Characteristics of Ginsenoside Rg3-Mediated Brain Na\(^+\) Current Inhibition


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ABSTRACT

We demonstrated previously that ginsenoside Rg3 (Rg3), an active ingredient of Panax ginseng, inhibits brain-type Na\(^+\) channel activity. In this study, we sought to elucidate the molecular mechanisms underlying Rg3-induced Na\(^+\) channel inhibition. We used the two-microelectrode voltage-clamp technique to investigate the effect of Rg3 on Na\(^+\) currents (I\(\text{Na}\)) in Xenopus laevis oocytes expressing wild-type rat brain Na\(^+\)-V1.2 \(\alpha\) and \(\beta\)1 subunits, or mutants in the channel entrance, the pore region, the lidocaine/tetrodotoxin (TTX) binding sites, the S4 voltage sensor segments of domains I–IV, and the Ile-Phe-Met inactivation cluster. In oocytes expressing wild-type Na\(^+\) channels, Rg3 induced tonic and use-dependent inhibitions of peak I\(\text{Na}\). However, Rg3 treatment inhibited the peak and plateau I\(\text{Na}\) in the IFMQ3 mutant, indicating that Rg3 inhibits both the resting and open states of Na\(^+\) channel. Neutralization of the positive charge at position 859 of voltage sensor segment II abolished the Rg3-induced activation voltage shift and use-dependent inhibition. These results reveal that Rg3 is a novel Na\(^+\) channel inhibitor capable of acting on the resting and open states of Na\(^+\) channel via interactions with the S4 voltage-sensor segment of domain II.

Na\(^+\) channels are transmembrane proteins that consist of a pore-forming \(\alpha\) subunit and auxiliary \(\beta1\), \(\beta2\), and \(\beta3\) subunits (Catterall, 1987; Goldin, 1995; Wang et al., 2003). The \(\alpha\) subunit is composed of four homologous domains (I–IV), each composed of six \(\alpha\)-helical transmembrane segments (S1–S6). Among them, the S4 segment acts as the voltage-sensing apparatus of the Na\(^+\) channel (Hodgkin and Huxley, 1952). The pore-forming \(\alpha\) subunit is responsible for voltage-dependent increases in Na\(^+\)-selective permeability. These changes trigger the inward Na\(^+\) current (I\(\text{Na}\)), which initiates axonal and somatic action potentials in nerve and muscle fibers and may also be involved in axonal information transfer for intraneuronal or interneuronal communications (Hodgkin and Huxley, 1952; Stuart and Sakmann, 1994). Na\(^+\) channels can exist in resting (closed), open (active), or inactivated states and transition among the various states in response to time- and voltage-dependent signaling (Hodgkin and Huxley, 1952). Various drugs can exhibit differential affinities to the specific Na\(^+\) channel states. For example, lidocaine (a local anesthetic) and phenytoin (an anticonvulsant) show a low affinity for the resting state and a higher affinity for the inactivated state, whereas flecainide (an-

ABBREVIATIONS: Rg3, 20-S-protopanaxadiol-3-O-\(\beta\)-d-glucopyranosyl (1→2)-\(\beta\)-glucopyranoside; TTX, tetrodotoxin; IFMQ3, Ile1488-Phe1489-Met1490 mutated to I1488Q-F1489Q-M1490Q; DPH, diphenylhydantoin.
Ginseng, the root of Panax ginseng C.A. Meyer, is well known in herbal medicine as a tonic and restorative agent. The main molecular ingredients responsible for the actions of ginseng are the ginsenosides (also called ginseng saponins), which are amphiphilic molecules comprising a hydrophobic backbone of aglycone (a hydrophobic, four-ring, steroid-like structure) linked to hydrophilic carbohydrate side chains consisting of monomers, dimers, or tetramers (Fig. 1). The ginsenosides are classified as protopanaxadiol or protopanaxatriol, according to the positions of the carbohydrate moieties at carbons -3, -6, and -20, which can be either free or connected to sugar rings (Nah, 1997). We recently demonstrated that ginsenoside Rg3 (20-S-protopanaxadiol-3-[O-β-d-glucopyranosyl (1→2)-β-glucopyranoside]) (Rg3), one of the active ingredients in Panax ginseng, inhibits voltage-dependent brain Na⁺ channel activity expressed in Xenopus laevis oocytes (Jeong et al., 2004; Kim et al., 2005). However, no previous work has examined the underlying mechanisms by which Rg3 regulates Na⁺ channel currents.

We herein sought to characterize ginsenoside-mediated Na⁺ channel regulation in an X. laevis oocyte gene expression system. This model system has few endogenous ion channels (Dascal, 1987) and allows heterologous expression of ion channels for various biochemical studies (Choi et al., 2002, 2003; Sala et al., 2002). We expressed brain Na⁺ channels by intraoocyte injection of cRNAs encoding the NaV1.2 α and β1 subunits (Pugsley and Goldin, 1998; Pugsley et al., 2000) with or without various mutations and examined the changes in Iₜ₊ in response to Rg3 treatment. We found that Rg3 caused both tonic and use-dependent inhibitions of the peak Iₜ₊ after low- and high-frequency stimulations. We further found that mutations in the channel pore entrance, pore region, lidocaine binding sites, tetrodotoxin (TTX) binding sites, and S4 voltage sensor segments of domains I–IV of the Na⁺ channel had no effect on Rg3-induced tonic inhibition of peak Iₜ₊. However, when inactivation cluster Ile1488-Phe1489-Met1490 was mutated to Ile1488-Q-Phe1489-Q-Met1490Q (IFMQ3) (West et al., 1992) to create an inactivation-deficient mutant, Rg3 treatment inhibited both the peak and nonactivating plateau Iₜ₊ levels of this mutant, indicating that Rg3 regulates the resting and open states of the expressed Na⁺ channel. A single Lys-to-Gln mutation at residue 859 (K859Q) within the S4 voltage sensor segment of domain II abolished the Rg3-induced shift of the activation voltage and use-dependent inhibition, although changes in the other domains did not. Taken together, these results show for the first time that Rg3 acts as a novel Na⁺ channel blocker by interacting with the S4 voltage-sensor segment of domain II.

**Experimental Procedures**

**Materials.** The 20(S)-ginsenoside Rg3 (Fig. 1) was kindly provided by the Korean Ginseng Cooperation (Taehon, Korea). The cDNA for the rat brain Na⁺ channel NaV1.2 α subunit was kindly provided by Dr. A. L. Goldin (University of California, Irvine, CA) and that for the Na⁺ channel β1 subunit was kindly provided by Dr. T. Zimmer (Friedrich Schiller University, Jena, Germany). Other agents were purchased from Sigma (St. Louis, MO).

**Preparation of X. laevis Oocytes and Microinjection.** X. laevis frogs were purchased from Xenopus I (Ann Arbor, MI). Their care and handling was 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, and ovarian follicles were removed. The oocytes were separated by treatment with collagenase and agitation for 2 h in Ca²⁺-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin. Stage V–VI oocytes were collected and stored in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES, pH 7.5) supplemented with 0.5 mM theophylline and 50 µg/ml gentamicin. This oocyte-containing solution was maintained at 18°C with continuous gentle shaking and was renewed everyday. Electrophysiological experiments were performed within 5 to 6 days of oocyte isolation, with chemicals applied to the bath. For Na⁺ channel experiments, 40 nl of cRNAs encoding the NaV1.2 α and β1 subunits was injected into the animal or vegetal pole of each oocyte one day after isolation, using a 10-µl WVR microdispenser (WVR Scientific, San Francisco, CA) fitted with a tapered glass pipette tip (15–20 µm in diameter; Choi et al., 2003).

**Site-Directed Mutagenesis of NaV1.2 and in Vitro Transcription of Na⁺ Channel Subunit cDNAs.** The substitution mutations of single or triplet amino acids were performed using the QuikChange XL site-directed mutagenesis kit (Stratagene, La jolla, CA), along with Pfu DNA polymerase and mutated sense and antisense primers. Overlap extension of the target domain by sequential polymerase chain reactions was carried out according to the manufacturer’s recommended protocol. The final PCR products were transformed to Escherichia coli strain DH5α, screened by PCR and confirmed by DNA sequencing of the target region. The mutant DNA constructs were linearized at the 3’ end by NotI digestion, and run-off transcripts were prepared using methylated cap analog m'7G(5')ppp(5')G. The cRNAs were prepared using the mMessage mMACHINE transcription kit (Ambion, Austin, TX) with T7 RNA polymerase. The absence of degraded RNA was determined by denaturing agarose gel electrophoresis followed by ethidium bromide staining. Likewise, recombinant plasmids containing wild-type NaV1.2 α or β1 subunit cDNA inserts were linearized by digestion with the appropriate restriction enzymes, and cRNAs were obtained using the mMessage mMachine in vitro transcription kit (Ambion) with SP6 RNA or T7 polymerases. The final cRNA products were resuspended at a concentration of 1 µg/µl in RNase-free water and stored at −80°C until use (Choi et al., 2003).

**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
(the diameter and height of the upper well were 8 and 3 mm, respectively, and those of the lower well were 6 and 5 mm, respectively) and gluing plastic meshes (-0.4-mm grid diameter) onto the bottom of the upper well. A perfusion inlet (-1 mm in diameter) was drilled through the wall of the lower well, and a suction tube was placed on the edge of the upper well. For experiments, a single oocyte was placed on the net separating the upper and lower wells. The net grids helped anchor the oocyte in place during the electrophysiological recordings. The oocyte was then impaled with two microelectrodes filled with 3 M KCl (0.2-0.7 MΩ) and electrophysiological experiments were carried out at room temperature using an oocyte clamp (Warner Instruments, Hamden, CT). Stimulation and data acquisition were controlled with a pClamp 8 (Axon Instruments, Union City, CA) (Choi et al., 2003). For most of the electrophysiological experiments on Na+ channel activity, oocytes were clamped at a holding potential of -100 mV and the membrane potential was depolarized to -10 mV for 100 ms every 5 s. Linear leak currents were corrected by means of the leak subtraction procedure (Jeong et al., 2005).

The voltage-dependence of Na+ channel activation was calculated by measuring the peak current at test potentials ranging from -50 mV to +50 mV evoked in 5-mV increments. The conductance (gNa) was calculated according to the equation, gNa = INa/(Vt - Vh), where INa is the peak amplitude of the Na+ current, Vt is the test potential, and Vh is the reversal potential for Na+. The conductance-voltage curves were drawn according to the equation gNa = 1/[1 + exp ((Vh - V0.5)/k)]. The conductance-voltage curve was calculated according to the equation, gNa = 1/[1 + exp ((Vh - V0.5)/k)], where Vh is the depolarized potential, V0.5 is the half-maximal value of the Na+ current at which gNa is 0.5, k is the slope factor (potential required for an e-fold change). The voltage-dependence of Na+ channel inactivation was determined using 200 ms conditioning prepulses ranging from -60 mV to +20 mV from a holding potential of -100 mV in 5-mV increments, followed by a test pulse to -10 mV for 5 ms. The peak INa was normalized to its respective maximum value (INa(max)) and plotted as a function of the prepulse potential.

The steady-state inactivation curves were drawn according to the equation, INa/INa(max) = 1/[1 + exp ((Vh - V0.5)/k)], where Vh is the prepulse potential, V0.5 is the potential at which INa is 0.5 INa(max), and k is the slope factor.

The frequency-dependent effect of Rg3 was examined using a protocol in which 50 depolarizing pulses of 10-ms duration and 10 Hz frequency were applied to -10 mV from a holding potential of -100 mV. The protocol was run in the absence (control) and presence of 10 or 100 μM Rg3. The current amplitude of each pulse was normalized to the peak maximal current (pulse number 1) and plotted as a function of pulse number.

The kinetics of the Rg3 blockade of IFM033, which is the fast-inactivation-deficient mutant of Na1.2, were examined by clamping oocytes at -100 mV in ND96 solution. A single 500-ms depolarizing pulse to -10 mV was applied, and the INa was recorded. Different concentrations of Rg3 (10, 30, 100, or 300 μM) were perfused into the bath for 1 min, and a second, single depolarizing pulse from -100 to -10 mV was given. The data were individually fitted to either a single [1 - Aslow × exp(-t/τslow)] or double [1 - Afast × exp(-t/τfast)] + [1 - A slow × exp(-t/τ slow)] exponential equation, in which Afast and A slow represent the proportion of current decaying with time constants τfast and τslow, respectively, and t is the time interval. If we assume that a first-order relationship describes the dependence of the blocking rate constant for binding (kB) can be obtained by fitting the τfast values with the equation: τfast = kfast × [Rg3] + τ0 (Lansman et al., 1986). Recovery from open channel blockade was measured at a holding potential of -100 mV with a 10-ms depolarizing prepulse to -10 mV (P1) followed by a variable recovery period from 10 to 500 ms, subsequently followed by a 10-ms test pulse to -10 mV (P2).

Data Analysis. To obtain the concentration-response curve of the effect of Rg3 on INa, the peak amplitudes at different concentrations of Rg3 were plotted and then fitted to the following Hill equation using the Origin software (OriginLab Corp, Northampton, MA): y = A × exp(b × (x - C))/[1 + exp(b × (x - C))], where y is the peak INa at given concentration of Rg3, b is the maximal peak INa, EC50 is the concentration of Rg3 producing a half-maximum effect, A is the concentration of Rg3, and b is the interaction coefficient. All values are presented as means ± S.E.M. The differences between the means of control and treatment values were determined using an unpaired Student’s t test. A value of P < 0.05 was considered statistically significant.

Results

The Effect of Rg3 on Peak INa in Oocytes Expressing Na1.2. The effect of Rg3 on currents from brain Na+ channels expressed in X. laevis oocytes was examined. INa was recorded by two-electrode voltage clamping of oocytes injected with cRNAs encoding Na1.2 α and β1 subunits. Oocytes were held at -100 mV, and INa was elicited by depolarization to -10 mV at a low frequency (0.2 Hz). This procedure minimized the use-dependent blockade and allowed evaluation of whether Rg3 produced a tonic blockade of peak INa (Pugsley and Goldin, 1998; Pugsley et al., 2000). For comparison, we also examined the effect of lidocaine on the peak INa. As shown in Fig. 2A, the depolarizing voltage step induced a large inward INa with rapid inactivation. Application of Rg3 (100 μM) or lidocaine (1000 μM) inhibited the peak INa by 64 ± 7 and 41 ± 10%, respectively (Fig. 2A, inset), indicating that both agents induced a tonic inhibition of the Na+ current.

Concentration-Dependent Inhibition of INa by Rg3 or Lidocaine. The current-voltage relationships were assessed in the absence or presence of Rg3 with voltage steps ranging from -50 to +50 mV evoked from a holding potential of -100 mV every 5 s. As shown in Fig. 2B, addition of Rg3 caused a voltage-dependent reduction in peak INa, with a more pronounced reduction noted at lower voltage ranges. In addition, Rg3 treatment shifted the threshold voltage of channel opening and the voltage of the peak INa to more depolarized values compared with the control. However, Rg3 had no significant effect on the kinetics of current decay. As shown in Fig. 2C, the inhibitory effect of Rg3 on peak INa was dose-dependent up to 300 μM, with an estimated IC50 value of 32 ± 6 μM. The Hill coefficient was 1.1 ± 0.4, indicating that one molecule of Rg3 seemed sufficient to block one Na+ channel. We also tested the effect of lidocaine on peak INa and found that it was dose-dependent up to 3000 μM (Fig. 2C). The IC50 was 966 ± 37 μM, which is consistent with previous report (Pugsley and Goldin, 1998). These findings indicate that Rg3 was more potent than lidocaine by approximately 33.2-fold. It is noteworthy that the IC50 value for Rg3-mediated Na+ current inhibition was 2-fold higher than that for Rg3-induced inhibition of Na+ influxes triggered by acetylcholine treatment in bovine adrenal chromaffin cells (Tachikawa et al., 1995) and 8-fold higher than that for Rg3-induced inhibition of Na+ influxes mediated by NMDA receptor (Kim et al., 2002). These discrepancies in IC50 values might reflect the differential affinity of Rg3 for ion channels or receptors, suggesting that NMDA or nicotinic acetylcholine receptors might be more sensitive to Na+ channels to low concentrations of Rg3.

The Effects of Rg3 on the Activation and Inactivation of Na1.2. We next examined the effects of Rg3 on the voltage-dependence of Na+ channel steady-state activation and...
inactivation. First, the effect of Rg3 on Na\(^+\) channel activation was determined by a conductance transformation of the peak current-voltage relationship (Fig. 3, A and B), with the curves representing the best data fit using the Boltzmann function. There was a significant depolarizing shift of the half-maximal activation voltage (\(V_{g0.5}\)). The \(V_{g0.5}\) was -28.9 ± 0.57 mV in control experiments and -17.8 ± 0.21 mV in Rg3-treated oocytes (\(P < 0.01\), compared with control, \(n = 10\)). However, the slope factor (\(k_g\)) was not significantly different, yielding values of 4.6 ± 0.5 mV under control conditions and 4.8 ± 0.2 mV after Rg3 treatment. We then investigated the effect of Rg3 on voltage-dependent Na\(^+\) channel inactivation by plotting the normalized peak \(I_{Na}\) against the conditioning prepulse voltage (Fig. 3, C and D) and then fitting the data to the Boltzmann function. There was no significant difference in the half-maximal inactivation voltage (\(V_{h0.5}\)) and the slope factor (\(k_h\)) between control and Rg3 treatment groups; \(V_{h0.5}\) was -28.9 ± 0.57 mV under control conditions and -26.9 ± 0.68 mV, respectively, and \(k_h\) was 7.2 ± 0.4 and 8.1 ± 0.68 mV, respectively (\(n = 10\)). These findings indicate that Rg3 affects the steady-state activation but not inactivation of the Na\(^+\) channel.

**Use-Dependent Blockade of Na\(_V\)1.2 by Rg3 and Lidocaine.** Because Na\(^+\) channel blockers such as lidocaine and other antiarrhythmic drugs exhibit use-dependent inhibition (Hondegham and Kazung, 1984), we tested whether Rg3 behaved in the same way, using Rg3 concentrations shown to induce minimal \(I_{Na}\) blockade (10 \(\mu\)M) and marked \(I_{Na}\) blockade (100 \(\mu\)M) in our initial tonic block experiments. \(I_{Na}\) was elicited by 20-ms pulses from -100 to -10 mV for 50 times at 10 Hz. Each peak \(I_{Na}\) was normalized to the first pulse peak \(I_{Na}\). Under control conditions, there was a slight reduction in peak \(I_{Na}\), whereas treatment with 10 and 100 \(\mu\)M Rg3 induced use-dependent inhibitions of peak \(I_{Na}\) values by 11 ± 1 and 15 ± 2%, respectively (\(n = 9\) each; Fig. 4A). Lidocaine (1000 \(\mu\)M) treatment also induced a use-dependent inhibition...
of the peak $I_{Na}$ by 35 ± 2% ($n = 8$ each) (Fig. 4A). Thus, Rg$_3$ and lidocaine both seemed to induce use-dependent inhibitions of $I_{Na}$.

**The Effect of Rg$_3$ on Peak $I_{Na}$ at Different Holding Potentials.** The effect of Rg$_3$ on peak $I_{Na}$ at different holding potentials was examined. Diphenylhydantoin (DPH), which is a well known anticonvulsant that preferentially binds to the inactivated Na$^+$ channel (Valenzuela et al., 1996), did not significantly affect the peak $I_{Na}$ evoked at −10 mV from a holding potential of −110 mV (6 ± 1% inhibition by 100 μM, $n = 10$) (Fig. 5A). However, at a more depolarized holding potential of −50 mV, DPH dramatically reduced the peak $I_{Na}$ (86 ± 7% inhibition by 100 μM, $n = 10$). This indicates that the blockade of peak $I_{Na}$ by DPH is highly sensitive to the membrane potential and that DPH has a much higher affinity to the inactivated state than to the resting state of the Na$^+$ channel as shown by Kuo and Bean (1994) (Fig. 5, A and C, left). We then tested the effect of Rg$_3$ on the peak $I_{Na}$ at different holding potentials. In contrast to the action of DPH, the inhibitory effect of 100 μM Rg$_3$ was not significantly affected by changes in the holding potential (78 ± 14% and 69 ± 10% inhibition at −110 and −50 mV, respectively; $n = 15$ each), indicating that the inhibitory effect of Rg$_3$ on the peak $I_{Na}$ is independent of the membrane holding potential (Fig. 5, B and C, right).

**Lidocaine and TTX Do Not Prevent Rg$_3$-Induced Inhibition of Peak $I_{Na}$.** We performed occlusion experiments using lidocaine and TTX, which are well known Na$^+$ channel blockers, to determine whether Rg$_3$ shares a common binding site or pathway with lidocaine or TTX. As shown in Figs. 6B and D, single applications of 30 μM Rg$_3$, 1000 μM lidocaine and 1 nM TTX inhibited the peak $I_{Na}$ values by 42 ± 8, 25 ± 5, and 44 ± 3%, respectively. Cotreatment of Rg$_3$ with lidocaine produced an additive inhibition of peak $I_{Na}$ by 88 ± 4%, whereas cotreatment of Rg$_3$ with TTX produced an additive inhibition of peak $I_{Na}$ by 80 ± 4%. These results suggest that Rg$_3$ regulates Na$^+$ channels by acting on different site(s) from those of lidocaine and TTX.

**The Effect of Rg$_3$ on Mutant Na$^+$ Channels.** To gain insight into the mechanism(s) by which Rg$_3$ inhibits peak

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**Fig. 3.** The effect of Rg$_3$ on steady-state activation and inactivation of $I_{Na}$ in wild-type and K859Q mutant channels. A, effect of Rg$_3$ on steady-state activation and inactivation of $I_{Na}$ in wild-type channels. The voltage dependence of conductance was compared in the absence (●) and presence (○) of 100 μM Rg$_3$. Inactivation was measured using a two-pulse protocol in which oocytes were held at −100 mV and depolarized to potentials from −60 to +20 mV for 200 ms, followed by a test-pulse to −10 mV for 10 ms to determine channel availability. Inactivation curves are shown in the absence (●) and presence of 100 μM Rg$_3$ (○). B, representative current traces for the activation and inactivation of wild-type channels were obtained as described under Experimental Procedures. The indicated traces are presented for clarity. C, the effect of Rg$_3$ on the steady-state activation and inactivation of $I_{Na}$ in the K859Q mutant. D, representative current traces for the activation and inactivation of the K859Q mutant channel were obtained as described. The indicated traces are represented for clarity. Data represent the means ± S.E.M. ($n = 10–11$ group). The curves represent a two-state Boltzmann function.
I\textsubscript{Na}, we examined the requirement of different Na\textsuperscript{+} channel \(\alpha\) subunit protein domains using site-directed mutagenesis. We constructed the following six mutant types: 1) mutation of the channel pore entrance of the S6 segment of domain I by replacement of residue Tyr401 with cysteine (Y401C) or threonine (Y401T); 2) mutation at the channel pore sites by replacement of Glu942 with glutamine (E942Q), Glu945 with glutamine (E945Q), or Asp927 with asparagine (D927N) (Kontis and Goldin, 1993); 3) mutation of the lidocaine binding sites by replacement of residues Phe1764 and/or Tyr1771 with alanine (F1764A or Y1771A and F1764A-Y1771A, respectively) (Ragsdale et al., 1994; 1996); 4) mutation of the extracellular and intracellular TTX binding sites by replacement of Phe385 with cysteine (F385C), serine (F385S), tyrosine (F385Y), or methionine (F385M), or replacement F387 with glycine (F387G), threonine (F387T) or glutamine (F387Q) (Noda et al., 1989; Terlau et al., 1991); 5) mutation of the S4 voltage-sensor segments of domains I to IV by replacement of residues Lys226, Lys859, Arg1312, or Arg1638 with glutamine (K226Q, K859Q, R1312Q, or R1638Q) (Kontis et al., 1997); and 6) creation of a fast inactivation gating-deficient mutant (IFMQ3) (West et al., 1992).

Representative traces were obtained from oocytes expressing channel pore, TTX binding site, and voltage-sensor mutants in the absence or presence of Rg3 (Fig. 7A). Rg3 treatment induced tonic inhibition of the peak \(I\textsubscript{Na}\) in all mutants (Fig. 7 and Table 1). The concentration-response relationship for peak \(I\textsubscript{Na}\) inhibition by Rg3 in the different kinds of mutants was determined (Fig. 7B), and the data were fitted using the Hill equation. The Hill coefficients and \(V\text{max}\) values for the mutants were not significantly different from those of the wild-type channels. However, the IC\textsubscript{50} values from mutants F385M, E387Q, Y401C, and K859Q were significantly higher than that of the wild-type channel (**, \(P<0.01\) compared with wild-type channel) (Table 1). Because mutations of Phe1764 or Tyr1771 to alanine (F1764A or Y1771A) are resistant to lidocaine-induced tonic and use-dependent blockades (Ragsdale et al., 1994, 1996), we examined whether these mutations could affect the Rg3-mediated tonic and use-dependent inhibitions of peak \(I\textsubscript{Na}\). Although lidocaine treatment induced only a slight inhibition of peak \(I\textsubscript{Na}\) in oocytes expressing the F1764A, Y1771A, and F1764A-Y1771A mutants after low- and high-frequency stimulations (Fig. 8, A, C, and E, low-frequency stimulations; B, D, and F, high-frequency stimulations), Rg3 treatment induced wild-type inhibition levels of peak \(I\textsubscript{Na}\) in the mutants after in both low- and high-frequency stimulations (Fig. 8). The IFMQ3 mutant, which lacks fast inactivation, showed a slower decay and a sizable persistent noninactivating or plateau current at the end of a 500-ms depolarizing pulse to \(-10\) mV in the absence of Rg3, which is consistent with a previous report (West et al., 1992). In the IFMQ3 mutant, Rg3 treatment induced dose-dependent inhibitions of peak and plateau \(I\textsubscript{Na}\) (Fig. 9A). The plateau \(I\textsubscript{Na}\) showed a larger change; the IC\textsubscript{50} values of Rg3 acting on the on peak and plateau \(I\textsubscript{Na}\) in this mutant were 38 ± 3 and 14 ± 4 \(\mu\)M, respectively (approximately a 3-fold difference). These results suggest that Rg3 interacts more readily with the open state of the Na\textsuperscript{+} channel versus the resting state (Fig. 9B). Finally, we examined whether the IFMQ3 mutant showed any changes in the inhibitory effect of
Rg3 on oocytes subjected to high-frequency stimulation. We observed that Rg3 treatment induced a 2- to 3-fold larger use-dependent inhibition in IFMQ3 mutant channels versus wild-type channels; 10 and 100 μM Rg3 induced 17 ± 1 and 45 ± 4% use-dependent inhibitions of $I_{Na}$, respectively ($n = 11$) (Fig. 4, A and B). In contrast, Rg3 did not induce additional use-dependent inhibitions in the other tested mutants (data not shown). Collectively, these results further demonstrate that Rg3 uses different binding site(s) from those of lidocaine and TTX and that Rg3 blocks the resting and open state of brain Na$^{+}$ channels.

A Point Mutation in the S4 Voltage-Sensor Segment of Domain II of NaV1.2 Abolishes the Rg3-Induced Voltage Shift of Na$^{+}$ Channel Activation. As shown in Fig. 3, A and B, Rg3 treatment strongly depolarized the Na$^{+}$ channel activation voltage, suggesting that Rg3 might modify its activation gating. The S4 segments comprise four homologous domains (I–IV) of the Na$^{+}$ channel and are believed to act as the voltage-sensing apparatus (Kontis et al., 1997). To investigate whether mutations in the voltage-sensor segments of the Na$^{+}$ channel affect the Rg3-induced depolarization of the Na$^{+}$ channel activation voltage, we constructed four different mutants in the S4 segments of domain I to IV (Table 1) (Kontis et al., 1997) and examined their influences on the Rg3-induced voltage shift of the Na$^{+}$ channel activation curve. Our results revealed that replacing Lys859 (domain II) with glutamine (K859Q) abolished the Rg3-induced voltage shift, although the mutation itself resulted in a depolarizing shift of the activation curve by $-10$ mV compared with wild-type channel (Fig. 3, C and D). The half-maximal activation voltage ($V_{g0.5}$) was $-10.9 ± 0.4$ mV and the slope factor ($k_g$) was $5.1 ± 0.3$ mV in the Rg3 control, but only $-9.8 ± 0.4$ mV and $5.3 ± 0.4$ mV in the mutant (Fig. 3, C and D). The other tested mutants did not show significant alterations of the activation curve (data not shown), indicating that the Lys859 residue of domain II may play an important role in the Rg3-induced modification of voltage-dependent Na$^{+}$ channel activation.

A Single Point Mutation in the S4 Voltage-Sensor Segment of Domain II of NaV1.2 in Both Wild-Type and IFMQ3 Mutant Channels Abolishes Rg3-Induced Use-Dependent Inhibition. We next examined whether the K859Q mutation affects the Rg3-induced use-dependent inhibition of Na$^{+}$ channels, because this mutation abolished Rg3-induced modification of Na$^{+}$ channel activation gating. Rg3 treatment did not produce use-dependent inhibition in either K859Q or IFMQ3-K859Q mutants (Fig. 4, C and D), suggesting that the Lys859 residue of the S4 voltage sensor

Fig. 5. Rg3-induced $I_{Na}$ inhibition is independent of the holding potential. A, representative traces of $I_{Na}$ in the absence or presence of 30 or 100 μM phenytoin (DPH). Each oocyte was held at $-50$ or $-100$ mV and stepped to $-10$ mV for 100 ms every 5 s. B, representative traces of $I_{Na}$ in control oocytes or those treated with 30 or 100 μM Rg3. Each oocyte was held at $-50$ or $-100$ mV and stepped to $-10$ mV for 100 ms every 5 s. C, left, histograms for the inhibition of $I_{Na}$ by 30 or 100 μM DPH at different holding potentials. C, right, histograms for the inhibition of $I_{Na}$ by 30 or 100 μM Rg3 at different holding potentials. Data represent the means ± S.E.M. ($n = 10–11$ group).
segment of domain II might play an important role in Rg3-induced use-dependent inhibition of the IFMQ3 mutant. These mutations also significantly increased the IC50 values by 1.5-fold compared with wild-type in terms of tonic inhibition (*, P < 0.01, compared with wild-type; Table 1). It is noteworthy that lidocaine treatment produced wild-type level use-dependent inhibition in the K859Q mutant (Fig. 4C), suggesting that the Rg3 site is not related with the action of lidocaine. In contrast, when we examined whether the mutation in the S4 voltage-sensor segment of domain II affected Rg3-induced open-channel blocking pattern, we found that Rg3 treatment inhibited both peak and plateau $I_{\text{Na}}$ levels to comparable degrees in the IFMQ3-K859Q and IFMQ3 mutants (compare Fig. 9, C and A). The IC50 values of the IFMQ3-K859Q mutant were 56 ± 6 and 20 ± 1 M for the peak and plateau $I_{\text{Na}}$ levels, respectively (Fig. 9D and Table 1), which was significantly higher than that for the IFMQ3 mutant in terms of peak (1.5-fold) but not plateau $I_{\text{Na}}$ inhibition (**, P < 0.01, compared with IFMQ3 mutant, Table 1). These results indicate that the K859Q mutant might decrease the affinity of Rg3 for the IFMQ3 mutant to a comparable degree as that seen in the wild-type but that the K859Q mutation did not affect the Rg3-induced open channel blocking pattern.

**Developmental Rate of Rg3-Induced Open Channel Blockade.** We examined the ability of various concentrations of Rg3 to block IFMQ3 (Fig. 10A). Untreated control traces showed that during a depolarizing pulse to −10 mV, the IMFQ3 current decayed exponentially with a single, slow time constant ($\tau_{\text{slow}}$) of 585.4 ± 2.7 ms ($n = 9$). Currents elicited after Rg3 treatment decayed exponentially with two distinct time constants. In the presence of low concentration of Rg3 (10 μM), the currents decayed with a slow time constant that was similar to that of the control, whereas treatment with higher concentrations of Rg3 such as 30 to 300 μM induced a concentration-dependent fast exponential component to the curves ($\tau_{\text{fast}}$), which represented the Rg3 block ($\tau_{\text{m}}$). The time constants of the fast components were 464.5 ± 6.4, 235.1 ± 7.8, 64.1 ± 14.2, and 51.8 ± 9.4 ms in samples treated with 10, 30, 100, and 300 μM Rg3, respectively. This result indicates that Rg3 also interacts with the open state of the Na+ channel. A plot of the reciprocal of $\tau_{\text{m}}$ against drug concentration (Lansman et al., 1986) was used to approximate the drug-channel interaction kinetics. The relationship between $1/\tau_{\text{m}}$ and the concentration of Rg3 was determined with a straight line representing the best fit to the equation $1/\tau_{\text{m}} = k_{\text{on}}[\text{Rg3}] + l$. (Fig. 10B). The slope of the line is the apparent binding constant ($k_{\text{on}}$), which equals 0.062 ± 0.009 × 106 M$^{-1}$s$^{-1}$, and the intercept $l$ equals 1.89 ± 0.78 s$^{-1}$. The latter value is close to the reciprocal of $\tau_{\text{slow}}$ (1.71 ± 0.41 s$^{-1}$), indicating that in the IFMQ3 mutant, a residual inactivation determines the intercept in the absence of Rg3.

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**Fig. 6.** Lidocaine and TTX do not interfere with the action of Rg3. A, C, peak inward current amplitudes elicited by 100-ms depolarizations to −10 mV from a holding potential of −100 mV, evoked every 5 s. Rg3 (30 μM) was first applied during the period indicated by the solid bars, and then lidocaine (1000 μM) was applied in absence or presence of Rg3 (30 μM) as indicated by the solid bars. B, traces are representatives of six separate oocytes from three different frogs. Inset, the histograms show the percentage blockade of $I_{\text{Na}}$ by Rg3, lidocaine (Lido), or Rg3 + lidocaine (Lido). Data represent the means ± S.E.M. ($n = 10–13$/group). C and D, experiments were performed as above with TTX (1 nM) used in place of lidocaine. Data represent the means ± S.E.M. ($n = 10–12$/group).
This suggests that the unbinding constant ($k_{off}$) is likely to be very small.

**Rg3 Treatment Slows Recovery from Inactivation.** Because Rg3 blocked the open state of the IFMQ3 channel (Fig. 9) and exhibited an additional use-dependent block compared with the wild-type channel (Fig. 4B), we examined whether the inhibitory effect of Rg3 on $I_{Na}$ was derived from a delayed recovery of the channel from the open channel block. Current traces were recorded in the absence (Fig. 10C, control, top) and presence (Fig. 10C, bottom) of 100 μM Rg3 with a recovery time interval of 10 ms between pulses. Recovery from open channel block was analyzed as shown in Fig. 10D. After a 10-ms prepulse (P1), recovery from open channel block was assessed using a test pulse (P2) after increasing recovery intervals (Fig. 10C and D, inset). The IFMQ3 Na$^+$ channel block was assessed using a test pulse (P2) after a recovery time interval of 10 ms between pulses. Recovery from open channel block was analyzed as shown in Fig. 10D. After a 10-ms prepulse (P1), recovery from open channel block was assessed using a test pulse (P2) after increasing recovery intervals (Fig. 10C and D, inset). The IFMQ3 Na$^+$ channels were found to recover rapidly, probably because of a slow inactivation associated with the mutation (Fig. 10D, < 50 ms, C). In contrast, Rg3 treated channels (Fig. 10D, ○) showed a delayed recovery from open channel block (up to 260 ms). This slow recovery seems to underlie the enhanced use-dependent inhibition produced by Rg3.

**Discussion**

Ginseng has long been used as a treatment for a wide variety of ailments, and some of the purported effects of this root have been documented in laboratory studies (Nah, 1997). Although the beneficial effects and functional mechanisms of ginsenosides have not been fully elucidated, accumulating evidence suggests that they may target the ion channels involved in neuronal excitability. Ginsenosides have been shown to affect several ion channels found at presynaptic and postsynaptic sites in the nervous system (Nah and McCleskey, 1994; Nah et al., 1995; Kim et al., 1998, 2002; Choi et al., 2002, 2003; Sala et al., 2002), and their effects are closely coupled to the inhibition of neurotransmitter release (Tachikawa et al., 1995; Kudo et al., 1998). We demonstrated recently that Rg3 stereospecifically inhibits voltage-dependent brain Na$^+$ currents and that the carbohydrate portion of Rg3 plays a key role in the inhibition of Na$^+$ currents (Kim et al., 2005). However, very little is known about the molecular mechanism(s) underlying Rg3-induced Na$^+$ channel modulation.

Herein, we characterized Rg3-induced channel regulation of brain Na$^+$ channels (Na$\alpha_{1,2}$) expressed in X. laevis oocytes. Our results revealed four major findings. First, Rg3 produced a tonic inhibition of the peak $I_{Na}$ via an interaction with the resting state of the Na$^+$ channel (Fig. 2). Second, Rg3 induced a large depolarizing shift in the steady-state activation of the Na$^+$ channel (Fig. 3). Third, Rg3 produced a use-dependent block of the Na$^+$ channel after high-frequency stimulation, indicating that Rg3 could exert an inhibitory effect on the open state of the Na$^+$ channel (Fig. 4). Fourth, the inhibitory effect of Rg3 on the peak $I_{Na}$ was independent of the holding potential, indicating that Rg3 might have a

### Table 1

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<th>Type</th>
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<th>$V_{max}$</th>
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$^*P < 0.01$ compared with wild-type Na$^+$ channel.

$^{**}P < 0.01$ compared with IFMQ3 mutant.

**Fig. 7.** Effect of Rg3 on various mutant Na$^+$ channels. The site-directed mutants were generated at the channel pore, the lidocaine or TTX binding sites, and the S4 voltage sensor segment of domains I and II, as described under Experimental Procedures. A, traces are representative of six separate oocytes from three different frogs for analysis of channels with mutations in the channel pore, TTX interaction site, and S4 voltage sensor segment of domain II. B, representative concentration-response curves for the effect of Rg3 on various mutants. 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.
lower affinity for the inactivated state of the Na⁺ channel (Fig. 5).

To examine the molecular mechanism by which Rg₃ regulates brain Na⁺ channel activity, we used site-directed mutagenic methods similar to those previously used to identify drug- or toxin-Na⁺ channel interaction site(s) (Cestele and Catterall, 2000). We used six different types of Naᵥ1.2 mutants to assess the sites at which Rg₃ interacts with Na⁺ channels: 1) mutations in the channel pore entrance of the S6 segment of domain I; 2) mutations in the pore region of domain II; 3) mutations in the lidocaine binding sites; 4) mutations in the TTX binding sites; 5) mutations in the S4 voltage-sensor segments of domains I, II, III, and IV; and 6) mutations in the inactivation cluster. The pore site(s) are unlikely to be the target for Rg₃, because the inhibitory potency of Rg₃ on Na⁺ channel activity was not altered in cells injected with pore site mutants (Fig. 7 and Table 1). In addition, it does not seem as though Rg₃ interacts with the binding sites for lidocaine or TTX (Ragsdale et al., 1994, 1996), because the inhibitory potency of Rg₃ on Na⁺ channel activity was not altered in cells injected with the F385C, F385S, F385Y, F385T, F385M, E387G, E387T, E387Q, F1764A, Y1771A, or F1764A-Y1771A mutants, which harbor changes in the lidocaine or TTX binding sites (Fig. 7 and Table 1). In addition, the inhibitory effect of Rg₃ on the peak $I_{Na}$ seemed additive in the presence of lidocaine or TTX in

![Fig. 8](image-url). The effect of Rg₃ or lidocaine on F1764A, Y1771A, and F1764A-Y1771A mutant Na⁺ channels. The site-directed mutants F1764A, Y1771A, and F1764A-Y1771A were constructed as described under Experimental Procedures. A, C, and E, traces are representatives of six separate oocytes from three different frogs for the F1764A (A), Y1771A (C), and F1764A-Y1771A (E) mutants, respectively. B, D, and F, Rg₃ but not lidocaine inhibited peak $I_{Na}$ in a use-dependent manner in the F1764A (B), Y1771A (D), and F1764A-Y1771A (F) mutants. Insets, only sweeps 1 and 50 are shown for control (○), 1000 μM lidocaine (⊙), and 100 μM Rg₃ (▼). The other sweeps were omitted for clarity. Data represent the means ± S.E.M. (n = 9–10/group).
occlusion experiments (Fig. 6, C and D), providing further evidence that Rg₃ uses a separate binding site. Finally, we tested the possibility that the hydrophobic cluster related with Na⁺ channel inactivation might be involved in the Rg₃-induced inhibition of IₐNa. In the IFMQ3 mutant channel, which lacks fast inactivation (West et al., 1992), Rg₃ treatment inhibited the nonactivating plateau IₐNa to a greater degree than the peak IₐNa, providing further evidences that Rg₃ inhibits IₐNa in both the open and resting states of the Na⁺ channel.

Two other lines of evidence that support the possibility that Rg₃ also inhibits open Na⁺ channels are the observations that 1) the inhibitory effect of Rg₃ on peak IₐNa is not dependent on membrane holding potentials and 2) Rg₃ does not shift the steady-state inactivation curve in wild-type Na⁺ channels (Figs. 3 and 5), which does occur in many drugs (e.g., antiarrhythmic agents and anticonvulsants) that act on inactivated Na⁺ channels (Willow et al., 1985). Moreover, Rg₃ treatment induced an additional use-dependent block of IₐNa in the IFMQ3 mutant compared with wild-type channel, indicating that Rg₃ might prefer to bind and block the open state of the Na⁺ channel. Similar use-dependent open channel blockades have been observed in the case of disopyramide, RSD921, tetracaine, and flecainide (Cahalan, 1978; Grant et al., 1996; Pugsley and Goldin, 1999; Wang et al., 2003; Ramos and O’Leary, 2004).

We next used S4 voltage-sensor segment mutants (K226Q, K859Q, R1312Q, and R1368Q) to examine whether the voltage-sensor segment of the Na⁺ channel was involved in Rg₃-induced Na⁺ channel regulation (Noda et al., 1989). The K859Q mutation in domain II, but not K226Q, R1312Q, or R1638Q, significantly increased the IC₅₀ values by 1.5-fold compared with wild-type (*, P < 0.01, compared with wild-type; Table 1), indicating that this mutation might decrease the affinity of Rg₃ to Na⁺ channels. In addition, K859Q alone abolished the Rg₃-induced voltage shift in Na⁺ channel activation (Fig. 3C) and Rg₃-induced use-dependent but not tonic inhibition of IₐNa (Figs. 4C and 7). Although these results do not allow precise elucidation of the interaction between Rg₃ and Na⁺ channels, our data seem to indicate that the voltage-sensor S4 segment of domain II might be an important portion for Rg₃-induced Na⁺ channel regulation (Figs. 3C and 4C). It is unlikely that Rg₃ interacts nonspecifically with Na⁺ channels, because the 20(S)-ginsenoside Rg₃ used in the present study inhibits IₐNa but 20(R)-ginsenoside Rg₃ does not (Jeong et al., 2004) (Fig. 1). Moreover, modifications of the hydrophilic portion of Rg₃ by opening the cyclic glucoses or conjugating the glucoses with other hydrophobic molecule abolished the inhibitory effect of Rg₃ on peak IₐNa (Kim et al., 2005). These findings indicate that Rg₃ specifically modulates Na⁺ channel by interaction with unidentified site of Na⁺ channel. Further works will be necessary to determine specific interaction site(s) of Rg₃ with Na⁺ channel.

Based on our findings, it seems reasonable to speculate as...
to whether the in vitro Rg3-induced Na⁺ channel blockade could translate to in vivo pharmacological effects. Qian et al. (2005) and Xie et al. (2005) determined plasma concentration of Rg3 after administration of Rg3 via intravenous (5 mg/kg) or intragastric (10 mg/kg) routes in rats and found that plasma Rg3 peaked at 1 μg/ml 10 min after intravenous administration and 4 h after intragastric administration. These findings suggest that plasma-borne Rg3 might exert in vivo pharmacological effects. In addition, in rats, Bae et al. (2004) and Tian et al. (2005) showed that oral (100 mg/kg) or intravenous administration (5 and 10 mg/kg) of Rg3 exerted significant neuroprotective effects against focal cerebral ischemic injury by decreasing neurological deficit scores and reducing the infarct area compared with the control group. Rg3 also significantly improved mitochondrial energy metabolism, antagonized decreases in superoxide dismutase and glutathione-peroxidase activities, and increased malondialdehyde levels in a cerebral ischemia model (Tian et al., 2005). Glutathione-peroxidase activities, and increased malondialdehyde levels in a cerebral ischemia model (Tian et al., 2005). Glutathione-peroxidase activities, and increased malondialdehyde levels in a cerebral ischemia model (Tian et al., 2005).

Fig. 10. Kinetics of open channel blockade by Rg3 and recovery delay in IFMQ3 mutant Na⁺ channels. A, traces evoked from −100 mV to −10 mV in the absence (Con) and presence of various concentrations of Rg3, showing open channel blockade of IFMQ3. B, the rate of Rg3 block as a function of Rg3 concentration. A detailed description of the determination of tB can be found in the text. Data represent the means ± S.E.M. (n = 10–12/group). C, delayed recovery of IFMQ3 channels from open channel blockade by Rg3. Shown is a representative pair of current traces recorded in the absence (Con, top) and presence (Rg3, bottom) of 100 μM Rg3, with a recovery time interval of 10 ms between pulses. Channels recovered almost fully at this recovery interval in the absence of Rg3. In the presence of 100 μM Rg3, the test current measured during the second pulse (P2) was significantly reduced. D, recovery from inactivation or open channel blockade was assessed in detail using the paired-pulse voltage-clamp protocol shown in the inset. After oocytes were depolarized for 10 ms (P1), fractional recovery during a subsequent test pulse (P2) was assessed after an intervening recovery interval. Rg3 (●)-free channels recovered rapidly (<50 ms), whereas complete recovery from inactivation was delayed for 260 ms in the presence of Rg3 (○). Data represent the means ± S.E.M. (n = 9–10/group).

References


Lee et al.

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