Effects of Ginsenoside Rg3 on α9α10 Nicotinic Acetylcholine Receptor-Mediated Ion Currents

Byung-Hwan Lee,a Sun-Hye Choi,a Sung-Hee Hwang,a Hyeon-Joong Kim,a Sang-Mok Lee,a Hyoung-Chun Kim,b Hyewon Rhim,c and Seung-Yeol Nah*a

aDepartment of Physiology, College of Veterinary Medicine and Bio/Molecular Informatics Center, Konkuk University; Seoul 143–701, Korea: bNeuropsychopharmacology and Toxicology Program, College of Pharmacy, Kangwon National University; Chunchon 200–701, Korea: and cLife Science Division, Korea Institute of Science and Technology; Seoul 136–791, Korea.

Received November 21, 2012; accepted January 13, 2013

© 2013 The Pharmaceutical Society of Japan

The authors declare no conflict of interest.

Ginsenosides is a low molecular weight substance found in ginseng as one of the active ingredients. Ginsenosides, like other herbal medicines, has a wide range of neuropharmacological actions including neuroprotective effects. The α9α10 nicotinic acetylcholine receptor is one of numerous nicotinic acetylcholine receptors that exists as a heteropentameric form in auditory hair cells of the cochlea. In this study, we report the effects of ginsenosides on rat α9α10 nicotinic acetylcholine receptor-mediated ion currents using the two-electrode voltage clamp technique. Treatment with acetylcholine evoked inward currents (IACh) in oocytes heterologously expressing the α9α10 nicotinic acetylcholine receptor. Ginsenosides blocked IACh in order of potency of Rg3 > Rb3 > CK> Re=Rg2 > Rf> Rc > Rb > Rg with reversible manners, and the blocking effect of Rg3 on IACh was same after pre-application than co-application of Rg3. The half maximal inhibitory concentration (IC50) of Rg3 was 39.6±2.4 μM. Rg3-induced IACh inhibition was not affected by acetylcholine concentration and was independent of membrane holding potential. Although the inhibitory effect of Rg3 on IACh was abolished in oocytes expressing α9 subunit alone, indicating that the presence of α10 subunit might be required for Rg3-induced regulations of α9α10 nicotinic acetylcholine receptor channel activity. These results indicate that α10 subunit of α9α10 nicotinic acetylcholine receptor might play an important role in Rg3-induced regulation of the α9α10 nicotinic acetylcholine receptor.

Key words ginseng; ginsenoside; α9α10 nicotinic acetylcholine receptor; Xenopus oocyte

Nicotinic acetylcholine receptors are members of the Cys-loop family of ligand-gated ion channels, which also includes 5-hydroxytryptamine 3, γ-aminobutyric acid A, and glycine receptors.16) Sixteen different nicotinic acetylcholine receptor subunits are currently known, and subunits of nicotinic acetylcholine receptor α (α1–7, α9 and α10), β (β1–4), γ, δ and ε have been identified.27) Neuronal nicotinic acetylcholine receptors contain α2–6 subunits that are usually expressed as heteropentamers in combination with β2–4 subunits3–5) and are found in the central and peripheral nervous systems.6,7) In contrast, the α7 and α9 subunits can form homomeric receptors.5,8) In particular, the α9 subunits can form heteropentameric receptors in combination with the α10 subunits.9) Although many nicotinic acetylcholine receptor subunits are expressed in the central and peripheral nervous systems, the distributions of α9α10 nicotinic acetylcholine receptor are restricted to certain cell populations, such as leucocytes, pituitary, skin keratinocyte, sperm, and dorsal root ganglion.9–14) The α9α10 nicotinic acetylcholine receptor is also expressed in mammalian vestibular and cochlear mechanosensory hair cells but has not been detected in the brain.9,10) The α9α10 nicotinic acetylcholine receptor displays a biphasic response to concentrations of extracellular calcium and exists as a heteropentamer with a stoichiometry of (α9)2(α10)2.9,15) The α9α10 nicotinic acetylcholine receptor is also related to various diseases such as tinnitus, hearing loss and auditory processing disorders.16) It is known that blockage of α9α10 nicotinic acetylcholine receptor reduces inflammation-related nerve injury.17) Ginsenosides, one of ginseng components, is a substance of low molecular weight uniquely found in ginseng (Fig. 1A). Ginsenosides exhibits diverse biological activities in nervous systems, with neuropharmacological actions such as analgesia, neuroprotection against neurotoxins or excitatory amino acids.18,19) However, the cellular mechanisms of ginsenoside activity are relatively unknown, especially with regards to possible regulation of receptors or ion channels involved in synaptic transmission in nervous system.

In previous reports, we have shown that ginsenosides or ginsenoside metabolites regulates the Cys-loop family of ligand-gated ion channels, such as 5-hydroxytryptamine 3A, human glycine α1 and nicotinic acetylcholine receptors. For example, the application of ginsenoside Rg2 inhibits 5-hydroxytryptamine- and glycine-induced peak inward currents (I5HT and IGly) of mouse 5-hydroxytryptamine 3A and human glycine α1 receptor channels expressed in Xenopus laevis oocytes. Ginsenoside Rg3 inhibits I5HT in a competitive and voltage-independent manner through interaction with amino acids locate in channel pore region, whereas ginsenoside Rg3 had no effects on wild-type α7 nicotinic acetylcholine receptors but inhibited mutant α7 nicotinic acetylcholine receptor at channel pore region.20,21) As noted above, the α9α10 nicotinic acetylcholine receptor plays an important role in auditory systems and contains the same Cys-loop as 5-hydroxytryptamine 3A and glycine receptors, which are all pentameric ligand-gated ion channels. However, relatively little is known about the effects of ginsenosides on α9α10 nicotinic acetylcholine receptor channel activity.

In this study, we investigated the effects of ginsenosides on the α9α10 nicotinic acetylcholine receptor channel activity.
regulation in Xenopus oocytes. We initially expressed rat α9 and α10 nicotinic acetylcholine receptor cRNAs in Xenopus oocytes. We examined the effect of ginsenosides on acetylcholine evoked inward currents (IACh) and found that ginsenoside Rg3 (Rg3) was most potent for the inhibition of IACh in oocytes expressing α9α10 nicotinic acetylcholine receptor. Inhibition of IACh by Rg3 was concentration dependent, reversible and voltage independent. Moreover, inhibition of Rg3 on IACh was non-competitive with acetylcholine. Rg3-induced inhibition of IACh was abolished in oocytes expressing α9 subunit alone. In the present study, we demonstrate that Rg3 is a novel agent that regulates the α9α10 nicotinic acetylcholine receptor through interaction with α10 subunit.

MATERIALS AND METHODS

Materials Ginsenosides (Fig. 1) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Ginsenosides was dissolved in dimethyl sulfoxide (DMSO) as a stock solution and was diluted with bath medium before use. The cDNAs for the rat α9 and α10 nicotinic acetylcholine receptors (Gene bank ID: NM_022930 and NM_022639) were used.

Preparation of Xenopus laevis Oocytes and Microinjection Xenopus laevis frogs were purchased from Xenopus I (Ann Arbor, MI, U.S.A.). Animal care and handling were in accordance with the highest standards of Konkuk University guidelines. To isolate oocytes, frogs were anesthetized with an aerated solution of 3-amino benzoic acid ethyl ester, and the ovarian follicles were removed. The oocytes were separated with collagenase 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-ethansulfonic acid (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 medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES, pH 7.5) supplemented with 50 µg/mL gentamicin. The solution containing the oocytes was maintained at 18°C with continuous gentle shaking and was replaced daily. Electrophysiological experiments were performed 3 to 6 d after oocyte isolation. For α9α10 nicotinic acetylcholine receptor experiments, oocytes were injected with both α9 and α10 nicotinic acetylcholine receptor-encoding cRNAs (40 nL, a 1:1 molar ratio) into the animal or vegetal pole of each oocyte one day after isolation, using a 10-µL microdispenser (VWR Scientific, West Chester, PA, U.S.A.) fitted with a tapered glass pipette tip (15 to 20 µm in diameter).

cRNA Preparation of the Rat α9α10 Nicotinic Acetylcholine Receptor The cDNA constructs were linearized at the 3′ ends by digestion with NotI, and run-off transcripts were prepared using the methylated cap analogue m⁷G(5′)ppp(5′)G. The cRNAs were prepared using a mMessage mMachine transcription kit (Ambion, Austin, TX, U.S.A.) with T7 RNA polymerase. The absence of degraded RNA was confirmed by denaturing agarose gel electrophoresis followed by ethidium bromide staining. The final cRNA products were re-suspended at a concentration of 1 µg/µL in RNase-free water and stored at −80°C.
Data Recording

A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings, as previously reported. \(^{20}\) A single oocyte was superfused continuously with ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), and 5 mM HEPES, pH 7.5) in the absence or presence of acetylcholine or ginsenosides during recording. Both voltage and current microelectrodes were filled with 3 mM KCl and had a resistance of 0.2 to 0.7 MΩ. Two-electrode voltage-clamp recordings were obtained at room temperature using an Oocyte Clamp (OC-725C, Warner Instruments) and were digitized using d igidata 1200A (Molecular devices, Sunnyvale, CA, U.S.A.). Stimulation and data acquisition were controlled using pClamp 8 software (Molecular devices). For electrophysiological experiments, the oocytes were clamped at a holding potential of −80 mV, and 1.5 s voltage steps were applied from −120 to +50 mV to assess the relationship between current and voltage. Linear leak and capacitance currents were corrected by means of the leak subtraction procedure. In all experiments, we incubated oocytes with the Ca\(^{2+}\)-chelator 1,2-bis(2-aminophenoxy)ethane-\(N,N,N′,N′\)-tetraacetic acid ace toxymethyl ester (BAPTA-AM; 100 \(\mu\)M) for 3 to 4 h prior to electrophysiological recording to avoid activation of the endogenous Ca\(^{2+}\)-sensitive Cl\(^−\) currents. \(^{9}\)

Data Analysis

To obtain the concentration-response curve for the effect of Rg\(_3\) on the inward \(I_{ACh}\) in oocytes expressing \(\alpha_9\alpha_{10}\) nicotinic acetylcholine receptor, \(I_{ACh}\) was plotted as a function of different concentrations of Rg\(_3\). Origin software (OriginLab Corp., Northampton, MA, U.S.A.) was used to fit the plot to the Hill equation: \(I/I_{\text{max}} = 1/[1 + ([A]/IC_{50})^{nH}]\), where \(I_{\text{max}}\) was the maximal current obtained from each IC\(_{50}\) value of acetylcholine in receptors, IC\(_{50}\) was the concentration of Rg\(_3\) required to decrease the response by 50\%, [A] was the concentration of Rg\(_3\), and nH was the Hill coefficient. All values were presented as the mean±S.E.M. The differences between the means of the 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 to be statistically significant.

RESULTS

Effect of Rg\(_3\) on \(I_{ACh}\) in Oocytes Expressing \(\alpha_9\alpha_{10}\) Nicotinic Acetylcholine Receptor

The addition of acetylcholine (10 \(\mu\)M) to the bathing solution induced a large \(I_{ACh}\) in oocytes injected with rat \(\alpha_9\alpha_{10}\) nicotinic acetylcholine receptor cRNAs (Fig. 2A). In H\(_2\)O-injected control oocytes, the application of acetylcholine did not induce any inward currents (data not shown). Ginsenosides (100 \(\mu\)M each) also had no effect in oocytes expressing the \(\alpha_9\alpha_{10}\) nicotinic acetylcholine receptor at a holding potential of −80 mV (data not shown). However, the co-application of ginsenosides (100 \(\mu\)M each) with acetylcholine (10 \(\mu\)M) for 30 s inhibited \(I_{ACh}\) in oocytes expressing the \(\alpha_9\alpha_{10}\) nicotinic acetylcholine receptor (Fig. 2A, \(n = 10–14\) from three different frogs). Thus, the co-application of ginsenosides Rb\(_1\), Rb\(_2\), Rc, Re, Rf, Rg\(_1\), Rg\(_2\), Rg\(_3\) or ginsenoside metabolite CK with acetylcholine inhibited \(I_{ACh}\) by 22.9±3.0, 56.4±4.8, 27.9±3.2, 43.1±5.6, 35.5±4.4, 5.5±1.5, 44.1±3.0, 70.6±6.6, or 58.5±5.1\% (Fig. 2B). Interestingly, the pre-application of Rg\(_3\) (100 \(\mu\)M) alone for 30 s before
co-application with acetylcholine (10 µM) or co-application of Rg3 with acetylcholine induced almost the same inhibition of \( I_{\text{ACB}} \) (70.6±6.6% and 74.6±4.6%) (Figs. 3A, B). To determine the concentration-dependent effect of Rg3, we experimented with different concentrations of Rg3. Co-application with Rg3 for 30 s inhibited \( I_{\text{ACB}} \) by 0.5±1.2, 3.7±1.6, 15.8±2.6, 34.7±6.0, 69.0±5.1, and 80.9±4.8% at 1, 3, 10, 30, 100, and 300 µM, respectively, in oocytes expressing the α9\(\alpha10 \) nicotinic acetylcholine receptor (Figs. 3C, D). The IC\(_{50}\) of \( I_{\text{ACB}} \) was 39.6±4.9 µM for co-application in oocytes expressing the α9\(\alpha10 \) nicotinic acetylcholine receptor (n=10 or 11, with samples taken from three different frogs for each point).

**Current–Voltage Relationship in Rg3-Mediated α9\(\alpha10 \) Nicotinic Acetylcholine Receptor Regulation** In experiments examining the current–voltage (I–V) relationship, the membrane potential was held at −80 mV, and a voltage ramp was applied from −120 to +50 mV for 1.5 s. Leakage correction was executed by subtraction of the I–V curve obtained by the same voltage protocol before the application of acetylcholine. The application of acetylcholine to the bathing medium induced a mainly inward current at negative voltages and an outward current at positive voltages (Fig. 4A). Co-application of Rg3 with acetylcholine decreased both inward and outward currents. The reversal potentials were −8.8±1.6 mV and −10.5±1.9 mV with application of acetylcholine alone and co-application of Rg3 with acetylcholine in oocytes expressing the α9\(\alpha10 \) nicotinic acetylcholine receptor. The co-application of Rg3 with acetylcholine did not affect α9\(\alpha10 \) nicotinic acetylcholine receptor channel properties; Rg3 did not alter the reversal potential of the α9\(\alpha10 \) nicotinic acetylcholine receptor (Fig. 4A). In addition, the inhibitory effect of Rg3 (40 and 100 µM) on \( I_{\text{ACB}} \) was independent of the membrane-holding potential (Fig. 4B). Forty and one hundred micromole Rg3 inhibited \( I_{\text{ACB}} \) by 41.9±3.8, 43.2±5.6, 40.5±2.8, 41.8±5.4, and 69.1±7.0, 69.3±4.7, 73.7±5.9, 62.4±8.9% at membrane-holding potentials of −120, −90, −60, −30 mV, respectively, in oocytes expressing the α9\(\alpha10 \) nicotinic acetylcholine receptor (n=8 to 11, from three different frogs).

**Non-competitive Inhibition of α9\(\alpha10 \) Nicotinic Acetylcholine Receptor Channel Currents by Rg3** To further study the mechanism by which the co-application of Rg3 inhibits \( I_{\text{ACB}} \) in oocytes expressing the α9\(\alpha10 \) nicotinic acetylcholine receptor, we analyzed the effect of Rg3 on \( I_{\text{ACB}} \) evoked by different acetylcholine concentrations (Figs. 5A, B). Co-application of Rg3 of 100 µM for 30 s with various concentrations of acetylcholine did not significantly shift the concentration–response curve of acetylcholine to the right (EC\(_{50}\) values were changed from 10.9±0.6 to 14.9±1.5 µM, *p<0.08, while the Hill coefficient changed from 1.5 to 1.7) in oocytes expressing the α9\(\alpha10 \) nicotinic acetylcholine receptor. Thus, the inhibitory effect of Rg3 on \( I_{\text{ACB}} \) was not affected by increasing concentrations of acetylcholine in the range of 1 to 300 µM acetylcholine (Fig. 5B). These results indicate that Rg3 inhibited \( I_{\text{ACB}} \) in a non-competitive manner and inhibition of \( I_{\text{ACB}} \) by Rg3 was not related to the acetylcholine-binding site.

**Effects of Rg3 on \( I_{\text{ACB}} \) in Oocytes Expressing α9 Subunit Alone** Since α9 subunit can form homomeric receptors, we next examined the effects of Rg3 on \( I_{\text{ACB}} \) in oocytes expressing α9 subunit alone. Interestingly, as shown in Fig. 6A, Rg3 had no effect on \( I_{\text{ACB}} \) in oocytes expressing α9 subunit alone even with high concentration of Rg3 compared to α9\(\alpha10 \) nicotinic acetylcholine receptors. However, we could not observe any acetylcholine-induced inward currents in oocytes expressing α9 subunit data not shown). These results show that co-expressions of α9 and α10 subunits of nicotinic acetylcholine receptors are required for Rg3-induced regulation of α9\(\alpha10 \) nicotinic acetylcholine receptors. Furthermore, the present study shows that α10 subunit of nicotinic acetylcholine receptor might play an important role in Rg3-induced α9\(\alpha10 \) nicotinic acetylcholine receptor regulation.

**Effects of Extracellular Ca\(^{2+}\) on Rg3-Mediated Inhibition of \( I_{\text{ACB}} \)** Channels of the α9\(\alpha10 \) nicotinic acetylcholine receptor are known to be permeable and have a biphasic response to extracellular Ca\(^{2+}\), in contrast to other ligand-gated ion channels. We examined whether Rg3-mediated inhibition of \( I_{\text{ACB}} \) was related to extracellular Ca\(^{2+}\) concentration. As shown in Figs. 7A and 7B, \( I_{\text{ACB}} \) was potentiated with extracellular Ca\(^{2+}\). Next we examined the effects of various concentrations of Rg3 on \( I_{\text{ACB}} \) in the absence of extracellular Ca\(^{2+}\). As shown in Fig. 7C, the removal of extracellular Ca\(^{2+}\) from ND96 in the presence of 0.1 mM EGTA did not decrease the inhibitory effects of Rg3 on \( I_{\text{ACB}} \). Thus, the inhibitory
The effects of Rg3 (100 µM) on I_{ACH} did not change in ND96 and Ca^{2+}-free ND96. Next, we examined whether the inhibitory effects of Rg3 on I_{ACH} are affected by various concentrations of extracellular Ca^{2+}. We found that the inhibitory effects of Rg3 (100 µM) on I_{ACH} were not affected by varying concentrations of extracellular Ca^{2+} (Fig. 7C). These results show that the presence of extracellular Ca^{2+} may not relate Rg3-mediated inhibition of I_{ACH} and that extracellular Ca^{2+} does not play a
role in Rg3-mediated regulation of the α9α10 nicotinic acetylcholine receptor.

**DISCUSSION**

α9α10 Nicotinic acetylcholine receptors are abundantly expressed in auditory system and is related to various auditory-related diseases such as tinnitus, hearing loss and auditory processing disorders. Inhibitions of α9α10 nicotinic acetylcholine receptor attenuates inflammation-related nerve injury in auditory systems. Accumulating evidences have shown that the ginsenosides protects the central nervous system against excitatory amino acids- or neurotoxins-induced brain damage through regulations of various ion channels or ligand-gated ion channels. However, the effects of ginsenosides in the nervous system are not fully understood. Furthermore, ginsenosides’ molecular mechanisms and ability to exhibit various beneficial effects are relatively unknown at the cellular level. In previous studies, we have demonstrated that ginsenosides regulates subsets of nicotinic acetylcholine receptor channel activity such as α3β4 and other heteromeric nicotinic acetylcholine receptors. Interestingly, Rg3 had no effects on homomeric α7 nicotinic acetylcholine receptor. Although expression of the α9α10 nicotinic acetylcholine receptor is limited in several tissues, little is known about the effects of Rg3 on the α9α10 nicotinic acetylcholine receptor.

In the present study, we examined the effects of Rg3 on the α9α10 nicotinic acetylcholine receptor heterologously expressed in *Xenopus* oocytes. We found that: (1) pre- or co-application of Rg3 with acetylcholine inhibited $I_{ACh}$ in a reversible and concentration-dependent manner; (2) inhibition of $I_{ACh}$ by Rg3 pre-application with acetylcholine was independent of the concentration of acetylcholine or membrane-holding potential; (3) $I_{ACh}$ inhibition by Rg3 was non-competitive and Rg3 had no effects on $I_{ACh}$ in oocytes expressing α9 subunit alone; and (4) extracellular Ca$^{2+}$ did not play a role in inhibitory effect of Rg3 on $I_{ACh}$. These results show the possibility that α10 subunit might play a role in Rg3-induced regulation of α9α10 nicotinic acetylcholine receptor channel activity.

Previous reports have shown that ginsenosides regulates heteromeric nicotinic acetylcholine receptors expressed in oocytes. However, ginsenosides Rg3 had no effects on homomeric α7 nicotinic acetylcholine receptor. Instead, Rg3 inhibited mutant homomeric α7 nicotinic acetylcholine receptor, which was mutated at channel pore regions. These studies showed that ginsenosides regulate nicotinic acetylcholine receptor channel activity through interaction with amino acids in channel pore region. Similarly, we have reported that Rg3 inhibits 5-hydroxytryptamine 3A receptor-gated ion currents through interactions with amino acids at channel pore region with both non-competitive and voltage-independent manners. Thus, the main target of ginsenosides in regulation of ligand-gated ion channels might be channel pore region rather than ligand binding site(s).

In the present study examining how Rg3 regulates α9α10 nicotinic acetylcholine receptor-gated ion currents, we found that Rg3 inhibited $I_{ACh}$ in *Xenopus* oocytes expressing the α9α10 nicotinic acetylcholine receptor. We could observe $I_{ACh}$ in oocytes expressing α9 but not α10 subunit alone, indicating that α9 but not α10 subunit alone could form homomeric α9 nicotinic acetylcholine receptor channels but the currents...
were not large as α9α10 nicotinic acetylcholine receptor co-expression. As shown in Fig. 6, Rg3 had no effect on IαCh in oocytes expressing α9 subunit alone, showing that Rg3 could not exert its effect on homomeric nicotinic acetylcholine receptors. Interestingly, when α10 nicotinic acetylcholine subunit was co-expressed with α9 nicotinic acetylcholine receptor, Rg3 exhibited inhibitory effects on IαCh. These results show the possibility that α10 nicotinic acetylcholine subunit might play an important role in Rg3-induced α9α10 nicotinic acetylcholine receptor regulation and that co-expressions of both subunits might provide Rg3 binding site(s) through the induction of conformational changes of receptor proteins. However, further studies will be required to elucidate the role of α9 or α10 subunit in Rg3-induced α9α10 nicotinic acetylcholine receptor regulation.

In conclusion, we found that Rg3 inhibited IαCh of α9α10 nicotinic acetylcholine receptor in a concentration-dependent, non-competitive, and voltage-independent manner. Moreover, inhibition of Rg3 on IαCh was not observed in homomeric α9 nicotinic acetylcholine receptor. These results indicate that ginsenosides are a novel agent acting on the α9α10 nicotinic acetylcholine receptor and that ginsenosides-mediated IαCh regulation of the α9α10 nicotinic acetylcholine receptor could provide a molecular basis for the pharmacological actions of ginseng in the nervous system.

Acknowledgements This work was supported by the Basic Science Research Program (2011-0021144) and the Priority Research Centers Program through the National Research Foundation of Korea (NRF), which is funded by the Ministry of Education, Science, and Technology (2012-0006686) and by the BK21 project fund to S.-Y. Nah.

REFERENCES

7) Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar N, Valera S, Barkas T, Ballivet M. A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX. Neuron, 5, 847–856 (1990).