Resveratrol Inhibits GABA$_C$ ρ Receptor-Mediated Ion Currents Expressed in *Xenopus* Oocytes

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Resveratrol is a phytoalexin found in grapes, red wine, and berries. Resveratrol has been known to have many beneficial health effects, such as anti-cancer, neuroprotective, anti-inflammatory, and life-prolonging effects. However, relatively little is known about the effects of resveratrol on the regulation of ligand-gated ion channels. We have previously reported that resveratrol regulates subsets of homomeric ligand-gated ion channels such as those of 5-HT$_{3A}$ receptors. The γ-aminobutyric acid$_C$ (GABA$_C$) receptor is mainly expressed in retinal bipolar cells and plays an important role in visual processing. In the present study, we examined the effects of resveratrol on the channel activity of homomeric GABA$_C$ receptor expressed in *Xenopus* oocytes injected with cRNA encoding human GABA$_C$ ρ subunits. Our data show that the application of GABA elicits an inward peak current (I_{GABA}) in oocytes that express the GABA$_C$ receptor. Resveratrol treatment had no effect on oocytes injected with H$_2$O or with GABA$_C$ receptor cRNA. Co-treatment with resveratrol and GABA inhibited I_{GABA} in oocytes with GABA$_C$ receptors. The inhibition of I_{GABA} by resveratrol was in a reversible and concentration-dependent manner. The IC$_{50}$ of resveratrol was 28.9±2.8 μM in oocytes expressing GABA$_C$ receptor. The inhibition of I_{GABA} by resveratrol was in voltage-independent and non-competitive manner. These results indicate that resveratrol might regulate GABA$_C$ receptor expression and that this regulation might be one of the pharmacological actions of resveratrol on the nervous system.

Key Words: GABA$_C$ receptor, Ligand-gated ion channel, Resveratrol, *Xenopus* oocyte

INTRODUCTION

γ-Aminobutyric acid (GABA) receptors are members of the Cys-loop family with ligand-gated ion channels, and the Cys-loop family includes proteins such as 5-hydroxytryptamine 3 (5-HT3), nicotinic acetylcholine, and glycine receptors [1]. The structure of the ligand-gated ion channels is similar to that of the pentameric ion channels. In addition, the ion channels make channel pores through the transmembrane 2 (TM2) domain [1]. The GABA receptors are classified into 3 types of receptors: GABA$_A$, GABA$_B$, and GABA$_C$. The GABA$_A$ and GABA$_C$ receptors function as anion selective channels that are permeable to chloride

ions, whereas GABA$_B$ receptors are G-protein-coupled receptors [2,3]. GABA$_A$ receptors are composed of heteropentameric anion channels composed of α, β, γ, δ, ε, θ and π subunits; however GABA$_C$ receptors can form homopentameric or heteropentameric anion channels by ρ subunits alone or with α1 and γ2 [2-4]. The GABA$_A$ receptor is predominantly expressed in the central nervous system [5,6], whereas GABA$_C$ receptors are mainly expressed in retinal bipolar cells [7,8] and have a lower abundance in cerebellum [9] and hippocampus [10]. They play an important role in vision, sleep, cognition, and memory [11].

Resveratrol is a phytoalexin found in grapes, red wine, and other berries (Fig. 1A) and is also produced as an anti-fungal chemical by plants [12]. The concentration of resveratrol in red wine is as high as 0.2∼5.8 mg/l [13]. Resveratrol exhibits diverse physiological and pharmacological activities, such as anti-cancer, chemopreventive, antiviral, cardio-protective, anti-aging, anti-inflammatory, and life-prolonging effects [13-15]. Resveratrol also has neuroprotective effects, and it attenuates neurodegenerative disorders such as Alzheimer's disease [16]. It also attenuates neuronal cell death caused by *in vitro* or *in vivo* brain hypoxia or ischemic conditions [17,18]. Although ac-

ABBREVIATIONS: GABA, γ-aminobutyric acid; Res, resveratrol; I_{GABA}, GABA-mediated inward current.

cumulating evidence indicates that resveratrol has diverse beneficial properties, including protective effects on the nervous systems, relatively little is known about its effects on cells, especially with respect to the regulation of receptors involved in synaptic transmission.

Recently, we demonstrated that resveratrol regulates 5-HT$_{3A}$ receptor expression [19]. In the present study, we examined the effects of resveratrol on homomeric GABA$_C$ receptor channel activity and found that resveratrol inhibits GABA$_C$ receptor channel activity in a concentration-dependent, voltage-independent, and non-competitive manner. These results indicate that resveratrol might play a role in the regulation of GABA$_C$ receptor channel activities.

METHODS

Materials

The cDNAs for human GABA$_C$ receptor ρ 1 subunit were purchased from Thermo Fisher Scientific Inc. (Wyman Street Waltham, MA, USA). Fig. 1A shows the structure of resveratrol. Resveratrol used in this study was dissolved in dimethyl sulfoxide (DMSO) as previously reported [20] and was diluted with bath medium before use. Resveratrol was stored in the dark because it is light sensitive, and a fresh stock solution was prepared for every experiment. The final DMSO concentration was less than 0.1%. Resveratrol and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Oocyte preparation

Xenopus laevis care and handling were performed in accordance to the guide for the *Care and Use of Laboratory Animals*, published by NIH, USA. Frogs underwent surgery only twice, separated by an interval of at least 3 weeks. Frogs were anesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester for oocyte isolation. Oocytes were separated by collagenase treatment along with gentle shaking for 2 h in a CaCl$_2$-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 mM sodium

Fig. 1. Chemical structure of resveratrol and effect of resveratrol (Res) on GABA$_C$ receptors expressing oocytes. (A). Application of resveratrol (100 μM) for 1 min had no effect on I_{GABA} expression in oocytes expressing GABA$_C$ receptors (B).

pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Only stage 5 or 6 oocytes were collected and maintained at 18°C, with continuous gentle shaking in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, and 5 mM HEPES; pH 7.5) supplemented with 0.5 mM theophylline and 50 μg/ml gentamycin. All the solutions were changed daily. All the experiments were performed within 2~4 days following the isolation of the oocytes [21].

Oocyte recording

A single oocyte was placed in a small Plexiglas net chamber (0.5 ml) and was constantly superfused with ND96 medium in the presence or absence of GABA 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) with Digidata 1200A. For most of the electrophysiological experiments, the oocytes were clamped at a holding potential of -80 mV. For the current-voltage relationship, voltage ramps were applied from -100 to +40 mV for 300 ms.

cRNA preparation of GABA$_C$ receptor ρ 1 and microinjection

A recombinant plasmid containing a human GABA$_C$ receptor ρ 1 cDNA insert was linearized by digestion with the appropriate restriction enzymes. The cRNAs from linearized templates were obtained by using an *in vitro* transcription kit (mMessage mMachine; Ambion, Austin, TX) 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 use. Oocytes were injected with H$_2$O or mouse glycine α 1 receptor cRNAs (5~10 ng) by using a Nanoject Automatic Oocyte Injector (Drummond Scientific, Broomall, PA). The injection pipette was pulled from the glass capillary tubing used for the recording electrodes, and the tip was broken to obtain an outer diameter of approximately 20 μm [21]. The final cRNA products were resuspended with RNase-free water at a concentration of 1 μg/μl and stored at -80°C.

Data analysis

To obtain the concentration-response curve for GABA-induced current in the presence of resveratrol, the observed peak amplitudes were normalized and plotted, and then fitted to the Hill equation, described below, by using Origin software (Northampton, MA): $y/y_{max}=[A]^n/([A]^n+[IC_{50}]^n)$, where y represents percent (%) inhibition at a given concentration of resveratrol, y_{max} represents percent (%) maximal inhibition, IC_{50} is the concentration of resveratrol producing half-maximum inhibition of the control response to GABA, [A] is the concentration of resveratrol, and n is the interaction coefficient. All the values are presented as the mean± S.E.M. The differences between mean values of control and that of resveratrol treatment data were analyzed using unpaired Student's *t* test and one-way ANOVA test. A value of p$<$0.05 was considered statistically significant.
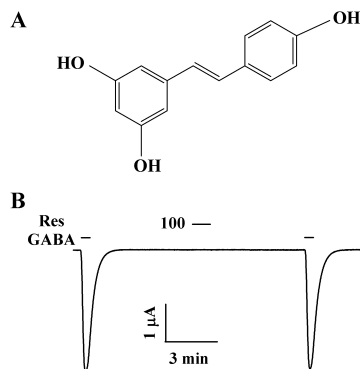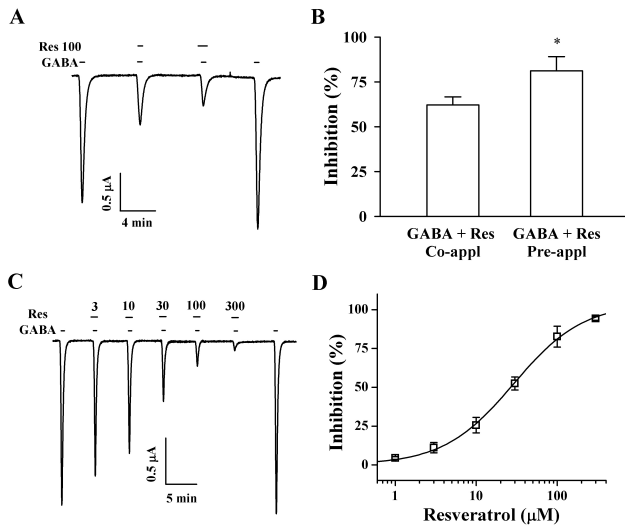
Fig. 2. Effect of Res on I_{GABA} expression in oocytes that express GABA_C receptors. (A) GABA (2 μM) was first applied and then GABA was co- or pre-applied with Res (100 μM). Thus, co- and pre- application of Res with GABA inhibited I_{GABA}. The resting membrane potential of oocytes was approximately -35 mV, and oocytes were voltage clamped at a holding potential of -80 mV prior to drug application. Traces are representative of 6 separate oocytes from 3 frogs. (B) Pre-application of Res inhibited I_{GABA} more potently than that inhibited by co-treatment. (C) I_{GABA} in GABA_C receptors expressing oocytes was elicited at -80 mV holding potential, with indicated time in the presence of 2 μM GABA and with the indicated pre-treatment concentration of Res that was applied before GABA application. (D) I_{GABA} % inhibition induced by Res treatment was calculated using the average of peak that the inward current elicited by GABA treatment before Res application and of the peak inward current elicited by GABA treatment after pre-treatment of Res before GABA. The continuous line shows the curve fitted according to the equation. Each point represents the mean±S.E.M. (n=9~12 from 3 frogs).

Fig. 3. Time-dependent effects of pre-application of Res on I_{GABA} in oocytes that express GABA_C receptors. (A) Res (100 μM)-mediated inhibition on I_{GABA} is pre-application-time dependent. Traces represent 6 separate oocytes from 3 frog batches. I_{GABA} in GABA_C receptor-expressing oocytes was elicited at a holding potential of -80 mV for the indicated Res pre-application time prior to drug application. (B) Res-mediated inhibition of I_{GABA} was almost saturated after 30 s of pre-application. The resting membrane potential of the oocytes was approximately -35 mV, and the oocytes were voltage-clamped at a holding potential of -80 mV. Each point represents the mean±S.E.M. (n=9~12/group).

RESULTS

Effect of resveratrol on I_{GABA} in oocytes that express homomeric GABA_C receptors

The addition of GABA to the bathing solution induced a large inward current in oocytes injected with GABA_C receptor, indicating that functional GABA_C receptors were expressed by the oocytes (Fig. 1B). Resveratrol itself had no effect on oocytes with GABA_C receptors at a holding potential of -80 mV (Fig. 1B). Interestingly, pre-treatment of resveratrol induced a much larger inhibition of I_{GABA} than that after co-treatment (Fig. 2A and B, n=9 from 3 frogs). The inhibition of I_{GABA} by resveratrol in oocytes with GABA_C receptors was reversible (Fig. 2A). Thus, these results show that resveratrol may regulate GABA_C receptors channel activity, although resveratrol itself had no effect on GABA_C receptor channel activity.

Concentration-dependent effect of resveratrol on I_{GABA} in oocytes with GABA_C receptor

Since pre-treatment of resveratrol enhanced the inhibition on I_{GABA} in oocytes that expressed GABA_C receptor as compared to the I_{GABA} inhibition in co-treated oocytes, we
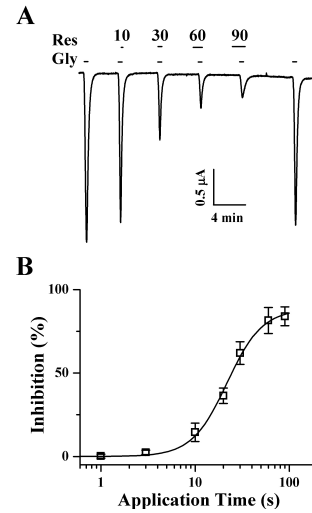
examined the effects of resveratrol on I_{GABA} after the pre-treatment of resveratrol with GABA. In concentration-response experiments, the pre-treatment of resveratrol with GABA inhibited I_{GABA} in a concentration-dependent manner in oocytes with GABA_C receptor (Fig. 2C). The IC_{50} of I_{GABA} was 28.9±2.8 μM for oocytes that expressed GABA_C receptors (n=9~12 from 3 frogs) (Fig. 2D). Since the pre-application of resveratrol showed a stronger decrease of I_{GABA} than that observed for the co-application of resveratrol, we examined the time-dependent effects of resveratrol pre-application. As shown in Fig. 3A, the pre-application of resveratrol enhanced I_{GABA} inhibition and was time-dependent, while the time-dependent effects of resveratrol were almost saturated at 30 sec pre-application (Fig. 3B).

Current-voltage relationship and voltage-independent inhibition of I_{GABA} in oocytes that express GABA_C receptors mediated by resveratrol

As shown in Fig. 4, the current-voltage relationship induced by GABA with voltage steps from -100 to $+40$ mV showed a slight rectification at positive potentials in oocytes with GABA_C receptors. The reversal potential of GABA_C receptors was Vr=-25.3±1.8 mV. (mean±S.E.M., n= 6 from 3 frogs). Pre-treatment with resveratrol and GABA did not modify the potential reversal of GABA_C receptor with a reduction of I_{GABA} (n=6 from 3 frogs). The inhibitory effect of resveratrol on I_{GABA} in oocytes that express GABA_C receptors was independent of the membrane holding potential (Fig. 4B). Thus, resveratrol inhibited I_{GABA} by 75.0±5.1, 79.6±3.1, 76.3±2.7, and 79.1±4.2% at -120, -90, -60, and -30 mV membrane holding potential in oocytes with GABA_C receptor, respectively (Fig. 4B; n=9~12, from 3
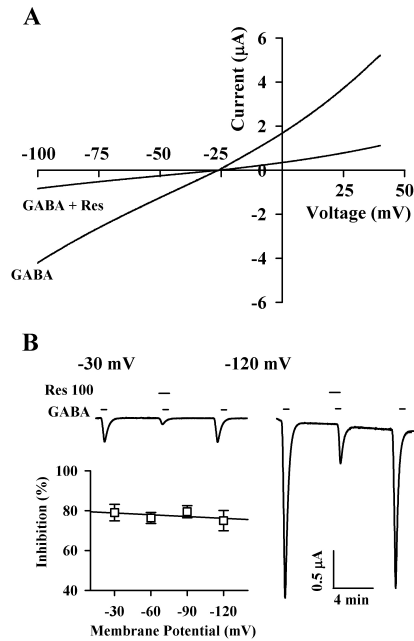
A

B

Fig. 4. Current-voltage relationship and voltage-independent inhibition by Res. (A) Current-voltage relationships of I_{GABA} inhibition by Res in GABA$_C$ receptor-expressing oocytes. Representative current-voltage relationships were obtained using voltage ramps of −100 to +40 mV for 300 ms at a holding potential of −80 mV. Voltage steps were applied before and after application of 2 μM GABA in the absence or presence of 100 μM Res. (B) Voltage-independent inhibition of I_{GABA} in the GABA$_C$ receptors by Res. *Inset*; the values were obtained from the receptors in the presence or absence of 100 μM Res at the indicated membrane holding potentials.

A

B

Fig. 5. Concentration-dependent effects of GABA on Res-mediated inhibition of I_{GABA}. (A) The representative traces were obtained from the GABA$_C$ receptor-expressing oocytes. I_{GABA} expression shown in the upper and lower panels were elicited at a holding potential of −80 mV by GABA at concentrations of 1 μM and 30 μM GABA respectively. (B) Concentration-response relationship of GABA with GABA$_C$ receptors treated with GABA (0.3~30 μM) alone or with GABA plus pre-application of 30 μM or 100 μM Res. The I_{GABA} of oocytes expressing the GABA$_C$ receptors was measured using the indicated concentration of GABA in the absence (□) or presence of 30 μM (○) or 100 μM (△) Res. Oocytes were exposed to GABA alone or to GABA with Res. Oocytes were voltage-clamped at a holding potential of −80 mV. Each point represents mean±S.E.M. (n=9~12/group).

frogs).

Noncompetitive inhibition of GABA$_C$ receptors by resveratrol

To further study the mechanism by which resveratrol inhibits I_{GABA} in oocytes with GABA$_C$ receptors, we analyzed the effect of 100 μM resveratrol on I_{GABA} evoked by various GABA concentrations on oocytes with GABA$_C$ receptors (Fig. 5). Pre-application of 30 or 100 μM resveratrol with different concentrations of GABA did not shift the dose-response curve of GABA to the right (ED$_{50}$, from 2.2±0.3 to 2.4±0.1 and 3.1±0.2 μM and Hill coefficient, from 1.76 to 1.87 and 1.55) in oocytes expressing GABA$_C$ receptors, indicating that resveratrol regulates GABA$_C$ receptor channel activity in a non-competitive manner (n=9~12 from 3 frogs; Fig. 5).

DISCUSSION

In the present study, we demonstrated that (1) co- or pre-treatment with resveratrol and GABA inhibited I_{GABA} expression in human GABA$_C$ receptor-expressing oocytes in a reversible and concentration-dependent manner. (2) I_{GABA} inhibition caused by resveratrol occurred in a non-competitive and voltage-independent manner in GABA$_C$ receptor-expressing oocytes, indicating that resveratrol could
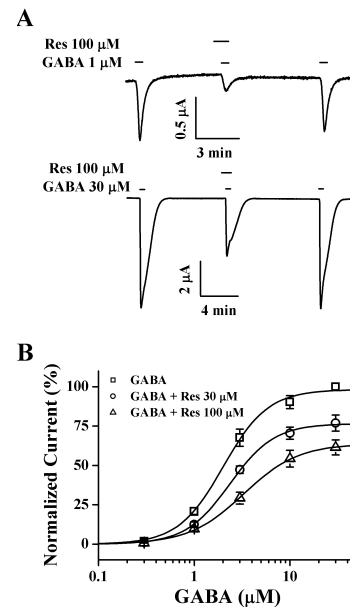
be associated with the inhibitory regulator of I_{GABA} in GABA$_C$ receptor-expressing oocytes (Fig. 4 and 5).

However, our data was insufficient for elucidating the mechanisms for resveratrol inhibition of I_{GABA} in GABA$_C$ receptor-expressing oocytes. The possibility that resveratrol may act as an open channel blocker of GABA$_C$ receptors seems unlikely because the inhibitory effect of resveratrol on I_{GABA} in oocytes expressing GABA$_C$ receptors was not voltage-dependent (Fig. 4). It is known that open channel blockers such as local anesthetics or hexamethonium are strongly voltage dependent, due to the charge that they carry in the transmembrane electrical field [22-24].

Another possibility may be that resveratrol is a competitive inhibitor of GABAc receptors and inhibits the receptors by interacting with the receptor-binding site(s). Competition experiment data showed that the presence of resveratrol did not change the concentration of GABA in oocytes that express GABA$_C$ receptors without changing the Hill coefficient (Fig. 5). Thus, the non-competitive modulation of GABA$_C$ receptor channel activity of resveratrol shows that resveratrol might have other binding site(s) or interaction site(s), such as those of a non-competitive inhibitor, on the GABA$_C$ receptors.

Finally, the third possibility may be that resveratrol has binding sites that enable the regulation of GABA$_C$ receptor. In previous reports, we have demonstrated that the regulatory effects of resveratrol on homomeric 5-HT$_3$ receptor

channel activities were attenuated or abolished by site-directed mutations of amino acid residues of pre-transmembrane domain of 5-HT$_3$ receptor [19]. On the basis of data from this study and that of the previous reports, we speculate that resveratrol achieves its effect through direct interactions with GABA$_C$ receptors. Further studies are required to identify resveratrol binding site(s) on the GABA$_C$ receptors.

Previous studies have shown that the effects of resveratrol on nervous system might be mediated by ligand-gated ion channels. For example, resveratrol-mediated neuroprotection against brain ischemia is inhibited by N-methyl-D-aspartate (NMDA) receptor antagonist [25]. Resveratrol also attenuates kainite-induced epilepsy [26]. In addition, resveratrol suppresses catecholamine secretion by inhibiting the $\alpha 3\beta 4$ nicotinic acetylcholine receptor in adrenal medullary cells [27]. In addition, resveratrol potentiates 5-HT$_{3A}$ receptor channel activity [19]. Thus, although resveratrol is known as a neuroprotective agent, the mechanisms of resveratrol-mediated regulation of receptor or ion channel activities at the cellular level are poorly understood.

GABA$_C$ receptor is expressed in retina, thalamus, hippocampus, pituitary gland, and gut [7-10,28-30]. Its role may include visual processing, regulation of sleep-waking rhythms, pain perception, memory, learning, regulation of hormones, and neuroendocrine gastrointestinal secretion. Thus, although GABA$_C$ receptor channel activity might be closely related with the regulation of visual processing and other brain functions, we currently do not understand the inhibition of GABA$_C$-receptor-mediated ion currents by resveratrol and its association with GABA$_C$ receptor-related functions in nervous system. Further studies are required to determine how in $vitro$ resveratrol-mediated inhibition of I_{GABA} is linked to GABA$_C$ receptor-related in $vivo$ pharmacology in the nervous system.

In summary, we found that resveratrol, an active ingredient found in grapes, inhibits the GABA$_C$ receptor-mediated ion currents by interacting with sites that are distinct from the GABA-binding site(s), and that these results further indicate that the resveratrol-mediated GABA$_C$ receptor regulation might be the cellular basis for its effects on the nervous system.

ACKNOWLEDGEMENTS

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