A Role for the Val291 Residue within the Transmembrane Domain 2 in Diltiazem- and TMB-8 [3,4,5-Trimethoxybenzoic Acid 8-(Diethylamino)octyl Ester]-Mediated 5-Hydroxytryptamine Type 3A Receptor Regulations

Byung-Hwan LEE,^{a,#} Sun-Hye CHOI,^{a,#} Mi Kyung PYO,^a Tae-Joon SHIN,^a Sung-Hee HWANG,^a Bo-Ra KIM,^a Jun-Ho LEE,^b Hyewhon RHIM,^c Hyoung-Choon KIM,^d and Seung-Yeol NAH^{*,a}

^aDepartment of Physiology, College of Veterinary Medicine, Konkuk University; Seoul 143–701, Korea: ^bDepartment of Physiology, College of Oriental Medicine Kyung-Hee University; Seoul 130–701, Korea: ^cBiomedical Research Center, KIST; Seoul 136–791, Korea: and ^dNeurotoxicology Program, College of Pharmacy, Korea Institute of Drug Abuse, Kangwon National University; Chunchon 200–701, South Korea. Received November 21, 2008; accepted February 3, 2009

Previous reports have shown that diltiazem and TMB, calcium channel antagonists, inhibit 5-hydroxytryptamine type 3A (5-HT_{3A}) receptor-mediated currents (I_{5-HT}) in cell lines and in heterologously expressed *Xenopus* oocytes. In the present study, we sought to elucidate the molecular mechanisms underlying diltiazem- and TMB-induced 5-HT_{3A} receptor regulations. We used the two-microelectrode voltage clamp technique to investigate the effect of diltiazem and TMB on 5-HT-mediated ion currents 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, diltiazem and TMB dose-dependently inhibited peak I_{5-HT} with an IC_{50} of 71.4 ± 4.9 and 4.5 ± 0.3 μ M, respectively. Among various mutants of TM2, mutation V291A greatly attenuated and abolished the TMB- and diltiazem-induced inhibition of peak I_{5-HT} , respectively. Mutation V291A also induced constitutively active ion currents in the absence of 5-HT. Diltiazem and TMB inhibited constitutively active ion currents in a dose-dependent manner. The IC_{50} values of constitutively active ion currents in V291A receptors were 165.3 ± 11.1 and 6.6 ± 0.5 μ M for diltiazem and 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), respectively. Results of site-directed mutagenesis experiments suggest that the Val291 residue could be a candidate for common interaction site for diltiazem- and TMB-8-mediated 5-HT_{3A} receptor regulations.

Key words diltiazem; 5-hydroxytryptamine type 3A receptor; common interaction site; 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester

The 5-hydroxytryptamine type 3A (5-HT_{3A}) receptors are cationic ion channels that may be activated by serotonin or its agonists. 5-HT₃ receptors are one of the Cys-loop family of ligand-gated ion channels, such as 5-HT₃, GABA_A, glycine and nicotinic acetylcholine (nACh) receptors.^{1,2)} The 5-HT₃ receptors consist of a homomeric pentamer; each subunit includes a large N-terminal extracellular domain, four hydrophobic transmembrane domains (TM1–TM4) and an extracellular C-terminal domain (Fig. 1C). 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.^{1,2)} Recently, site-directed mutagenesis has been used to characterize the detailed actions and binding sites of various drugs and toxins that regulate 5-HT₃ receptors.^{3,4)} Amino acids 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 could form a gating region.^{5–7)} However, the precise mechanisms by which the binding of serotonin or its agonists leads to channel opening and gating are still poorly understood at the molecular level.¹⁾ Recent studies showed that various agents exert their pharmacological effects by targeting 5-HT₃ receptors.^{3,4,8,9)}

Among them, diltiazem and 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), which are also known as L-type calcium channel antagonists,¹⁰⁾ regulate 5-HT_{3A} and both muscle- and neuronal-type AChR channel activi-

ties.^{11,12)} Diltiazem and TMB-8 act as open-channel blockers in the regulation of 5-HT_{3A} and muscle- and neuronal-type nicotinic ACh receptor channel activities. Thus, Hargreaves *et al.* (1996) showed in ligand-binding assays that the inhibition of [³H]m-chlorophenylbiguanidin binding by diltiazem is mediated by a site that is distinct from the agonist-binding site and inhibits 5-HT_{3A} receptor function.¹³⁾ On the other hand, Sun *et al.* (1999) have shown by radioligand-binding assays that TMB-8 acts as a competitive antagonist of the mouse 5-HT_{3A} receptor, and that TMB-8 is a potent noncompetitive antagonist of the nicotinic ACh receptor as it binds with micromolar affinity to the resting, open channel and desensitized nicotinic ACh receptor.¹⁴⁾ In contrast, Dang *et al.* (2000) have reported that TMB-8 is a kind of open channel blocker,⁸⁾ as is diltiazem.¹⁵⁾ Thus, although these reports show a possibility that diltiazem and TMB-8 may share a similar pathway or use common regulatory modes in their regulations on 5-HT_{3A} receptors, it is not clearly demonstrated whether diltiazem and TMB-8 show overlapping interaction site(s) in 5-HT₃ receptor channel regulations.

Here, we investigated whether the inhibitory effects of diltiazem and TMB-8 on 5-HT₃ receptor channel currents are affected when amino acids, which form the putative pore-lining face in TM2, are mutated. For this, we used a *Xenopus* oocyte gene expression system. This model system has few endogenous ion channels¹⁶⁾ and allows heterologous expression of 5-HT_{3A} receptor channel activities.^{17–19)} We expressed wild-type or various TM2-mutant 5-HT_{3A} receptors

* To whom correspondence should be addressed. e-mail: synah@konkuk.ac.kr

These authors contributed equally to this work.

(Fig. 1C, box) by intraoocyte cRNA injection, and examined the 5-HT-elicited ion currents (I_{5-HT}) in these oocytes in the absence and presence of diltiazem or TMB-8. In wild-type receptors, diltiazem and TMB-8 inhibited the peak I_{5-HT} in a dose-dependent manner. Oocytes expressing V291A mutant receptors almost abolished diltiazem-induced inhibition of peak I_{5-HT} , whereas oocytes expressing the V291A mutant receptor showed a great attenuation of TMB-8-induced inhibition of I_{5-HT} . Mutation V291A also showed constitutively active ion currents in the absence of 5-HT. Diltiazem and TMB-8 inhibited the constitutively active ion currents in a dose-dependent manner. Collectively, these results show a possibility that Val291 in the channel gating region of TM2 may be a candidate for a common interaction residue for diltiazem- and TMB-8-mediated 5-HT_{3A} receptor regulations.

MATERIALS AND METHODS

Materials Mouse 5-HT_{3A} receptor cDNA was kindly provided by Dr. D. Julius (University of California San Francisco, CA, U.S.A.).¹⁹⁾ TMB-8, diltiazem and the other agents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) and their chemical structure is shown in Figs. 1A and B.

Preparation of *Xenopus* Oocytes and Microinjection *Xenopus laevis* frogs were purchased from Xenopus I (Ann Arbor, MI, U.S.A.). 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 treat-

ment followed by agitation for 2 h in a Ca²⁺-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), 2.5 mM sodium pyruvate, 100 units/ml penicillin and 100 μ g/ml streptomycin. Stage V–VI oocytes were collected and stored in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES, pH 7.5) supplemented with 0.5 mM theophylline and 50 μ g/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 d 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 μ l VWR microdispenser (VWR Scientific, San Francisco, CA, U.S.A.) fitted with a tapered glass pipette tip that was 15–20 μ m in diameter.¹⁷⁾

Site-Directed Mutagenesis of 5-HT_{3A} Receptors and *in Vitro* Transcription of 5-HT_{3A} Receptor cDNAs The substitution mutation of amino acids was performed using Pfu DNA polymerase (QuickChange™ Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, U.S.A.) and mutated sense and antisense primers. Sequential polymerase chain reaction (PCR) was used for overlapping extension at the target domain, as described in the provided manual. Final PCR products were transformed into *Escherichia coli* strain DH5 α , 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 *SalI* digestion, and run-off transcripts were prepared using the methylated cap analog, m⁷G(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, U.S.A.). The final cRNA products were resuspended at 1 μ g/ μ l with RNase-free water and stored at –80 °C until use.¹⁷⁾ The absence of degraded RNA was confirmed by denaturing agarose gel electrophoresis followed by ethidium bromide staining.

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 (ca. 0.4 mm grid diameter) onto the bottom of the upper well. The perfusion inlet (ca. 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 M Ω). Recordings were performed in ND96 solution at room temperature. In experiments using mutant 5-HT_{3A} receptors, ND96 solution containing 300 μ M niflumic acid (NFA), a Ca²⁺-activated Cl[–] channel blocker (White *et al.*,²⁰⁾ was used to exclude any contributions of Cl[–] currents. In the indicated experiments,

Fig. 1. Structures of Diltiazem and TMB-8, the Primary Amino Acid Sequence of the Channel-Lining Regions (TM2) of the Mouse 5-HT_{3A}

(A, B) Structure of diltiazem and TMB-8. (C) Partial amino acid sequence of the TM2 region of the cloned mouse 5-HT_{3A} receptors. The box indicates amino acid residues that were mutated in the present study.

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, U.S.A.) and stimulation and data acquisition were controlled by pClamp 8 (Axon Instruments, Union City, CA, U.S.A.).¹⁷

Data Analysis To obtain concentration–response curves of the effect of diltiazem or TMB on I_{5-HT} , the peak amplitudes at different concentrations of diltiazem or TMB were plotted and then fitted to the following Hill equation using the Origin software (OriginLab Corp, Northampton, MA, U.S.A.): $\text{Response} = E_{\max} - E_{\min} / 1 + (IC_{50} / [A])^{n_H} + E_{\min}$, where $E_{\max}$ and $E_{\min}$ are maximal and minimal responses, respectively. $[A]$ is concentration of diltiazem or TMB and n_H is the Hill coefficient. IC_{50} is the concentration of diltiazem or TMB required to decrease the response by 50%. All values are presented as means $\pm$ 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.

RESULTS

Effect of Diltiazem and TMB-8 on I_{5-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 diltiazem and TMB-8 on the 5-HT-stimulated inward current (I_{5-HT}) mediated by wild-type mouse 5-HT_{3A} receptors expressed in *Xenopus* oocytes. Addition of 1 μ M 5-HT to the bath solution of oocytes maintained at a holding potential of -80 mV elicited inward currents (Fig. 2A). H₂O-injected oocytes did not elicit any inward currents in the presence of 5-HT and MDL-72222, a selective 5-HT_{3A} re-

ceptor antagonist, blocked I_{5-HT} (data not shown), indicating that the 5-HT_{3A} receptors were functionally expressed in this system.^{17–19} Co-application of diltiazem (Dil) to this system inhibited the peak I_{5-HT} in a dose-dependent manner, blocking I_{5-HT} by 0.4 ± 1.2 , 2.0 ± 0.6 , 10.2 ± 0.5 , 24.6 ± 2.2 , 60.3 ± 2.0 , 81.2 ± 2.3 and $94.6 \pm 1.1\%$ at 1, 3, 10, 30, 100, 300 and 1000 μ M, respectively, in oocytes expressing wild-type 5-HT_{3A} receptors (Figs. 2A, D). Co-application of TMB-8 to this system also inhibited the peak I_{5-HT} in a dose-dependent manner, blocking I_{5-HT} by 0.3 ± 0.3 , 3.1 ± 0.6 , 16.2 ± 4.4 , 37.0 ± 2.3 , 71.4 ± 1.1 , 86.3 ± 1.7 and $96.2 \pm 0.6\%$ at 0.1, 0.3, 1, 3, 10, 30 and 100 μ M, respectively, in oocytes expressing wild-type 5-HT_{3A} receptors (Figs. 3A, D). Pretreatment of diltiazem or TMB-8 did not induce further significant inhibition of I_{5-HT} compared to co-application of both agents (data not shown). The IC_{50} s for diltiazem and TMB-8 were 71.4 ± 4.9 and 4.5 ± 0.3 μ M, respectively, and the Hill coefficients were 1.2 ± 0.1 and 1.1 ± 0.1 , respectively, in oocytes expressing wild-type 5-HT_{3A} receptors (Tables 1, 2).

Diltiazem- or TMB-8-Induced Inhibitions of Peak I_{5-HT} Are Abolished or Attenuated in Mutations S290A, V291A, F292A, I294A, I295A and V296A in TM2 Previous studies showed that diltiazem and TMB-8 inhibit 5-HT_{3A} receptor channel activity as open channel blockers and in competitive and non-competitive manners.^{8,14} These reports support a possibility that both agents regulate 5-HT_{3A} receptor channel activity through interaction with unidentified receptor site(s). Based on this, we hypothesized that there are common interaction site(s) with diltiazem and TMB-8. Since TM2 forms the pore region of 5-HT_{3A} receptors, and mutations in this region affect the inhibition of I_{5-HT} in response to anesthetics, cadmium and the open channel blocker,^{3,4,7} we investigated the effect of diltiazem and TMB-8 on I_{5-HT} in oocytes expressing 5-HT_{3A} receptors that contain site-

Fig. 2. Concentration-Dependent Effects of Diltiazem (Dil) on I_{5-HT} in Wild-Type and Additional Mutants of S290A and V291A

(A–C) I_{5-HT} was elicited in oocytes expressing wild-type or mutant receptors at a holding potential of -80 mV for the indicated time in the presence of 1 μ M 5-HT, and then the indicated concentrations of diltiazem were co-applied with 5-HT. (D) Concentration–response curves were generated for the effect of diltiazem on 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 1 (mean $\pm$ S.E.M.; $n = 9–12$ oocytes for each point).

Fig. 3. Concentration-Dependent Effects of TMB-8 on I_{5-HT} in Wild-Type and Additional Mutants of L287A and V291A

(A–C) I_{5-HT} was elicited in oocytes expressing wild-type or various mutant receptors at a holding potential of -80 mV for the indicated time in the presence of 1 μ M 5-HT, and then the indicated concentrations of TMB-8 were co-applied with 5-HT. (D) Concentration–response curves were generated for the effect of TMB-8 on 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 2 (mean $\pm$ S.E.M.; $n = 9–12$ oocytes for each point).

Table 1. Effect of Diltiazem on Wild-Type and Various Mutant 5-HT_{3A} Receptors Expressed in *Xenopus* Oocytes

	IC ₅₀	V _{max}	n _H
Wild-type	71.4±4.9	98.2±2.2	1.2±0.1
L287A	20.1±2.6	101.2±3.4	1.2±0.1
G288A	NC	NC	NC
Y289A	NC	NC	NC
S290A	64.1±6.0	97.3±3.0	1.2±0.1
V291A	334.2±15.1**	9.9±1.6**	1.7±0.6
F292A	144.5±14.5*	53.0±4.0**	1.6±0.4
L293A	26.0±2.7*	79.2±2.3*	1.2±0.1
I294A	103.8±7.8*	95.3±4.8	1.3±0.2
I295A	170.4±3.6**	49.2±0.3**	2.3±0.1*
V296A	214.4±3.6**	84.0±1.1*	2.2±0.1*
^a V291A	165.3±11.1	96.6±2.1	1.2±0.1

Values represent the mean±S.E.M. ($n=9-15$ /group). Currents were elicited at a holding potential of -80 mV. IC₅₀ (μ M), V_{max}, and Hill coefficient values were determined as described in Materials and Methods. IC₅₀ (μ M), V_{max}, and Hill coefficient values of the constitutively active ion currents in oocytes expressing ^aV291A were determined in the absence of 5-HT. NC, no current. * $p<0.05$, ** $p<0.005$ compared with wild-type 5-HT_{3A} receptors.

Table 2. Effect of TMB-8 on Wild-Type and Various Mutant 5-HT_{3A} Receptors Expressed in *Xenopus* Oocytes

	IC ₅₀	V _{max}	n _H
Wild-type	4.5±0.3	97.9±1.9	1.1±0.1
L287A	4.4±0.4	95.3±2.9	1.5±0.2
G288A	NC	NC	NC
Y289A	NC	NC	NC
S290A	11.1±1.3*	102.8±4.4	1.1±0.1
V291A	21.2±7.9**	39.1±1.9**	0.9±0.3
F292A	6.3±0.9	98.0±4.2	1.0±0.1
L293A	4.4±0.9	96.1±5.8	1.1±0.2
I294A	3.8±0.2	98.5±1.1	1.4±0.1
I295A	13.0±0.6**	100.9±2.1	1.5±0.1
V296A	21.1±2.5**	95.4±4.6	1.1±0.1
^a V291A	6.2±0.5	94.9±2.0	1.0±0.1

Values represent the mean±S.E.M. ($n=9-15$ /group). Currents were elicited at a holding potential of -80 mV. IC₅₀ (μ M), V_{max}, and Hill coefficient values were determined as described in Materials and Methods. IC₅₀ (μ M), V_{max}, and Hill coefficient values of the constitutively active ion currents in oocytes expressing ^aV291A were determined in the absence of 5-HT. NC, no current. * $p<0.05$, ** $p<0.005$ compared with wild-type 5-HT_{3A} receptors.

directed mutations in TM2 residues ranging from L287 to V296 (Fig. 1C, box). The constructed mutants included replacement of residues L287, G288, Y289, S290, V291, L293, I294, I295 and V296 with alanine, generating L287A, G288A, Y289A, S290A, V291A, L293A, I294A, I295A and V296A, respectively. Among these mutants, oocytes expressing G288A and Y289A failed to show I_{5-HT} following 5-HT stimulation.

We first examined the effects of diltiazem (Dil) on these currents. Figure 2 shows representative traces of peak I_{5-HT} in the presence or absence of diltiazem in oocytes expressing wild-type, S290A and V291A receptors. At a holding potential of -80 mV, the inhibitory effect of diltiazem on peak I_{5-HT} was almost abolished in oocytes expressing the V291A *versus* wild-type receptors, with the blockade of diltiazem-induced inhibition decreasing in the order of V291A>V296A>I295A>F292A>I294A (Table 1). Representative concentration–response relationships for peak I_{5-HT} inhibition by diltiazem in five different mutant receptors were fitted using the Hill equation (Fig. 2D, smooth lines). The

Hill coefficients did not substantially differ from the wild-type values in oocytes expressing mutant receptors, while the V_{max} values differed significantly from wild-type in oocytes expressing V291A, F292A, I295A, and V296A (* $p<0.05$, ** $p<0.005$; Table 1). Most of these mutants, including V291A, V296A, I295A and F292A, had significantly higher IC₅₀ values than that of wild-type channels (* $p<0.05$, ** $p<0.005$; Table 1).

We next examined the effects of TMB-8 on these currents. Figure 3 shows representative traces of peak I_{5-HT} in the presence or absence of TMB-8 in oocytes expressing wild-type, L287A and V291A mutated receptors. At a holding potential of -80 mV, the inhibitory effect of TMB-8 on peak I_{5-HT} was greatly attenuated in oocytes expressing the V291A *versus* wild-type receptors, with the blockade of TMB-8-induced inhibition decreasing in the order of V291A>V296A>I295A>S290A (Table 2). Representative concentration–response relationships for peak I_{5-HT} inhibition by TMB-8 in four different mutant receptors were fitted using the Hill equation (Fig. 3D, smooth lines). The Hill coefficients did not significantly differ from the wild-type values in oocytes expressing S290A, V291A, I295A and V296A, whereas the V_{max} values significantly differed from wild-type in oocytes expressing only V291A (* $p<0.05$, ** $p<0.005$; Table 2). Most of these mutants, including S290A, V291A, I295A and V296A, had significantly higher IC₅₀ values than the wild-type (* $p<0.05$, ** $p<0.005$; Table 2). In addition, since V291A receptors exhibited the strongest effects on diltiazem and TMB-8 activity among various mutant receptors, these results suggest a possibility that diltiazem and TMB-8 may share this site.

Diltiazem and TMB-8 Inhibit Constitutively Active Ion Currents in the V291A Mutation

In addition to the alterations of regulatory modes of 5-HT_{3A} receptor channel activity in several mutant receptors, including V291A, by diltiazem and TMB-8, we also found that mutation V291A induced constitutively active ion currents even in the absence of 5-HT.^{8,21} The average amplitude of these constitutively active ion currents was -1581 ± 223 nA ($n=15$ from five different frogs) (Figs. 4A, B). To further examine 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), an 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 they are mainly cation currents traveling through the constitutively open channels (Fig. 4A). Since treatment of oocytes expressing V291A receptors with diltiazem or TMB-8 alone inhibited the constitutively active ion currents in the absence of 5-HT (Figs. 4B, C), we next examined the effects of diltiazem and TMB-8 on the constitutively active V291A receptor-mediated ion currents. The results revealed that diltiazem dose-dependently inhibited the constitutively active ion currents by 0.1 ± 0.5 , 2.0 ± 0.7 , 4.2 ± 0.9 , 12.6 ± 2.8 , 31.8 ± 5.2 , 65.8 ± 2.8 , 88.4 ± 1.4 , and $92.5\pm3.2\%$ at 1, 3, 10, 30, 100, 300, 1000 and 3000 μ M, respectively. Similarly, TMB-8 dose-dependently inhibited the constitutively active ion currents by 0.3 ± 0.8 , 4.8 ± 1.2 , 12.6 ± 3.0 , 31.9 ± 5.3 , 56.5 ± 4.3 , 82.0 ± 5.9 , 88.1 ± 2.6 , and $93.4\pm4.9\%$ at 0.1, 0.3, 1, 3, 10, 30, 100, and 300 μ M, respectively. The

Fig. 4. Diltiazem and TMB-8 Inhibit Constitutively Active Ion Currents in Mutation V291A

(A) The replacement of Na^+ with NMDG abolished the constitutively active ion currents in the presence of $300 \mu\text{M}$ niflumic acid (NFA), a Ca^{2+} -activated Cl^- channel blocker. (B) The representative traces show that diltiazem inhibits constitutively active ion currents in a concentration-dependent manner. The period of drug application is indicated by the solid bars. (C) Experiments were performed as above with various concentrations of TMB-8. Traces (B, C) are representative of six separate oocytes from three different frogs. Data represent the means $\pm$ S.E.M. ($n=10$ – 12 /group). Oocytes expressing V291A mutant receptors were holding at -80 mV . (D) Concentration-response curves for the effect of diltiazem and TMB-8 on constitutively active V291A mutant receptors (mean $\pm$ S.E.M.; $n=12$ – 15 oocytes each).

IC_{50} values of diltiazem and TMB-8 were 165.3 ± 11.1 and $6.5 \pm 0.5 \mu\text{M}$, respectively. The IC_{50} value for TMB-8 was about 24-fold smaller than that of diltiazem in the inhibition of constitutively active ion currents mediated by V291A mutant receptors. Interestingly, we also found that the recovery time after TMB-8 treatment was much slower than that of diltiazem (Figs. 4B, C). The difference in recovery kinetics of diltiazem and TMB-8 suggests that TMB-8 might have a high affinity for 5-HT_{3A} receptor compared to diltiazem.

TMB-8 Occludes Diltiazem-Induced Inhibition of Constitutively Active Ion Currents in Mutation V291A To further investigate how diltiazem and TMB-8 inhibits $I_{5\text{-HT}}$, we performed competition experiments between diltiazem and TMB-8. To simplify the comparison between the suppressive effects of diltiazem, TMB-8, and their mixture on $I_{5\text{-HT}}$, we assumed that if diltiazem and TMB-8 act through the same interaction sites, the inhibitory effects produced by the mixture of diltiazem and TMB-8 should be less than the sum of their individual effects. In contrast, if diltiazem and TMB-8 act through different interaction sites, the inhibitory effects produced by the mixture of diltiazem and TMB-8 should be equal or close to the sum of their individual effects. As shown in Fig. 5, a single application of diltiazem (Dil) ($100 \mu\text{M}$) and TMB-8 ($3 \mu\text{M}$) inhibited the constitutively active currents by 28.9 ± 2.9 and 21.6 ± 0.6 %, respectively ($n=7$ – 9), and co-treatment of diltiazem with TMB-8 produced an additive inhibition of the constitutively active currents by 52.3 ± 2.5 % ($n=7$ – 9). In a series of experiments, TMB-8 ($10 \mu\text{M}$) inhibited $I_{5\text{-HT}}$ by 52.2 ± 2.7 % ($n=7$ – 9), and co-treatment of diltiazem with TMB-8 produced an additive inhibition of the constitutively active currents by 76.2 ± 4.1 % ($n=7$ – 9). However, different results were observed with higher concentrations of TMB-8. Thus, $30 \mu\text{M}$ TMB-8, which

Fig. 5. TMB-8 Interferes with the Action of Diltiazem in Mutation V291A

(A) Diltiazem ($100 \mu\text{M}$) was first applied during the period indicated by the solid bars, and then TMB-8 ($3 \mu\text{M}$) was applied in the presence of diltiazem as indicated by solid bars. The histograms show the percent blockade of the constitutively active ion currents by diltiazem, TMB-8, or diltiazem (Dil)+TMB-8. Data represent the means $\pm$ S.E.M. ($n=8$ – 10 /group). (B) Experiments were performed as above with TMB-8 ($30 \mu\text{M}$) used in place of diltiazem. Traces (A, B) represent six separate oocytes from three different frogs. Data represent the means $\pm$ S.E.M. ($n=10$ – 12 /group). Oocytes were holding at -80 mV . (C) Competition experiments between diltiazem (Dil) and TMB-8. The suppressive effect on $I_{5\text{-HT}}$ by diltiazem at the indicated concentrations was occluded by the indicated concentration of TMB-8. Diltiazem or/and TMB-8 were applied for 30 s. The effects on $I_{5\text{-HT}}$ of diltiazem co-applied with TMB-8 were much different from the sum of the suppressive effects when they were applied individually. Data represent the means $\pm$ S.E.M. ($n=6$ – 9 /group).

is almost a saturating concentration, inhibited $I_{5\text{-HT}}$ by 81.3 ± 4.3 % ($n=7$ – 9). When $30 \mu\text{M}$ TMB-8 and $100 \mu\text{M}$ diltiazem were co-applied, $I_{5\text{-HT}}$ was inhibited by 83.9 ± 4.6 % ($n=6$ – 7), which was not much different from the inhibition with TMB-8 alone. Thus, a high concentration of TMB-8 occludes diltiazem-mediated $I_{5\text{-HT}}$ inhibition. Again, these results show that diltiazem regulates the constitutively active currents of the V291A mutant receptor by sharing an overlapping site with TMB-8.

DISCUSSION

Diltiazem and TMB-8, which are used widely as calcium channel antagonists, are known to inhibit 5-HT_{3A} receptor channel currents ($I_{5\text{-HT}}$) as noncompetitive open channel blockers. They have also been shown to be noncompetitive open channel blockers of muscle ($\alpha 1\beta 1\delta\gamma$) or neuronal ($\alpha 7$ and $\alpha 4\beta 2$) AChRs.^{22,23} Thus, although mounting evidence supports the possibility that diltiazem and TMB-8 may share a similar pathway or use common regulatory modes in their regulations on 5-HT_{3A} receptors, it has not been directly demonstrated whether diltiazem and TMB-8 achieve their effects through a similar pathway or show overlapping interaction site(s) in 5-HT_{3A} receptor channel regulation.

The present study has further explored the influences of mutations of amino acid residues in TM2 of 5-HT_{3A} receptors on the inhibition of $I_{5\text{-HT}}$ mediated by both agents. In this

regard, our principle findings were three-fold. First, TMB-8 was about 16 times more potent than diltiazem in the inhibition of I_{5-HT} in wild-type. Second, the mutation of amino acid residues such as V291, I295 and V296 in TM2 commonly affected diltiazem- and TMB-mediated 5-HT_{3A} receptor channel regulations, but the V291A receptor exhibited the greatest effects on diltiazem and TMB-8 in changes of IC_{50} and V_{max} values compared with wild-type. Third, mutation V291A induced constitutively active ion currents in the absence of 5-HT, as previously reported.²¹⁾ Diltiazem and TMB inhibited those constitutively active ion currents in a dose-dependent manner. TMB-8 was also about 25 times more potent than diltiazem in the inhibition of constitutively active ion currents. These results support the possibility that Val291 in the channel gating region of TM2 may play an important role as a common residue in the diltiazem- and TMB-8-mediated 5-HT_{3A} receptor regulations.

As shown in Fig. 1C, for the conserved Val291 residue of the 5-HT_{3A} receptor, the present and previous studies have also shown that mutations of Val291 to other amino acids affect the inhibition of I_{5-HT} in response to anesthetics, cadmium, ginsenoside Rg₃ and TMB-8.^{3,4,7,8,18)} Thus, Val291 of the 5-HT_{3A} receptor could be useful targets for studying the receptor-related pharmacology. This residue could also be used for drug developments such as anti-emetic agent if diltiazem or TMB-8 derivatives are selective for 5-HT_{3A} receptor.^{3,18)} In addition to the Val291 amino acid residue of the 5-HT_{3A} receptor, we saw that mutations at residues F292, I294, I295 and V296 caused significant changes of IC_{50} values in diltiazem-mediated concentration–response curves. We could also see that mutations at residues S290, I295 and V296 caused significant changes of IC_{50} values in TMB-8-mediated concentration–response curves. Thus, it appears that I294A receptors only affected diltiazem, but not TMB-8, action, whereas the S290A receptors only affected TMB-8, but not diltiazem, action. Interestingly, mutations of amino acid residues such as V291, I295 and V296 to alanine commonly influenced diltiazem- and TMB-mediated 5-HT_{3A} receptor channel regulation, and the mutation of the V291 residue most potently affected diltiazem and TMB-8 action, suggesting that this residue likely forms the main interaction or overlapping sites for diltiazem and TMB in the 5-HT_{3A} receptor channel protein.

Diltiazem and TMB-8 are known as open channel blockers of the 5-HT_{3A} receptor.^{5,6)} The present results also support this notion. Using the V291A mutant receptor, we were also able to show that diltiazem and TMB-8 interact with the open state of the receptor channel. Thus, we found that diltiazem and TMB-8 inhibited constitutively active currents of the V291A mutant receptor. The currents were also abolished by replacing permeant Na⁺ in the extracellular solution with impermeant NMDG (Fig. 4A). These results indicate that diltiazem and TMB-8 inhibit mainly inward cation currents through open channels. The present findings, in conjunction with the previous work, strongly suggest that diltiazem and TMB-8 may specifically regulate 5-HT_{3A} receptor channel activity in the open state through an interaction with amino acid residues such as V291, I295 and V296 of TM2. However, in the case of TMB-8 we could not exclude other possibilities, since Sun *et al.* (1999) reported that TMB-8 acts as a competitive antagonist rather than open channel blocker in 5-

HT₃ receptor regulations at the ligand binding sites.¹⁴⁾ In the present study, we also observed changes of IC_{50} values in the presence or absence of 5-HT in the V291A receptor channel current inhibition by TMB-8. In the presence of 5-HT, the IC_{50} value was about 4.7 times higher than that of wild-type, whereas the IC_{50} value obtained in the absence of 5-HT, which shows constitutively active currents, was only 1.5 times higher than that of wild-type, suggesting that the presence of 5-HT may compete with TMB-8¹⁴⁾ and requires a higher concentration of TMB-8 for the same extent of inhibition of I_{5-HT} compared with the inhibition of constitutively active currents. In addition, we found that the mutation of Val291 to V291A did not abolish TMB-mediated inhibition of I_{5-HT} , although diltiazem action on this mutant receptor was almost abolished (Figs. 2C, 3C). Thus, the results suggest a possibility that TMB-8 regulates 5-HT_{3A} receptor channel activity through additional interaction site(s) including amino acid residues in TM2.

In summary, we have used site-directed mutagenesis to characterize diltiazem- and TMB-8-induced regulation of 5-HT_{3A} receptor channel activity. Using wild-type and the constitutively active V291A mutant receptor we have found evidence that diltiazem and TMB-8 share a common interaction site in the regulation of 5-HT_{3A} receptor channel activity. The data also suggest that amino acid residues V291, I295 and V296 in the TM2 region play important roles in the diltiazem- and TMB-8-mediated inhibition of 5-HT_{3A} receptor currents. These findings provide new insight into one of the molecular bases underlying diltiazem- and TMB-8-induced regulation of 5-HT_{3A} receptor channel activity in the nervous system.

Acknowledgements This work was supported by grants to S. Y. Nah from the BK21 project, the KOSEF funded by the MEST (R01-2008-000-10448-0) and by grant to H. Rhim from Korea Institute of Science and Technology Core-Competence Program funded by the MEST and KIST Core-Competence Program. We thank D. Julius for providing the mouse 5-HT_{3A} receptor cDNA.

REFERENCES

- 1) Keramidas A., Moorhouse A. J., Schofield P. R., Barry P. H., *Prog. Biophys. Mol. Biol.*, **86**, 161–204 (2004).
- 2) Lummis S. C., *Biochem. Soc. Trans.*, **32**, 535–539 (2004).
- 3) Zhang L., Hosoi M., Fukuzawa M., Sun H., Rawlings R. R., Weight F. F., *J. Biol. Chem.*, **277**, 46256–46264 (2002).
- 4) Hu X. Q., Zhang L., Stewart R. R., Weight F. F., *J. Biol. Chem.*, **278**, 46583–46589 (2003).
- 5) Reeves D. C., Goren E. N., Akabas M. H., Lummis S. C., *J. Biol. Chem.*, **276**, 42035–42042 (2001).
- 6) Panicker S., Cruz H., Arrabit C., Slesinger P. A., *J. Neurosci.*, **22**, 1629–1639 (2002).
- 7) Panicker S., Cruz H., Arrabit C., Suen K. F., Slesinger P. A., *J. Biol. Chem.*, **279**, 28149–28158 (2004).
- 8) Dang H., England P. M., Farivar S. S., Dougherty D. A., Lester H. A., *Mol. Pharmacol.*, **57**, 1114–1122 (2000).
- 9) Lopreato G. F., Banerjee P., Mihic S. J., *Mol. Brain Res.*, **118**, 45–51 (2003).
- 10) Spedding M., Paoletti R., *Pharmacol. Rev.*, **44**, 363–376 (1992).
- 11) Bencherif M., Eisenhour C. M., Prince R. J., Lippicello P. M., Lukas R. J., *J. Pharmacol. Exp. Ther.*, **275**, 1418–1426 (1995).
- 12) Houlihan L. M., Slater E. Y., Beadle D. J., Lukas R. J., Bermudez I., *Neuropharmacology*, **39**, 2533–2542 (2000).
- 13) Hargreaves A. C., Gunthorpe M. J., Taylor C. W., Lummis S. C., *Mol.*

- Pharmacol.*, **50**, 1284—1294 (1996).
- 14) Sun H., McCardy E. A., Machu T. K., Blanton M. P., *J. Pharmacol. Exp. Ther.*, **290**, 129—135 (1999).
- 15) Gunthorpe M. J., Lummis S. C., *J. Physiol.*, **519**, 713—722 (1999).
- 16) Dascal N., *CRC Crit. Rev. Biochem.*, **22**, 317—387 (1987).
- 17) Choi S., Lee J. H., Oh S., Rhim H., Lee S. M., Nah S. Y., *Mol. Cells*, **15**, 108—113 (2003).
- 18) Lee B. H., Jeong S. M., Lee J. H., Kim J. H., Kim D. H., Park C. S., Lee S. M., Nah S. Y., *Mol. Cells*, **18**, 115—121 (2004).
- 19) Maricq A. V., Peterson A. S., Brake A. J., Meyers R. M., Julius D., *Science*, **254**, 432—437 (1991).
- 20) White M. M., Aylwin M., *Mol. Pharmacol.*, **37**, 720—724 (1990).
- 21) Lee B. H., Lee Jun Ho, Lee S. M., Jeong S. M., Yoon I. S., Lee Joon Hee, Choi S. H., Pyo M. K., Rhim H., Kim H. C., Jang C. G., Lee B. C., Park C. S., Nah S. Y., *Neuropharmacology*, **52**, 1139—1150 (2007).
- 22) Bertrand D., Devillers-Thiéry, A., Revah F., Galzi J. L., Hussy N., Mulle C., Bertrand S., Ballivet M., Changeux J. P., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 1261—1265 (1992).
- 23) Buisson B., Bertrand D., *Mol. Pharmacol.*, **53**, 555—563 (1998).