutively active ion currents of the V291A mutant receptor (Fig. 6).

As regards open channel blockers, it is notable that, unlike diltiazem or TMB-8, Rg_3 is not a charged agent (Fig. 1A). The present results and previous study (Jeong et al., 2004) indicate that Rg_3-induced blockades are not strongly voltage-dependent. In contrast, the charged open channel blockers usually show strong voltage dependency, since the narrow constriction of the channel gate is located within the transmembrane electric field (Buisson and Bertrand, 1998). Therefore, it could not be excluded that the uncharged Rg_3 might bind or interact outside the 5-HT_{3A} receptor channel and might inhibit $I_{5\text{-HT}}$ in the open state of 5-HT_{3A} receptor via allosteric mechanisms. In this case, the mutations introduced within the channel gating pore conformational changes that affect the binding or interaction of Rg_3 with channel proteins, thus attenuating or abolishing Rg_3-induced $I_{5\text{-HT}}$ inhibition. Further studies will be necessary to determine whether Rg_3 could be a novel candidate of uncharged open channel blocker.

Since the 5-HT_3 receptors are closely associated with the nociceptive processes of visceral pain in both humans and animals and with anticancer agent-inducednausea and vomiting (Camilleri et al., 2000; Polati et al., 1997), it seems possible that Rg_3-mediated regulation of 5-HT_{3A} receptors might be

therapeutically relevant. Although we currently do not have direct evidence that Rg_3 could be used as therapeutic agent for alleviation of 5-HT_{3A} receptor-related clinical symptoms such as vomiting and visceral pain, previous reports have shown that ginsenosides can inhibit 5-HT_{3A} receptor-mediated ion currents (Min et al., 2003), attenuate cisplatin-induced nausea and vomiting behavior in ferrets, and mitigated acetic acid-induced visceral hypersensitivity in rats (Kim et al., 2005a; Kim et al., 2005b). These findings, in conjunction with the present results, suggest that ginsenosides- and Rg_3-mediated $I_{5\text{-}HT}$ inhibition could be one of the mechanisms underlying the alleviation of 5-HT_{3A} receptor-mediated clinical symptoms in vivo.

In summary, we herein used site-directed mutagenesis to characterize Rg_3-induced regulation of 5-HT_{3A} receptor channel activity. We have obtained evidences that amino acid residues V291, F292, and I295 in the TM2 region mutation are involved in Rg_3-mediated inhibition of 5-HT_{3A} receptor currents. We have further obtained evidences using V291A mutant receptor that Rg_3 regulates 5-HT_{3A} receptor channel gating activity in the open state. These novel findings provide new insights into one of the molecular bases underlying ginsenoside-induced regulation of ligand-gated ion channel activity in the nervous system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuropharm.2006.12.001.

References

Buisson, B., Bertrand, D., 1998. Open-channel blockers at the human alpha4-beta2 neuronal nicotinic acetylcholine receptor. Mol. Pharmacol. 53, 555–563.

Camilleri, M., Northcutt, A.R., Kong, S., Dukes, G.E., McSorley, D., Mangel, A.W., 2000. Efficacy and safety of alosetron in women with irritable bowel syndrome: a randomised, placebo-controlled trial. Lancet 355, 1035–1040.

Choi, S., Lee, J.H., Oh, S., Rhim, H., Lee, S.M., Nah, S.Y., 2003. Effects of ginsenoside Rg_2 on the 5-HT3A receptor-mediated ion current in *Xenopus* oocytes. Mol. Cells 15, 108–113.

Choi, S., Jung, S.Y., Lee, J.H., Sala, F., Criado, M., Mulet, J., Valor, L.M., Sala, S., Engel, A.G., Nah, S.Y., 2002. Effects of ginsenosides, active components of ginseng, on nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. Eur. J. Pharmacol. 442, 37–45.

Dang, H., England, P.M., Farivar, S.S., Dougherty, D.A., Lester, H.A., 2000. Probing the role of a conserved M1 proline residue in 5-hydroxytryptamine(3) receptor gating. Mol. Pharmacol. 57, 1114–1122.

Dascal, N., 1987. The use of *Xenopus* oocytes for the study of ion channels. CRC Crit. Rev. Biochem. 22, 317–387.

Gunthorpe, M.J., Lummis, S.C., 1999. Diltiazem causes open channel block of recombinant 5-HT3 receptors. J. Physiol. 519, 713–722.

Hargreaves, A.C., Lummis, S.C., Taylor, C.W., 1994. Ca^{2+} permeability of cloned and native 5-hydroxytryptamine type 3 receptors. Mol. Pharmacol. 46, 1120–1128.

Hu, X.Q., Zhang, L., Stewart, R.R., Weight, F.F., 2003. Arginine 222 in the pre-transmembrane domain 1 of 5-HT3A receptors links agonist binding to channel gating. J. Biol. Chem. 278, 46583–46589.

Jeong, S.M., Nah, S.Y., 2005. Ginseng and ion channels: are ginsenosides, active component of *Panax ginseng*, differential modulator of ion channels? J. Ginseng Res. 29, 19–26.

Jeong, S.M., Lee, J.H., Kim, J.H., Lee, B.H., Yoon, I.S., Lee, J.H., Kim, D.H., Rhim, H., Kim, Y., Nah, S.Y., 2004. Stereospecificity of ginsenoside Rg_3 action on ion channels. Mol. Cells 18, 383–389.

Keramidas, A., Moorhouse, A.J., Schofield, P.R., Barry, P.H., 2004. Ligand-gated ion channels: mechanisms underlying ion selectivity. Prog. Biophys. Mol. Biol. 86, 161–204.

Kim, H.S., Lee, J.H., Goo, Y.S., Nah, S.Y., 1998. Effects of ginsenosides on Ca^{2+} channels and membrane capacitance in rat adrenal chromaffin cells. Brain Res. Bull. 46, 245–251.

Kim, J.H., Lee, J.H., Jeong, S.M., Lee, B.H., Yoon, I.S., Choi, S.H., Nah, S.Y., 2005a. Effect of ginseng saponins on a rat visceral hypersensitivity model. Biol. Pharm. Bull. 28, 2120–2124.

Kim, J.H., Yoon, I.S., Lee, B.H., Choi, S.H., Lee, J.H., Jeong, S.M., Kim, S.C., Park, C.K., Lee, S.M., Nah, S.Y., 2005b. Effects of Korean red ginseng extract on cisplatin-induced nausea and vomiting. Arch. Pharm. Res. 28, 413–420.

Kim, S., Ahn, K., Oh, T.H., Nah, S.Y., Rhim, H., 2002. Inhibitory effect of ginsenosides on NMDA receptor-mediated signals in rat hippocampal neurons. Biochem. Biophys. Res. Commun. 296, 247–254.

Kudo, K., Tachikawa, E., Kashimoto, T., Takahashi, E., 1998. Properties of ginseng saponin inhibition of catecholamine secretion in bovine adrenal chromaffin cells. Eur. J. Pharmacol. 341, 139–144.

Lee, B.H., Jeong, S.M., Lee, J.H., Kim, J.H., Kim, D.H., Park, C.S., Lee, S.M., Nah, S.Y., 2004. Ginsenosides regulate ligand-gated ion channels from the outside. Mol. Cells 18, 115–121.

Lee, J.H., Jeong, S.M., Kim, J.H., Lee, B.H., Yoon, I.S., Lee, J.H., Choi, S.H., Kim, D.H., Rhim, H., Kim, S.S., Kim, J.I., Jang, C.G., Song, J.H., Nah, S.Y., 2005. Mol. Pharmacol. 68, 1114–1126.

Le Novere, N., Changeux, J.P., 1999. LGICdb: the ligand-gated ion channel database. Nucleic Acid Res. 27, 340–342.

Lopreato, G.F., Banerjee, P., Mihic, S.J., 2003. Amino acids in transmembrane domain two influence anesthetic enhancement of serotonin-3A receptor function. Mol. Brain Res. 118, 45–51.

Lummis, S.C., 2004. The transmembrane domain of the 5-HT3 receptor: its role in selectivity and gating. Biochem. Soc. Trans. 32, 535–539.

Maricq, A.V., Peterson, A.S., Brake, A.J., Meyers, R.M., Julius, D., 1991. Primary structure and functional expression of the 5HT3 receptor, a serotonin-gated ion channel. Science 254, 432–437.

Min, K.T., Koo, B.N., Kang, J.W., Bai, S.J., Ko, S.R., Cho, Z.H., 2003. Effect of ginseng saponins on the recombinant serotonin type 3A receptor expressed in *Xenopus* oocytes: implication of possible application as an antiemetic. J. Altern. Complement. Med. 9, 505–510.

Nah, S.Y., 1997. Ginseng: recent advances and trends. Kor. J. Ginseng Sci. 21, 1–12.

Panicker, S., Cruz, H., Arrabit, C., Suen, K.F., Slesinger, P.A., 2004. Minimal structural rearrangement of the cytoplasmic pore during activation of the 5-HT3A receptor. J. Biol. Chem. 279, 28149–28158.

Panicker, S., Cruz, H., Arrabit, C., Slesinger, P.A., 2002. Evidence for a centrally located gate in the pore of a serotonin-gated ion channel. J. Neurosci. 22, 1629–1639.

Polati, E., Verlato, G., Finco, G., Monsaner, W., Grosso, S., Gottin, L., Pinaroli, A.M., Ischia, S., 1997. Ondansetron versus metoclopramide in the treatment of postoperative nausea and vomiting. Anesth. Analg. 85, 395–399.

Reeves, D.C., Goren, E.N., Akabas, M.H., Lummis, S.C., 2001. Structural and electrostatic properties of the 5-HT3 receptor pore revealed by substituted cysteine accessibility mutagenesis. J. Biol. Chem. 276, 42035–42042.

Sala, F., Mulet, J., Choi, S., Jung, S.Y., Nah, S.Y., Rhim, H., Valor, L.M., Criado, M., Sala, S., 2002. Effects of ginsenoside Rg_2 on human neuronal nicotinic acetylcholine receptors. J. Pharmacol. Exp. Ther. 301, 1052–1059.

Tachikawa, E., Kudo, K., Kashimoto, T., Takahashi, E., 1995. Ginseng saponins reduce acetylcholine-evoked Na^+ influx and catecholamine secretion in bovine adrenal chromaffin cells. J. Pharm. Exp. Ther. 273, 629–636.

White, M.M., Aylwin, M., 1990. Niflumic and flufenamic acids are potent reversible blockers of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes. Mol. Pharmacol. 37, 720–724.

Zhang, L., Hosoi, M., Fukuzawa, M., Sun, H., Rawlings, R.R., Weight, F.F., 2002. Distinct molecular basis for differential sensitivity of the serotonin type 3A receptor to ethanol in the absence and presence of agonist. J. Biol. Chem. 277, 46256–46264.