Mutations Leu427, Asn428, and Leu431 Residues within Transmembrane Domain-I-Segment 6 Attenuate Ginsenoside-Mediated L-Type Ca\(^{2+}\) Channel Current Inhibitions

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Many lines of evidences have shown that Panax ginseng exhibits beneficial effects on cardiovascular systems. We previously demonstrated that ginsenoside Rg3 (Rg3), one of active ingredients of Panax ginseng, inhibits Ca\(^{2+}\) channel currents in a stereospecific manner and affects the steady-state activation but not inactivation. This points a possibility that Rg3 regulates Ca\(^{2+}\) channels through specific interaction site(s) for Ca\(^{2+}\) influx inhibition through Ca\(^{2+}\) channels. However, it was not known how Rg3 interacts with Ca\(^{2+}\) channel proteins. In the current study, we sought to identify these site(s) in Xenopus oocytes expressing cardiac wild-type and mutant L(\(\alpha_{1d}\))-type Ca\(^{2+}\) channels using the two-microelectrode voltage-clamp technique. To this end, we assessed how various point mutations of the L-type Ca\(^{2+}\) channel affected the Rg3 action. Mutations of L427R, N428R and L431K in transmembrane domain-I-segment 6 (IS6) of the channel significantly attenuated the Rg3 action and caused rightward shifts in dose–response curves. Rg3 treatment produced a negative shift in the inactivation voltage but did not alter the steady-state activation voltage, and none of the mutant channels affected the Rg3-induced negative shift of inactivation voltage. Rg3 had no effects on inactivation time constant in wild-type and mutant channels. These results indicate that Rg3 inhibition of L-type Ca\(^{2+}\) channel currents is attenuated by mutations of Leu427, Asn428 and Leu431 residues of the L-type Ca\(^{2+}\) channel significantly attenuated the Rg3 action and caused rightward shifts in dose–response curves. Rg3 treatment produced a negative shift in the inactivation voltage but did not alter the steady-state activation voltage, and none of the mutant channels affected the Rg3-induced negative shift of inactivation voltage. Rg3 had no effects on inactivation time constant in wild-type and mutant channels. These results indicate that Rg3 inhibition of L-type Ca\(^{2+}\) channel currents is attenuated by mutations of Leu427, Asn428 and Leu431 in transmembrane IS6 residues. Leu427, Asn428 and Leu431 residues of the L-type Ca\(^{2+}\) channel play important roles in the Rg3 effect on channel properties.

Key words Panax ginseng; ginsenoside Rg3; L-type Ca\(^{2+}\) channel; site-directed mutation

Voltage-gated Ca\(^{2+}\) channels belong to a structurally homologous superfamily of voltage-gated channels including K\(^{+}\) and Na\(^{+}\) channels. 1) There are at least five different voltage-gated Ca\(^{2+}\) channel subtypes, the L-, N-, P/Q-, R- and T-types, and their precise physiological and pharmacological functions are still under investigation. 2) Ca\(^{2+}\) channels are transmembrane proteins that consist of a pore-forming \(\alpha\) subunit and several auxiliary subunits. 3) The \(\alpha\) subunit is composed of four homologous domains (I—IV), each composed of six \(\alpha\)-helical transmembrane segments (S1—S6), and is responsible for voltage-dependent increases in Ca\(^{2+}\)-selective permeability. The inward Ca\(^{2+}\) currents through voltage-gated Ca\(^{2+}\) channels in presynaptic terminals are closely coupled to neurotransmitter release. 4) Ca\(^{2+}\) channels can exist in resting (closed), open (active), or inactivated states and \(\beta\) subunits could affect the various states in response to time- and voltage-dependent signaling. 5-7) Clinically relevant L-type Ca\(^{2+}\) channel antagonists can exhibit differential affinities to the L-type Ca\(^{2+}\) channel states. For example, diltiazem and phenylalkylamines show a low affinity for the resting state and a higher affinity for the inactivated or open state 5-7) and site-directed mutagenesis studies have allowed characterization of the detailed actions and binding sites of various Ca\(^{2+}\) channel antagonists. 8-10) Ginseng, the root of Panax ginseng C. A. MEYER, is a well-known herbal medicine. Currently, ginseng is consumed in many countries as a functional food or medicine for tonic or health maintenance. 8) Many lines of evidences have also shown that Panax ginseng exhibits beneficial effects on cardiovascular systems. The main ingredients responsible for the actions of ginseng are the ginsenosides. 9) We have shown that ginsenosides inhibit voltage-gated Ca\(^{2+}\) channels in various neuronal cells 10-14) and that ginsenoside Rg3 (Rg3) is a more potent inhibitor of voltage-gated Ca\(^{2+}\) channels than other ginsenosides in rat sensory neurons and heterolo-gously-expressed cells with stereospecific manner. 15) These findings show a possibility that ginsenosides specifically modulate Ca\(^{2+}\) channel activities by interaction with unidentified site of Ca\(^{2+}\) channels as do Ca\(^{2+}\) channel agonists or antagonists. The purpose of this study was to know the possible Rg3 interaction site(s) in heterologously expressed L-type Ca\(^{2+}\) channels. 11) In this study we chose cardiac L-type Ca\(^{2+}\) channel among various subtypes, since L-type Ca\(^{2+}\) channel antagonists are used for clinical treatments and their pharmacologies and interaction sites are relatively well-characterized than other subtypes. 10) We present evidences that site-directed mutations of amino acid residues Leu427, Asn428 and Leu431 in domain-I-segment 6 (IS6) greatly attenuate Rg3-mediated inhibition of the L-type Ca\(^{2+}\) channel currents.

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MATERIALS AND METHODS

Materials  Figure 1A gives the chemical structures of the 20(S)-Rg3, that was kindly provided by the Korea Ginseng Cooperation (Korea). cDNAs for the L (αC, αZδ, and β3)-type Ca2+ channel subunits were gifts from Dr. K. P. Campbell (University of Iowa, U.S.A.). Their care and handling were in accordance with the highest standards of institutional guidelines. For isolation of oocytes, frogs were anesthetized with an aerated solution of 3-amino benzoic acid ethyl ester, and the ovarian follicles were removed. The oocytes were separated with collagenase followed by agitation for 2 h in Ca2+-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM N-(2-hydroxyethyl)piperazine-N'-2 ethanesulfonic acid (HEPES), 2.5 mM sodium pyruvate, 100 units/ml penicillin, and 100 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 0.5 mM theophylline and 50 µg/ml gentamicin. The oocyte-containing solution was maintained at 18 °C with continuous gentle shaking and renewed every day. Electrophysiological experiments were performed within 5 to 6 d of oocyte isolation, with chemicals applied to the bath. For Ca2+ channel experiments, Ca2+ channel-encoding cRNAs (40 nl) were injected into the animal or vegetal pole of each oocyte 1 d after isolation, using a 10-µl microdispenser (VWR Scientific, West Chester, PA, U.S.A.) fitted with a tapered glass pipette tip (15—20 µm in diameter).14)

Site-Directed Mutagenesis of the L-Type (αC) Subunit, and in Vitro Transcription of αC, αZδ, and β3 cDNAs  Single or double amino acid substitutions were made using a QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.), along with Pyrococcus furiosus DNA polymerase and sense and antisense primers encoding the desired mutations. Overlay of cDNA during its polyadenylation chain reaction (PCR) was carried out according to the manufacturer’s protocol. The final PCR products were transformed into Escherichia coli strain DH5α, screened by PCR, and confirmed by sequencing of the target regions. The mutant DNA constructs were linearized at their 3’ ends by digestion with XhoI, and run-off transcripts were prepared using the methylated cap analog 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. Likewise, recombinant plasmids containing Ca2+ channel cDNA inserts were linearized by digestion with the appropriate restriction enzymes, and cRNAs were obtained using a mMessage mMachine in vitro transcription kit with SP6 RNA polymerase or T7 polymerase. The final cRNA products were resuspended at a concentration of 1 µg/µl in RNase-free water, and stored at −80 °C.14)

Data Recording  A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings. The chamber was constructed by milling two concentric wells into the chamber bottom (diameter/height: upper well: 8/3 mm, lower well: 6/5 mm) and gluing plastic meshes (ca. 0.4-mm grid diameter) onto the bottom of the upper well. A perfusion inlet (ca. 1-mm diameter) was formed in the wall of the lower well, and a suction tube was placed on the edge of the upper well. The oocyte was then placed on a net separating upper and lower wells, the grids of the net serving as dimples to keep it in place during electrophysiological recording. The oocytes were impaled with two microelectrodes filled with 3 m KCl (0.2–0.7 MΩ). The recording solution consisted of 10 Ba(OH)2, 90 NaOH, 2 KOH, 5 HEPES (pH 7.0 adjusted with methanesulfonic acid) and 0.3 niflumic acid (nm). The electrophysiological experiments were performed at room temperature with an Oocyte Clamp (OC-725C, Warner Instrument, Hamden, CT, U.S.A.), and stimulation and data acquisition were controlled with a pClamp 8 (Molecular Devices, U.S.A.). For most of the electrophysiological experiments the oocytes were clamped at a holding potential of −100 mV and depolarized to 10 mV for 1 s, every 10 s. For current–voltage relationships, voltage steps were applied from −60 to +60 mV with 10-mV increments evoked every 10 s for 500 ms.14)

Data Analysis  To obtain concentration–response curves of the effects of Rg3 on Ca2+ currents, the peak amplitudes at different concentrations of Rg3 were plotted, and Origin software (OriginLab Corp., Northampton, MA, U.S.A.) was used to fit the plot to the Hill equation: 

\[
y/n = \frac{[A]^{n_H}}{[A]^{n_H} + [IC_{50}]^{n_H}}
\]

where \(y/n\) is the peak current at a given concentration of Rg3, \(y\) is the maximal peak current, \(IC_{50}\) is the concentration of Rg3 producing a half-maximal effect, \(A\) is the concentration of Rg3, and \(n_H\) is the Hill coefficient. All values are presented as means±S.E.M. The significance of differences between mean control and treatment values was determined using Student’s t test. \(p<0.05\) was considered statistically significant.
RESULTS

Effects of Rg3 on \( I_{Ba} \) in Oocytes Expressing Wild-Type L-Type (\( \alpha_{1c} \)) \( Ca^{2+} \) Channels Inward \( Ba^{2+} \) currents (\( I_{Ba} \)) were recorded by the two-electrode voltage clamp technique from oocytes injected with cRNAs encoding the L-type \( Ca^{2+} \) ion channel \( \alpha_{1c} \) and auxiliary \( \alpha_{2\delta} \) and \( \beta_{3} \) subunits.\(^{19} \) The oocytes were held at \(-100 \) mV and \( I_{Ba} \) was elicited by depolarization to 10 mV every 10 s. As shown in Fig. 2A, the depolarizing voltage steps induced a large inward \( I_{Ba} \). Application of Rg3 to this system inhibited \( I_{Ba} \) in a concentration-dependent manner with an IC50 of 5.9±1.6 \( \mu M \), and a Hill’s coefficient of 1.1±0.3 (Fig. 3, Table 1).

Rg3-Induced Inhibition of \( I_{Ba} \) Is Significantly Attenuated by Mutations L427R, N428R, and L431K in Transmembrane Domain IS6 We first constructed channels altered at the benzothiazepine and dihydropyridine \( Ca^{2+} \) channel antagonist binding sites in transmembrane III6 and IVS6, respectively\(^3,17\) but found no effect on the activity of Rg3, although the inhibitory effects of diltiazem and nifedipine on \( I_{Ba} \) were greatly attenuated in these mutant channels (data not shown). Next, since \( Ca^{2+} \) channels exhibit some structural homologies with \( Na^{+} \) channels and Rg3 inhibits Na,1.2 and Na,1.4 channel currents by interacting with amino acid residues such as N418, L421 and L437 of \( \alpha_{1C} \) and \( \alpha_{2\delta} \) subunits, we also tested a possibility whether Rg3-induced inhibition of \( I_{Ba} \) are mediated through interactions with the analogous amino acids from Val426 to Gly432 in transmembrane domain IS6 of the L-type \( Ca^{2+} \) channels (Fig. 1B). The mutants constructed are listed in Table 1.

We first examined the effects of Rg3 on \( Ca^{2+} \) channel currents in oocytes expressing V426A, L427A, N428A, L429A, V430A, L431A, and G432A channels and observed that the mutation N428A significantly increased IC50 value compared to wild-type (Table 1). We therefore further changed L427, N428 and L431 into other amino acids, generating L427R, N428D, N428K, N428Q, N428R, N428S, L431K, and L431R, and again examined the effect of these changes on Rg3 action. Mutations L427R, N428R or L431K also attenuated Rg3-induced inhibition (Figs. 2B—D, Table 1), while mutations N428D and N428Q had no effect. Although mutations N428K and N428S increased IC50 values, their effects were not as great as those of L427R, N428R, and L431K (Table 1).

TABLE 1. Effect of Rg3 on the Wild-Type and Various Mutant L-Type Calcium Channels Expressed in Xenopus laevis Oocytes

<table>
<thead>
<tr>
<th></th>
<th>( V_{max} )</th>
<th>IC50</th>
<th>( n_{h} )</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>79.0±6.8</td>
<td>5.9±1.6</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>V426A</td>
<td>85.8±4.0</td>
<td>5.1±0.8</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>L427A</td>
<td>79.4±7.8</td>
<td>6.5±1.9</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>L427R</td>
<td>65.2±2.7</td>
<td>17.4±2.1*</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>N428A</td>
<td>85.8±13.3</td>
<td>13.8±6.7*</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td>N428D</td>
<td>88.3±5.5</td>
<td>8.9±1.8</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>N428K</td>
<td>78.5±6.9</td>
<td>12.5±3.0</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>N428Q</td>
<td>79.8±6.4</td>
<td>5.8±1.5</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>N428R</td>
<td>55.3±6.2*</td>
<td>24.5±8.0*</td>
<td>1.2±0.3</td>
</tr>
<tr>
<td>N428S</td>
<td>78.7±6.9</td>
<td>12.5±3.0</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>L429A</td>
<td>87.1±5.9</td>
<td>5.0±1.0</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>V430A</td>
<td>60.1±5.0*</td>
<td>9.8±2.0</td>
<td>1.5±0.4</td>
</tr>
<tr>
<td>L431A</td>
<td>75.9±3.5</td>
<td>4.8±0.7</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>L431K</td>
<td>62.1±1.7</td>
<td>21.4±1.7*</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>L431R</td>
<td>69.7±3.5</td>
<td>16.6±2.5*</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>G432A</td>
<td>77.1±11.1</td>
<td>6.2±2.5</td>
<td>1.1±0.5</td>
</tr>
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</table>

\( V_{max} \) maximal inhibition by Rg3, \( IC_{50} \) inhibition was determined as the % difference of \( I_{Ba} \) in the presence and absence of Rg3. Values are means±S.E.M. (\( n=8—10/\)group).

*\( p<0.01 \) compared with the wild-type \( Ca^{2+} \) channel.

Fig. 2. Effects of Rg3 on Wild-Type (WT) and the Representative Mutant \( Ca^{2+} \) Channels in IS6

During experiments, oocytes were injected with wild-type or mutant cRNAs coding for \( \alpha_{1c} \) and cRNAs coding for the auxiliary \( \alpha_{2\delta} \) and \( \beta_{3} \) subunits of \( \alpha_{1C} \) channel. Electrophysiological experiments were performed 5—6 d after injection of the cRNAs. Oocytes were clamped at a holding potential of \(-100 \) mV and depolarized to \(+10 \) mV for 1 s, evoked every 10 s. Currents were measured with \( 10 \) ms \( Ba^{2+} \). The representative traces are from six separate oocytes from three different frogs in the absence of Rg3 and with various concentrations of Rg3.
Concentration-Dependence of the Effect of Rg₃ on Mutant L-Type Ca²⁺ Channels in IS6  We fitted two representative concentration–response curves for \( I_{\text{Ba}} \) inhibition by Rg₃ of channels with substitutions in residue N428 (Fig. 3A, smooth lines) and of channels with three other amino acid substitutions, L427R, N428R or L431K, using the Hill equation (Fig. 3B, smooth lines). The Hill coefficients were not significantly different from the wild-type values, whereas the \( V_{\text{max}} \) values were significantly changed from 79±6.8 to 55.3±6.2% for wild-type and N428R, respectively (*p < 0.01) (Table 1). IC₅₀ values were 5.9±1.6, 17.4±2.1, 24.5±8.0 and 21.4±1.7 \( \mu M \) for wild-type, L427R, N428R and L431K, respectively. Thus, these mutants gave 3—4 fold higher IC₅₀ values than the wild-type channel (*p < 0.01) (Table 1). Since substitutions L427R, N428R or L431K resulted in increased resistance to Rg₃ and the effect was greatest for N428R, we also examined the effect of replacing the arginine with other amino acids. As shown in Fig. 3A and Table 1, resistance to Rg₃ increased in the order arginine >> lysine >> aspartic acid >> glutamine. Thus, it is unlikely that the physical properties of the substituted amino acids affect the resistance to Rg₃, since amino acids with charged side chains as well as those with hydrophobic side chains reduced Rg₃ inhibition of \( I_{\text{Ba}} \) (Table 1). We also compared the sensitivity of the L427R, N428R and L431K channels to Rg₃. As shown in Fig. 3B, this was in the order N428R > L431K > L427R.

Current–Voltage Relationships of Wild-Type and Mutant Ca²⁺ Channels in the Presence and Absence of Rg₃  Figure 4 shows representative current–voltage relationships in the presence and absence of 30 \( \mu M \) Rg₃ with voltage steps from −60 to +60 mV, every 10 s at −100 mV holding potentials, for wild-type and three different mutant Ca²⁺ channels. Rg₃ caused voltage-dependent inhibition of the peak \( I_{\text{Ba}} \) of the wild-type and mutant Ca²⁺ channels at more depolarized voltages than the resting state, but the L427R, N428R, and L431K channels were much more resistant to Rg₃ than the wild-type channel, indicating that these amino acids are important for the Rg₃ effect on the L-type Ca²⁺ channel (Fig. 3).

Rg₃ Does Not Affect Inactivation Rate in Wild-Type and N428R Mutant Channels  As shown in Fig. 2, mutant channels showed almost no inactivation during the step pulses, we examined the effects of Rg₃ on inactivation rate. We measured the time constant of inactivation of \( I_{\text{Ba}} \) through wild-type and N428R mutant channels in the absence or presence of Rg₃. Thus, the rate of current inactivation in individual cells expressing either wild-type or N428R channels was fit to either single- or double-exponential equations. Rg₃ did not show any significant changes on the fast (t-fast) and slow (t-slow) time constant in wild-type channel inactivation. The inactivation of N428R channels followed a single-exponential time course (Fig. 5B) in the absence or presence of Rg₃. Thus, the fast and slow time constant were not significantly accelerated in the presence of Rg₃ (p < 0.08, compared to the absence of Rg₃, \( n = 4 – 6 \)). Finally, Rg₃ had no significant effects on both depolarized wild-type and N428R channels in inactivation rate. We could also observe that Rg₃ had no significant effects on both L427R and L431K channels in inactivation rate (data not shown).

Effects of Rg₃ on the Activation and Inactivation of Wild-Type and Mutant Ca²⁺ Channels  We also examined the effects of Rg₃ on the voltage-dependence of Ca²⁺ channel steady-state activation and inactivation in wild-type and mutant Ca²⁺ channels. First, the effect of Rg₃ on Ca²⁺ channel activation was determined by conductance transformation of the peak current–voltage relationships (Fig. 6A), using curves representing the best fit with the Boltzmann function. The half maximal activation voltage (\( V_{\text{g50.5}} \)) values were −5.8±0.2, −4.3±0.2, −7.6±0.3, and −1.7±0.2 mV in control conditions for wild-type, L427R, N428R, and L431K, respectively, and −5.1±0.2, −1.5±0.3, −3.1±0.3, and 0.8±0.2 mV in 30 \( \mu M \) Rg₃-tREATED oocytes, respectively. Thus, Rg₃ induced slight but not significant depolarizing shifts of the \( V_{\text{g50.5}} \) at 30 \( \mu M \) in the mutants compared to the wild-type (*p < 0.05, \( n = 8 – 10 \)) (Fig. 6A, Table 2). The
Rg3, a calcium channel modulator, was examined as described (Lee et al., 2006). The depolarization to +10 mV from a holding potential of −100 mV in the absence (control, Con) or presence of 30 μM Rg3 (normalized to peak current). In the absence or presence of Rg3, wild-type and N428R channel inactivation was fit by a single or double exponential equations. (A and B) Only t-slow values were measured in N428R channels (B). #, not detected in N428R channels. In the presence of Rg3, wild-type channels display a slight but not significant increase of inactivation rate. Results are means±S.E.M. of individual fits (n=4—6). * , not detected in N428R channels.

Table 2. Effect of Rg3 on the Voltage-Dependence of Activation and Inactivation of Wild-Type and Mutant L-Type Calcium Channels

<table>
<thead>
<tr>
<th></th>
<th>Activation</th>
<th>Inactivation</th>
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<tbody>
<tr>
<td></td>
<td>Vg0.5, mV</td>
<td>Kg, nS</td>
</tr>
<tr>
<td>Wild-type</td>
<td>−5.8±0.2</td>
<td>3.9±0.2</td>
</tr>
<tr>
<td>(−Rg3)</td>
<td>−5.1±0.2</td>
<td>4.7±0.2</td>
</tr>
<tr>
<td>L427R (−Rg3)</td>
<td>−4.3±0.2</td>
<td>5.0±0.1</td>
</tr>
<tr>
<td>L427R (+Rg3)</td>
<td>−1.5±0.3</td>
<td>5.7±0.3</td>
</tr>
<tr>
<td>N428R (−Rg3)</td>
<td>−7.6±0.3</td>
<td>4.7±0.3</td>
</tr>
<tr>
<td>N428R (+Rg3)</td>
<td>−3.1±0.3</td>
<td>4.3±0.3</td>
</tr>
<tr>
<td>L431K (−Rg3)</td>
<td>−1.7±0.2</td>
<td>4.6±0.2</td>
</tr>
<tr>
<td>L431K (+Rg3)</td>
<td>0.8±0.2</td>
<td>4.5±0.2</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01, compared with the absence of Rg3. Values are means±S.E.M. (n=8—10/group).

Fig. 6. The Effect of Rg3 on Steady-State Activation and Inactivation of IiNa in Wild-Type and Mutant N428R Ca2+ Channels

(A) Effect of Rg3 on steady-state activation of IiNa in the wild-type and mutant channels was examined as described (Lee et al., 2006). The voltage-dependence of conductance was compared in the absence (−Rg3) and presence (+Rg3) of 30 μM Rg3. (B) 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 (−Rg3) and presence (+Rg3) of 30 μM Rg3. Data are means±S.E.M. (n=7—8/group). The curves represent two-state Boltzmann functions.

slope factors (Kg) were also not significantly different, with values of 3.9±0.2, 5.0±0.1, 4.7±0.3, and 4.6±0.2 mV under control conditions for wild-type, L427R, N428R, and L431K, respectively, and 4.7±0.2, 5.7±0.3, 4.3±0.3, and 4.5±0.2 for wild-type, L427R, N428R, and L431K in 30 μM Rg3-treated oocytes, respectively (Table 2).

Next, we investigated the effect of Rg3 on wild-type and mutant channel inactivation by plotting normalized peak IiNa against the conditioning pre-pulse voltage (Fig. 6B, Table 2), and fitting the data to the Boltzmann function. The half maximal inactivation voltage (Vh0.5) values were −7.8±0.5, −3.7±0.5, −1.1±0.3, and −6.7±0.7 mV in control conditions for wild-type, L427R, N428R, and L431K, respectively, and −13.5±0.1, −8.6±0.7, −7.3±0.3, and −14.6±0.5 mV for wild-type, L427R, N428R, and L431K in 30 μM Rg3-treated oocytes, respectively (Table 2). Thus, Rg3 induced a significant hyperpolarizing shift of the Vh0.5 at 30 μM in both wild-type and mutant channels (* p<0.05, ** p<0.01, compared with the wild-type in the absence of Rg3 in, n=8—10). However, the slope factors (Kh) were not significantly different, yielding values of 8.5±0.4, 10.4±0.5, 5.4±0.3, and 9.2±0.7 mV under control conditions for wild-type, L427R, N428R, and L431K, respectively, and 10.1±0.9, 10.3±0.6,
DISCUSSION

L-type Ca^{2+} channels are high voltage-activated Ca^{2+} channels that have a large conductance and long lasting current.\(^7\) They are mainly located in the heart and neurons and function to modulate heart muscle contraction and neurotransmitter release.\(^1\) Recent studies show that they function both to regulate membrane excitability and in signal transduction in the nervous system.\(^1\) Thus, L-type Ca^{2+} channels are main target by some drugs for hypertension and other cardiovascular dysfunction treatments.\(^16\)

We previously demonstrated that Rg3 regulates Ca^{2+} channel currents in a stereospecific manner (i.e., 20(R)-Rg3, but not 20(R)-Rg3)\(^{15}\) and that subsets of ginsenosides including Rg3 affect resting or tonic state of Ca^{2+} channels examined.\(^4\) However, little was known of how it interacted with L-type Ca^{2+} channel proteins. We have first examined whether Rg3 shares the overlapping sites with dihydropyridine (DHP) and diltiazem binding sites in the regulation of L-type Ca^{2+} channels\(^5,7\) but found that these sites did not affect Rg3 sensitivity (data not shown). In previous report, we have shown that Rg3 regulates Na_{1.2} and Na_{1.4} channel currents by interacting with amino acid residues such as N418, L421 and L437 of transmembrane domain IS6.\(^14\) However, little was known of how it interacted with L-type Ca^{2+} channel proteins. We have first examined whether Rg3 shares the overlapping sites with dihydropyridine (DHP) and diltiazem binding sites in the regulation of L-type Ca^{2+} channels\(^5,7\) but found that these sites did not affect Rg3 sensitivity (data not shown). However, little was known of how it interacted with L-type Ca^{2+} channel proteins. We have first examined whether Rg3 shares the overlapping sites with dihydropyridine (DHP) and diltiazem binding sites in the regulation of L-type Ca^{2+} channels\(^5,7\) but found that these sites did not affect Rg3 sensitivity (data not shown). In previous report, we have shown that Rg3 regulates Na_{1.2} and Na_{1.4} channel currents by interacting with amino acid residues such as N418, L421 and L437 of transmembrane domain IS6.\(^18\) Since Ca^{2+} channels show some structural homologies with Na^{+} channels, we also examined whether mutations of the analogous amino acids from Val426 to Gly432 in transmembrane domain IS6 of the L-type Ca^{2+} channel influences Rg3 action (Fig. 1B). We have found that mutation of Leu427, Asn428, or Leu431 residues of transmembrane domain IS6 attenuated Rg3-mediated L-type Ca^{2+} channel current inhibitions by shifting into rightward directions in dose–response curves (Fig. 3).

Ca^{2+} channel inactivation, which is characterized by attenuated Ca^{2+} influx during prolonged depolarization, or in decreased Ca^{2+} channel availability at depolarized holding potentials, is an important form of Ca^{2+} channel regulation by various Ca^{2+} channel antagonists.\(^2,20\) Interestingly, we could observe that the mutant channels showed different inactivation kinetics from that of wild-type channels as shown in Fig. 2. Especially, N428R and L431K showed almost no inactivation during the 10 s step pulses (Figs. 2C, D). At the same time, Rg3 inhibitions on \(I_{Na}\) in these mutants were greatly attenuated. Thus, these results show a possibility that changes in inactivation kinetics in mutant Ca^{2+} channels might also affect Rg3 inhibitions on \(I_{Na}\) without direct Rg3 interactions with the mutant channels. However, it is unlikely that the decreases in Rg3 inhibitions on \(I_{Na}\) in mutant channels are due to the changes in inactivation kinetics (i.e., slow of inactivation rate) in mutant channels (Figs. 2, 5B), since we could show that the presence of Rg3 does not significantly affect on inactivation rate in wild-type and mutant channels (Fig. 5). In addition, we could also observe that Rg3 induced a significant hyperpolarizing shift of the half-maximal inactivation voltages (\(V_{1/2}\)) in wild-type channels and that Rg3-mediated shift in the half-maximal inactivation voltages was still maintained in mutant channels (Fig. 6, Table 2). Thus, Rg3-mediated inhibition on \(I_{Na}\) in wild-type and the attenuations of the inhibitory effects of Rg3 on \(I_{Na}\) in mutant channels is independent on inactivation of L-type Ca^{2+} channels.

In previous reports we demonstrated that point mutation of specific amino acid residues in the Kv1.4 and Na^{+} channels, as well as in the 5-HT\(_3\)A receptors, almost abolished Rg3 sensitivity.\(^18,22,23\) The difference in the severity of the Rg3 effect on the L-type Ca^{2+} channel versus other channels and receptors may have two explanations; first the point mutations in the L-type Ca^{2+} channel may not be effective enough to confer complete resistance to Rg3. The other possibility is that the amino acid residues identified in the present study are only part of the interaction site for Rg3. However, we cannot exclude the possibility that substitutions L427R, N428R, or L431K cause a conformational change of the L-type Ca^{2+} channels and interfere allosterically with interaction of Rg3 with the channel.

Based on our findings on the Rg3 interaction sites in L-type Ca^{2+} channels, it seems reasonable to speculate on how these sites are related to the in vitro pharmacological effects of Rg3. Ginseng has many beneficial effects on the cardiovascular systems.\(^24\) Gao et al. (1992) have shown that ginsenoside administration via the intravenous route attenuates ischemic and reperfusion arrhythmia in rats, and Yang et al. (1999) showed that ginsenoside administration via the in traperitoneal route attenuates myocardial reperfusion arrhythmia in rats fed a high cholesterol diet.\(^25,26\) However, we do not have any direct evidence that Rg3-mediated L-type Ca^{2+} channel regulation can be used prophylactically or therapeutically against arrhythmia as other L-type Ca^{2+} channel can. More investigation is needed on the potential application of Rg3 to heart dysfunctions.

In summary, we have shown that mutation of Leu427, Asn428, or Leu431 residue in transmembrane domain IS6 significantly attenuated Rg3-mediated L-type Ca^{2+} channel current inhibitions. Since the Rg3-induced effects on Ca^{2+} signaling may be a major component of the in vivo protective and therapeutic actions of Panax ginseng, these findings may contribute to the explanation of how Rg3 exerts its one of beneficial effects on the cardiovascular systems.

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