Resveratrol Enhances 5-Hydroxytryptamine Type 3A Receptor-Mediated Ion Currents: The Role of Arginine 222 Residue in Pre-transmembrane Domain I

Byung-Hwan LEE, Sung-Hee HWANG, Sun-Hye CHOI, Tae-Joon SHIN, Jiyeon KANG, Sang-Mok LEE, and Seung-Yeol NAH*

Department of Physiology, College of Veterinary Medicine and Bio/Molecular Informatics Center, Konkuk University; Seoul 143–701, Korea. Received November 30, 2010; accepted January 18, 2011; published online January 28, 2011

Resveratrol, which is found in grapes, red wine, and berries, has many beneficial health effects, such as anti-cancer, neuro-protective, anti-inflammatory, and life-prolonging effects. However, the cellular mechanisms by which resveratrol acts are relatively unknown, especially in terms of possible regulation of receptors involved in synaptic transmission. 5-Hydroxytryptamine type 3A (5-HT3A) receptor is one of several ligand-gated ion channels involved in fast synaptic transmission. In the present study, we investigated the effect of resveratrol on mouse 5-HT3A receptor channel activity. 5-HT3A receptor was expressed in Xenopus oocytes, and the current was measured using a two-electrode voltage clamp technique. Treatment of resveratrol itself had no effect on the oocytes injected with H2O as well as on the oocytes injected with 5-HT3A receptor cRNA. In the oocytes injected with 5-HT3A receptor cRNA, co- or pre-treatment of resveratrol with 5-HT potentiated 5-HT-induced inward peak current (I_{5-HT}) with concentration-, reversible, and voltage-independent manners. The EC_{50} of resveratrol was 28.0 ± 2.4 μM. The presence of resveratrol caused a leftward shift of 5-HT concentration–response curve. Protein kinase C (PKC) activator or inhibitor had no effect on resveratrol action on I_{5-HT}. Site-directed mutations of pre-transmembrane domain 1 (pre-TM1) such as R222A, R222D, R222E, R222K, and R222T abolished or attenuated resveratrol-induced enhancement of I_{5-HT}, indicating that resveratrol might interact with pre-TM1 of 5-HT3A receptor. These results indicate that resveratrol might regulate 5-HT3A receptor channel activity via interaction with the N-terminal domain and these results further show that resveratrol-mediated regulation of 5-HT3A receptor channel activity might be one of cellular mechanisms of resveratrol action.

Key words resveratrol; serotonin; 5-hydroxytryptamine type 3A receptor; ligand-gated ion channel; Xenopus oocyte

Resveratrol is a phytoalexin found in grapes, red wine, and berries (Fig. 1A) and is also produced by plants as an anti-fungal chemical. The contents of resveratrol in grapes is about 0.002—0.004% in grape skin and seed and is about 0.2—5.8 mg/l in red wine. Resveratrol exhibits a variety of pharmacological activities, such as anti-cancer, neuro-protective, anti-inflammatory, and life-prolonging effects. With regards to the nervous system, resveratrol has shown neuro-protective effects. For example, resveratrol ameliorates neurodegenerative disorders such as Alzheimer’s disease. Resveratrol also prevents neuronal cell death from in vitro or in vivo brain hypoxia or ischemic conditions as an antioxidant. Although this large body of evidence indicates that resveratrol has diverse beneficial properties including protective effects on the nervous system, relatively little is known regarding its cellular mechanism of action especially with respect to possible regulation of receptors involved in synaptic transmission.

5-Hydroxytryptamine type 3 (5-HT3) receptor is a member of the Cys-loop family of ligand-gated ion channels, which also include nicotinic acetylcholine, γ-aminobutyric acid (GABA)A, and glycine receptors and has been reported to mediate rapid transient membrane depolarizing effect of 5-HT in the central and peripheral nervous system like nicotinic acetylcholine receptors for fast synaptic transmissions. The subunit of 5-HT3A receptor is composed of the N-terminal domain, four transmembrane domains (TM1 to TM4), and an intracellular domain (Fig. 1B), and finally, homomeric pentamers of each subunit consist of 5-HT3 receptor. The 5-HT binding sites are assumed to be located in the N-terminal domain at subunit-subunit interfaces (Fig. 1B). The physiological and pathological roles of 5-HT3 receptors involve pain transmission, analgesia, vomiting, irri-
table bowel syndrome, mood disorders, and drug abuse.12)

In this study, we first investigated the possible regulation of 5-HT3A receptor channel activity, and further identified interaction site(s) of resveratrol with on the 5-HT3A receptor channel expressed in Xenopus oocytes using site-directed mutagenesis methods. For this study, we expressed neuronal human 5-HT3A receptor cRNAs into Xenopus laevis oocytes and examined the effect of resveratrol on 5-HT-elicited inward peak currents (I5-HT). The reason we used this system was that: (1) Xenopus oocytes have been widely used as a tool to express membrane proteins encoded by exogenously administered cDNAs or cRNAs including receptors, ion channels, and transporters,13) and, (2) 5-HT3A receptor channels expressed in Xenopus oocytes by injection of 5-HT3A receptor cRNAs subunits are well studied and characterized.11) We report that co-treatment of resveratrol with 5-HT potentiates I5-HT in both a concentration-dependent and voltage-independent manner and that pre-TM1 plays an important role in resveratrol-mediated regulation of 5-HT3A receptor.

MATERIALS AND METHODS

Materials Figure 1A shows the structure of resveratrol. Resveratrol used in this study was dissolved in dimethyl sulfoxide (DMSO) as previously reported14) and were diluted with bath medium before use. Since resveratrol is sensitive to light, it was kept in dark and was prepared fresh stock solution at experiment every experiment. Final DMSO concentration was less than 0.1%. Resveratrol and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Oocyte Preparation Xenopus laevis care and handling were in accordance with the guide for the ‘Care and Use of Laboratory Animals,’ published by NIH, U.S.A. Frogs underwent surgery only twice, separated by an interval of at least 3 weeks. To isolate oocytes, frogs were anesthetized with an aerated solution of 3-amino benzoic acid ethyl ester. Oocytes were separated by treatment with collagenase, with gentle shaking for 2 h in CaCl2-free medium containing 82.5 NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM Na2HPO4, and 5 mM HEPES, pH 7.5 supplemented with 0.5 mM theophylline and 50 µg gentamycin/ml. All solutions were changed daily. All experiments were performed within 2–4 d following isolation of the oocytes.15)

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

cRNA Preparation of 5-HT3A Receptor and Microinjection Recombinant plasmid containing mouse 5-HT3A cDNA insert was linearized by digestion with appropriate restriction enzymes. The cRNAs from linearized templates were obtained by using an in vitro transcription kit (mMessage mMachine; Ambion, Austin, TX, U.S.A.) with a T3 polymerase. The RNA was dissolved in RNase-free water at 1 µg/µl, divided into aliquots, and stored at −80 °C until used. Oocytes were injected with H2O or mouse 5-HT3A receptor cRNAs (5–10 ng) by using a Nanoject Automatic Oocyte Injector (Drummond Scientific, Broomall, PA, U.S.A.). The injection pipette was pulled from glass capillary tubing used for recording electrodes and the tip was broken to 15–20 µm in diameter.15)

Site-Directed Mutagenesis of 5-HT3A Receptor and in Vitro Transcription of 5-HT3A Receptor The substitution mutation of single or three amino acids was performed by Pfu DNA polymerase (QuickChangeTM XL Site-Directed Mutagenesis Kit, STRATAGENE, U.S.A.) and mutated sense and antisense primers. The overlap extension at the target domain by sequential polymerase chain reaction was performed in accordance with the supplier instruction manual with some modifications. Final PCR products were transformed to Escherichia coli DH5α strain, and screened by PCR method. The mutations were confirmed by DNA sequencing analysis on the target region. The mutant DNA constructs were linearized at the 3’ end by SalI digestion, and run-off transcripts were prepared using methylated cap analog m7G(5')ppp(5')G and and RNAs were prepared using T3 RNA polymerase included in the mMessage mMachine transcription kit (Ambion). The final cRNA products were resuspended with RNase-free water at concentration of 1 µg/µl and stored at −80 °C.

Data Analysis To obtain the concentration–response curve for 5-HT-induced current in the presence of resveratrol, the observed peak amplitudes were normalized and plotted and then fitted to the Hill equation below using Origin software (Northampton, MA, U.S.A.). y/y max = [A]n/(ECn0 + [ECn0]), where y represents % potentiation at a given concentration of resveratrol, y max represents % of maximal inhibition, ECn0 is the concentration of resveratrol producing half-maximum inhibition of the control response to 5-HT, [A] is the concentration of resveratrol, and n is the interaction coefficient. All values are presented as the mean ± S.E.M. The differences between means of control and resveratrol treatment data were analyzed using unpaired Student's t test and one-way analysis of variance (ANOVA) test. A value of p<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

As shown in previous reports,15,16) the addition of 5-HT to bathing solution induced a large inward current (I5-HT) in oocytes injected with wild-type 5-HT3A receptor cRNA (Fig. 2A). I5-HT was blocked by selective 5-HT3A receptor antagonist, MDL-72222 (0.5 µM), and in H2O-injected control oocytes, treatment of 5-HT did not induce any inward current as previously shown (data not shown). Resveratrol (30 µM) itself had no effect in oocytes expressing 5-HT3A receptor at a holding potential of −80 mV (Fig. 1C). But pre- or co-treatment of resveratrol (30 µM) with 5-HT (1 µM) for 30 s enhanced I5-HT in oocytes expressing wild-type 5-HT3A receptor (Fig. 2A, n=10—12 from three different frogs). Thus, pre-
and co-treatment of resveratrol with 5-HT induced the enhancement of \( I_{\text{5-HT}} \) by 407.3±31.5 and 387.6±35.6%, respectively, indicating that resveratrol effect on \( I_{\text{5-HT}} \) was not affected by pre-treatment (Fig. 2B). In concentration-dependent experiments using resveratrol, co-treatment with resveratrol for 30s enhanced \( I_{\text{5-HT}} \) in a concentration-dependent manner in oocytes expressing wild-type 5-HT\(_{3A}\) receptors (Figs. 2C, D). Thus, resveratrol enhanced \( I_{\text{5-HT}} \) by 5.1±6.1, 34.2±13.2, 61.3±21.8, 290.2±44.6, 535.1±40.4 and 536.2±47.2% at 1, 3, 10, 30, 100, and 300 \( \mu \)M in oocytes expressing wild-type 5-HT\(_{3A}\) receptors, respectively. The EC\(_{50}\) of \( I_{\text{5-HT}} \) were 28.0±2.4 \( \mu \)M in oocytes expressing 5-HT\(_{3A}\) receptor (\( n = 10—12 \), from three different frogs for each point) (Fig. 2D).

To further investigate the mechanism by which resveratrol enhances \( I_{\text{5-HT}} \) in oocytes expressing 5-HT\(_{3A}\) receptor, we analyzed the effect of 30 \( \mu \)M resveratrol on \( I_{\text{5-HT}} \) evoked by different 5-HT concentrations in oocytes expressing 5-HT\(_{3A}\) receptor (Fig. 3A). Co-treatment of resveratrol for 30s with various concentrations of 5-HT significantly shifted the concentration–response curve of 5-HT to the left (EC\(_{50}\) from 1.95±0.1 to 0.74±0.03 \( \mu \)M, \( p < 0.05 \) and Hill coefficient, from 1.9 to 2.5) in oocytes expressing 5-HT\(_{3A}\) receptor. Thus, the presence of resveratrol with 5-HT (0.3 to 3 \( \mu \)M) further induced a potentiation of \( I_{\text{5-HT}} \) enhancements compared to 5-HT alone but at high concentration of 5-HT (10, 30 \( \mu \)M) resveratrol had no effect (Fig. 3A).

In experiments exploring the current–voltage relationship, the membrane potential was held at −80 mV and a voltage ramp was applied from −100 to +60 mV during 300 ms. In the absence of 5-HT, the inward current at −100 mV was <0.01 \( \mu \)A and the outward current at +60 mV was near 0.1 \( \mu \)A (data not shown). The addition of 5-HT to the bathing medium mainly induced an inward current at negative voltages and an outward current at positive voltages. Co-treatment of resveratrol with 5-HT increased both inward and outward currents. The reversal potential was near 0 mV in both 5-HT and 5-HT plus resveratrol conditions. This suggests that 5-HT induces the cation current.\(^{17}\) Also, co-treatment of resveratrol with 5-HT did not affect the 5-HT receptor channel property because resveratrol did not change the reversal potential of 5-HT\(_{3A}\) receptor (Fig. 3B). The enhancing effect of resveratrol on \( I_{\text{5-HT}} \) in oocytes expressing 5-HT\(_{3A}\) receptor was independent of the membrane holding potential (Fig. 3C). Co-treatment of resveratrol inhibited \( I_{\text{5-HT}} \) by 342.9±25.2, 294.7±34.6, 342.4±39.5, and 299.2±49.0% at −120, −90, −60, and −30 mV membrane holding potentials in oocytes expressing 5-HT\(_{3A}\) receptor, respectively (\( n = 10—12 \), from three different frogs).

The above results indicate that resveratrol may be a novel regulator of 5-HT\(_{3A}\) receptor channel. Furthermore, since protein kinase C (PKC) is involved in the regulation of 5-HT\(_{3A}\) receptor channel activity\(^{18}\) and resveratrol regulates...
PKC activity, we examined whether resveratrol-mediated enhancement of $I_{5\text{-HT}}$ is achieved through PKC regulation. To do this, we first treated oocytes with phorbol 12-myristate 13-acetate (PMA), a PKC activator before resveratrol and examined the effects of resveratrol on $I_{5\text{-HT}}$. As shown in Fig. 4A, treatment of PMA potentiated $I_{5\text{-HT}}$ as much as resveratrol. However, co-application of resveratrol and PMA with 5-HT did not induce further potentiation of $I_{5\text{-HT}}$ compared to summation of resveratrol- or PMA-mediated potentiation of $I_{5\text{-HT}}$, indicating that resveratrol-mediated enhancement of $I_{5\text{-HT}}$ is independent of PKC activation. Staurosporin, a PKC inhibitor, did not affect resveratrol action (Fig. 4B). In addition, co-application of staurosporin with resveratrol and PMA did not affect $I_{5\text{-HT}}$ (data not shown), indicating that resveratrol-mediated enhancement of $I_{5\text{-HT}}$ is independent of PKC activity.

Next, since the presence of resveratrol with 5-HT induced a potentiation of $I_{5\text{-HT}}$, resulting in enhancement of $I_{5\text{-HT}}$ as shown in Figs. 2 and 3, we investigated whether the effect of resveratrol on $I_{5\text{-HT}}$ is related to 5-HT 3A receptor facilitation by resveratrol and whether resveratrol-mediated enhancement of $I_{5\text{-HT}}$ is affected after site-directed mutations of 5-HT 3A receptor pre-TM1 of N-terminal domain, which is a known site for 5-HT3-mediated $I_{5\text{-HT}}$ and affected receptor antagonist-induced inhibition of $I_{5\text{-HT}}$. We chose these sites for site-directed mutations since mutation of the pre-TM1 of N-terminal domain facilitates 5-HT3-mediated $I_{5\text{-HT}}$ and affected receptor antagonist-induced inhibition of $I_{5\text{-HT}}$. Figures 5A—C show the representative traces in the absence or presence of resveratrol in each mutant of pre-TM1 of N-terminal domain sites. Interestingly, mutations of R222A, R222D, R222E, and R222T abolished resveratrol-induced enhancement of $I_{5\text{-HT}}$. As well, lysine R222K dramatically attenuated resveratrol action on $I_{5\text{-HT}}$. Figure 5D shows the concentration–response relationship for $I_{5\text{-HT}}$ enhancements by resveratrol in different mutants where the smooth lines represent the best fits of the data using the Hill equation with the parameters of the fits. Thus, $V_{\text{max}}$ values of R222A, R222D, R222E, R222K, and R222T at 10—300 μM resveratrol were significantly different from that of wild-type channel.

In the present study, we demonstrated that (1) pre- or co-treatment of resveratrol with 5-HT induced a potentiation of $I_{5\text{-HT}}$ in oocytes expressing 5-HT 3A receptor in a reversible and concentration-dependent manner, (2) the potentiation of
$I_{5\text{-HT}}$ by resveratrol co-treatment with 5-HT caused a leftward shift of the 5-HT concentration–response curve and was voltage-independent in oocytes expressing 5-HT$_{3\text{A}}$ receptor, and finally, (3) in site-directed mutagenesis experiments we found that mutations of N-terminal of pre-TM1 abolished resveratrol-induced potentiation of $I_{5\text{-HT}}$.

From the present results, however, we still have not been able to elucidate how resveratrol enhances $I_{5\text{-HT}}$ in oocytes expressing 5-HT$_{3\text{A}}$ receptors. One possible mechanism is that resveratrol may potentiate $I_{5\text{-HT}}$ via a membrane signal transduction pathway related with PKC. However, this may not be the case because the enhancing effect of resveratrol on $I_{5\text{-HT}}$ in oocytes expressing 5-HT$_{3\text{A}}$ receptors was not affected by PKC activator and PKC inhibitor, indicating that resveratrol action on 5-HT$_{3\text{A}}$ receptor channel activity is not related with PKC activity (Fig. 4).

The other possibility is that resveratrol may work as a novel agent related to facilitation of 5-HT binding to its binding site(s) in 5-HT$_{3\text{A}}$ receptor. In competition experiments, we observed that the presence of resveratrol significantly shifted the concentration curve of 5-HT to the left from 1.95±0.1 to 0.74±0.03 μM in oocytes expressing 5-HT$_{3\text{A}}$ receptor without significantly changing the Hill coefficient (Fig. 3A), suggesting that the presence of resveratrol might be related to receptor facilitation in the presence of 5-HT, resulting in potentiation of $I_{5\text{-HT}}$.

Supporting this notion is the fact that in contrast to wild-type 5-HT$_{3\text{A}}$ receptor, mutation of R222 to R222A or R222T, of which site is located in pre-TM1 (Fig. 1) and is known to facilitate receptor activation rate, nearly abolished resveratrol-induced potentiation of $I_{5\text{-HT}}$. Interestingly, we also observed that mutation of R222 to R222A, R222 to R222D, R222 to R222E, R222 to R222K, or R222 to R222T attenuated or abolished resveratrol-induced potentiation of $I_{5\text{-HT}}$, suggesting that hydrophobic or neutral amino acids (alanine or threonine), positively charged amino acids (lysine) or negatively charged amino acids (aspartate or glutamate) might not discriminate resveratrol action at pre-TM1. Altogether, both competitive modulation of 5-HT$_{3\text{A}}$ receptor channel activity and interaction with pre-TM1 site by resveratrol suggest that resveratrol might regulate 5-HT$_{3\text{A}}$ receptor channel activity via interactions with more than one site of 5-HT$_{3\text{A}}$ receptor, since it is known that 5-HT binds N-terminal interface of subunit assembly. However, we could not exclude the possibility that resveratrol enhances 5-HT$_{3\text{A}}$ receptor channel currents through an allosteric change induced by interaction with the unidentified 5-HT$_{3\text{A}}$ receptor channel protein besides pre-TM1. Further studies will be required to elucidate how resveratrol connects R222 at pre-TM1 with $I_{5\text{-HT}}$ enhancement in a competitive manner.

The previous reports have shown that resveratrol effects on nervous systems might be related with ligand-gated ion channels. For example, resveratrol-mediated neuroprotection against brain ischemia was blocked by N-methyl-D-aspartate (NMDA) receptor antagonist. Resveratrol also attenuated kainite-induced epilepsy. In addition, resveratrol inhibited catecholamine secretion by inhibiting α3β4 nicotinic acetylcholine receptor in adrenal medullary cells. Thus, although resveratrol mainly shows neuroprotective effects, there is still only a rudimentary understanding of how resveratrol regulates receptor or ion channel activities at the cellular level. In the present study, we found that pre-TM1 of 5-HT$_{3\text{A}}$ receptor plays an important role for resveratrol-mediated 5-HT$_{3\text{A}}$ receptor channel activity using site-directed mutagenesis method. The present findings that resveratrol enhances $I_{5\text{-HT}}$ in oocytes expressing 5-HT$_{3\text{A}}$ receptor show the possibility that resveratrol might be involved in 5-HT$_{3\text{A}}$ receptor-mediated physiological or pharmacological activity. Further study will be required to find out how in vitro resveratrol-mediated potentiation of $I_{5\text{-HT}}$ is linked to 5-HT$_{3\text{A}}$ receptor-related in vivo pharmacology in central and peripheral nervous systems.

In conclusion, we found that resveratrol, an active ingredient found in grapes, enhances 5-HT$_{3\text{A}}$ receptor-mediated ion currents by interacting with pre-TM1 of 5-HT$_{3\text{A}}$ receptor expressed in *Xenopus* oocytes, and these results further indicated that this regulation of 5-HT$_{3\text{A}}$ receptor channel activity by resveratrol might be a cellular basis of resveratrol action.

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