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Ginsentology III: Identifications of Ginsenoside Interaction Sites for Ion Channel Regulation

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Abstract : A ligand – whether an endogenous hormone, neurotransmitter, exogenous toxin or synthetic drug – binds to plasma membrane proteins (e.g., ion channels, receptors or other functional proteins) to exert its physiological or pharmacological effects. Ligands can also have functional groups, showing stereospecificity for interaction sites on their counterpart plasma membrane proteins. Previous reports have shown that the ginsenoside Rg_3, a bioactive ginsenoside, meets these criteria in that: 1) an aliphatic side chain of Rg_3 plays a role as a functional group, 2) Rg_3 regulates voltage- and ligand-gated ion channels in a stereospecific manner with respect to carbon-20, and 3) Rg_3 regulates subsets of ligand-gated and voltage-gated ion channels through specific interactions with identified amino acid residues inside the channel pore, in the outer pore entryway, or in toxin binding sites. Rg_3, therefore, could be a candidate for a novel ginseng-derived glycosidic ligand regulating ion channels and receptors. This review will examine how Rg_3 regulates voltage-gated and ligand-gated ion channels through interactions with its target proteins in the plasma membrane. Hopefully, this review will advance understanding of ginseng pharmacology at the cellular and molecular levels.

Key words : *Panax* ginseng, Ginsenoside Rg_3, Ion channels, A novel glycosidic ligand, A new paradigm

INTRODUCTION

In herbal and traditional medicine, people have long believed that ginseng might have interesting physiological and pharmacological effects. Currently, ginseng is consumed in many countries as a food or medicine for increased stamina or health maintenance.[1] In addition, much research using modern methods and technology has shown that the active ingredients of ginseng are ginsenosides, which exert many effects in nervous and non-nervous cells or tissues. Most cellular events initiated by biologically active compounds such as hormones, neurotransmitters, drugs and other ligands are through interactions with plasma membrane proteins. It is known that ginsenosides act through plasma membrane proteins when they are studied in *in vitro* or *in vivo* systems.[2] Up to now, however, it has been unclear how ginsenosides interact with plasma membrane proteins to cause downstream effects. Recent studies have shown that ginsenoside Rg_3 is the most active component among various ginsenosides in regulating various ion channels and receptors and that the effects of Rg_3 are closely related to its structure. Three main structure-activity relationships have been observed in Rg_3-mediated regulation of voltage-gated or ligand-gated ion channels. First, an intact two carbohydrate portion attached to carbon-3 of the Rg_3 backbone is required for full inhibition of voltage-gated Na^+ channels. The production of aglycone by removing this carbohydrate portion or Rg_3-derivatives by conjugation with other chemical agents causes a loss of Rg_3 action.[3] Second, there are two Rg_3 epimers at carbon-20: 20(*R*)-Rg_3 and 20(*S*)-Rg_3. Interestingly, only 20(*S*)-Rg_3 shows inhibitory effects on voltage-gated Ca^{2+}, K^+ and Na^+ channel currents.[4] Finally, an aliphatic side chain of Rg_3 acts as a functional group in ion channel regulation. Modification of the aliphatic side chain of Rg_3 resulted in large differences in channel regulation, i.e., removal or oxidation of the aliphatic side chain had no effect on Na^+ channel regulation, while an Rg_3-derivative produced by reduction of the aliphatic side chain was a more potent inhibitor of Na^+ channel currents than Rg_3 itself.[5] These results strongly

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support the hypothesis that Rg_3 inhibits various ion channel currents through interactions with ion channels or receptor proteins in the plasma membrane.

This review will examine how Rg_3 acts specifically on ion channels and receptors. The key method used to approach this question was site-directed mutagenesis to identify Rg_3 interaction sites in Rg_3-induced ligand-gated and voltage-gated ion channels. This method is widely used to identify defined interaction sites of various kinds of drugs and toxins, since a ligand that regulates a certain type of ion channel (i.e., K^+, Na^+ and other channels) usually achieves its effects through specific interactions or binding sites on the ion channel protein. For example, if point mutations in a specific amino acid in the receptor or channel abolish or attenuate the action of a ligand, it is typically thought that the ligand interacts with that specific amino acid(s).[6] Using this site-directed mutagenesis method and heterologous expression of ligand-gated and voltage-gated ion channels in *Xenopus laevis* oocytes, we will show how ginsenoside behaves similarly to other ligands.

Ginsenoside Rg_3 regulates 5-HT_{3A} receptor channel activity through interaction with amino acids at the channel pore of transmembrane domain 2 (TM2)

The 5-hydroxytryptamine type 3 (5-HT_3) receptor is a member of the cys-loop family of ligand-gated ion channels, including the nicotinic acetylcholine receptor, $GABA_A$ receptor and glycine receptors.[7, 8] The 5-HT_3 receptor is a cationic ion channel that allows Ca^{2+} and Na^+ to flow into cells, causing depolarization when the receptor is activated by serotonin or other agonists. Various agents exert their pharmacological effects by targeting 5-HT_3 receptors, which are mainly involved in vomiting and irritable bowel syndrome.[9-12] The structure of the 5-HT_3 receptor is well-characterized, consisting of a large extracellular N-terminal and intracellular C-terminal, and each subunit has four hydrophobic transmembrane domains (TM1-TM4). The assembly of four homomeric pentamers is required for full functional activity of the 5-HT_3 receptor.[13] Studies have shown that serotonin and agonist-binding sites are located in the N-terminal domain at the subunit-subunit interfaces, while the TM2 domains of the five subunits combine to form the channel pore.[7, 8] Recently, site-directed mutagenesis has been used to characterize the detailed actions and binding sites of various drugs and toxins that regulate the 5-HT_3 receptor.[11, 12] The substituted cysteine accessibility method has also been used to infer channel gating regions of this receptor. In particular, Reeves *et al.*(2001) and Panicker *et al.* (2002) revealed that amino acids from D274 to D298 in TM2 form the putative pore-lining face, and that residues D274, G276, E277, S280, T284, L285, L287, S290, V291, F292, L293, I295, and V296 appear to form a gating region that is susceptible to sulfhydryl reagents.[14, 15] This putative gating region in the 5-HT_{3A} receptor is similar to that previously identified for the nicotinic acetylcholine receptor.[16] The precise mechanisms by which the binding of serotonin or agonist leads to channel opening and gating, however, are still poorly understood at the molecular level.[7]

Previous studies have demonstrated that Rg_3, one of the ginsenosides that most potently regulates the 5-HT_3 receptor, is a non-competitive inhibitor of 5-HT_{3A} receptor-mediated ion currents, and that the inhibitory effect of Rg_3 on serotonin-induced currents ($I_{5\text{-}HT}$) is observed when it is applied extracellularly but not intracellularly.[17] Moreover, mutations in pre-TM1, which causes facilitation of 5-HT_{3A} receptor channel activity, not only abolish the inhibitory effects of 20(*R*)-Rg_3 on $I_{5\text{-}HT}$, but also greatly attenuate the action of 20(*S*)-Rg_3 on $I_{5\text{-}HT}$.[18] These results indicate that Rg_3 achieves its inhibitory effects through interactions with unidentified amino acids, which are not in the serotonin or agonist binding sites, in a stereospecific manner. It is not easy, however, to obtain further information about which amino acid residues could interact with Rg_3. Mutations in the TM2 region affect $I_{5\text{-}HT}$ inhibition in response to anesthetics, cadmium and the open channel blocker TMB-8, so it was thought that Rg_3 might interact with the 5-HT_3 receptor pore regions of TM2.[13] Based on this hypothesis, a series of mutant 5-HT_3 receptors in the TM2 region were constructed and screened one by one.

Unexpectedly, V291A, F292A and I295A mutations in the TM2 region greatly attenuated or abolished Rg_3-induced inhibition of peak $I_{5\text{-}HT}$. These results further suggest that Rg_3 is acting through 5-HT_3 receptor, and alterations in TM2 of the 5-HT_3 receptor could affect the action of Rg_3. In addition, the V291A mutation but not the F292A or I295A mutations induced constitutively active ion currents, with a decreased current decay rate. Rg_3 treatment of this mutant receptor accelerated the rate of current decay in a dose-dependent manner in the presence of 5-HT, suggesting that the presence of Rg_3 caused channel closure rather than opening. It was observed that Rg_3 and TMB-8, an open channel blocker, inhibited the constitutively active ion currents in a dose-dependent manner. Diltiazem, another open channel blocker, did not prevent Rg_3-induced inhibition of the constitutively active

ion currents in occlusion experiments.[13] These data provide the following further insights: 1) Rg_3 inhibits 5-HT_{3A} receptor channel activity through interactions with residues V291, F292 and I295 in the channel gating region of TM2, and 2) Rg_3 regulates 5-HT_{3A} receptor channel activity in the open state at different site(s) from those used by TMB-8 and diltiazem. Thus, Rg_3 inhibits the 5-HT_3 receptor in the open state through interactions with V291, F292 and I295. The identification of Rg_3 interacting sites in the 5-HT_3 receptor first supports the hypothesis that Rg_3 interacts with a plasma membrane protein to achieve its biological functions and further suggests the possibility that Rg_3 regulates the activity of other receptors or ion channels through interactions with other sites.

Ginsenoside Rg_3 regulates human voltage-gated Kv1.4 channel activity through interaction with amino acids at the channel outer pore entry

Voltage-gated K^+ (Kv) channels play critical roles in a wide variety of physiological processes, including the regulation of neurotransmitter release, neuronal excitability, heart rate, muscle contraction, hormone secretion, epithelial electrolyte transport, cell volume and cell proliferation in both neuronal and non-neuronal cells.[19] Kv channels consist of tetramers of pore-forming Kvα and auxiliary Kvβ subunits.[19] The Kvα subunit is composed of six α-helical transmembrane segments (S1-S6). The S4 segment acts as the voltage-sensing module of the K^+ channel,[19] while the pore-forming S5 and S6 segments constitute a selectivity filter and govern voltage-dependent increases in K^+ permeability. Site-directed mutagenesis studies using Kvα subunits have clarified the detailed actions and binding sites of various drugs that regulate Kv channel activity.[19] There are numerous Kv channel subtypes: some Kv channel α subunits exhibit transient A-type K^+ currents and N-type inactivation, while others exhibit long-lasting, delayed-rectifying C-type K^+ currents and C-type inactivation, depending on the channel conductance and gating characteristics.[20]

In previous studies, we found that subsets of Kv channel currents were not blocked by Rg_3 (data not shown), indicating that Rg_3 exhibited differential regulation of Kv channel subtypes. Previous reports have also shown that Rg_3 regulates human Kv1.4 channel activity in a stereospecific manner: 20(*S*)-Rg_3 but not 20(*R*)-Rg_3 inhibits Kv1.4 channel currents.[4] Based on these results, Rg_3 might regulate Kv1.4 channel activity through specific interaction site(s) in the Kv1.4 channel protein. Interestingly, there is a well-known phenomenon that some subsets of Kv channel currents, including Kv1.4 currents, are enhanced when the extracellular K^+ concentration is increased; this is called K^+ activation. The K^+ activation site, which is located at the outer pore entry, consists of several amino acids including lysine 531 (K531). Mutations at this site abolish K^+ activation. Some subsets of Kv channel currents, including Kv1.4, are also affected by extracellular and intracellular tetraethylammonium (TEA), which is a well-known K^+ channel blockers. The wild-type Kv1.4 channel, however, is nearly insensitive to TEA. Interestingly, one of the extracellular TEA binding sites also contains the K531 residue. Mutations in this K531 residue (e.g., K531Y) increased the sensitivity of the Kv1.4 channel to extracellular TEA.

Interestingly, it appears that the regulatory effect of Rg_3 on Kv1.4 channel activity are strongly dependent on the extracellular K^+ concentration by shifting Rg_3 concentration-response curve rightward, indicating that Rg_3 competes with extracellular K^+ for the same interaction site(s).[21] Moreover, although extracellular TEA treatment does not inhibit the wild-type Kv1.4 channel, extracellular TEA also competes with Rg_3 for the inhibition of Kv1.4 channel currents by shifting the Rg_3 concentration-response curve rightward.[21] These results suggest that Rg_3-mediated regulation of Kv1.4 channel activity might be achieved through common interaction site(s) for K^+ activation and TEA binding sites. Alternatively, the Rg_3 interaction site(s) might overlap with the K^+ activation site or TEA binding site. In order to test this possibility, various mutants in the Kv1.4 channel, including the K531 residue, were introduced. Mutants were made in the channel pore regions: channel pore sites (S510K, D513Q, V525L, and V535Q) and outer pore sites (K531A, P532A, I533A, T534A, V535A).[19] Channel mutants were also made in the *N*-glycosylation site (N353Q),[22] the voltage sensor site (R447C and R450C),[23] the voltage shift sites (L478F and G548P),[24] the pH sensitive site (H507Q)[25] and the C-type inactivation site (V560A).[26] The K531A mutant, located in one of the outer pores, significantly attenuated Rg_3 inhibition of Kv1.4 channel currents, while the other mutants had no significant effects. These results suggest the possibility that Rg_3 regulates Kv1.4 channel activity by interacting with Lys531, which is also known to be one of the K^+ activation sites and one of the extracellular TEA binding sites. Other mutant channels at the K531 residue such as K531Y, I533M, and K531Y-I533M showed that the K531Y but not I533M substitution and the K531Y-I533M double substitution nearly abolished Rg_3 inhibition of Kv1.4 channel currents.[25, 27] These results indicate that Rg_3-mediated regulation of Kv1.4 channel activity is closely

related to the Lys531 residue.

Homology and virtual docking model methods,[28-30] which give three dimensional configurations of how Rg_3 could bind to the Kv1.4 channel protein through various interactions such as hydrogen bonds or hydrophobic interactions, show that the carbohydrate portion of Rg_3 plays an important role in its interaction with the Kv1.4 channel. The second, but not the first, carbohydrate attached at carbon-3 of the Rg_3 backbone forms six hydrogen bonds with amino acids in the pore entryway of the Kv1.4 channel. Among the amino acids forming hydrogen bonds with Rg_3, K531 forms three bonds and threonine and histidine form the other hydrogen bonds. The K531Y mutant in the Kv1.4 channel, however, shows only two hydrogen bonds with Rg_3, indicating that mutation of K531 to K531Y induces a conformational change and results in only two hydrogen bonds with Rg_3. This results in the loss of Rg_3-mediated Kv1.4 channel regulation.

This three dimensional modeling technique also helps to identify the role of the Rg_3 backbone in the regulation of Kv1.4 channel. The Rg_3 backbone is located in the pore portion of the Kv1.4 channel,[21] and this could block the pore and interrupt K^+ efflux when the channel is stimulated by depolarization. The second carbohydrate portion of Rg_3 forms stable hydrogen bonds with amino acids in the outer pore entryway. Thus, the backbone of Rg_3 could acts as a physical plug or wedge in Rg_3-mediated Kv1.4 channel regulation. In conclusion, the use of a site-directed mutagenesis method allows for the confirmation and identification of the Rg_3 interaction site on the Kv1.4 channel. Homology and virtual docking model methods also provide three-dimensional configurations of how the carbohydrate portion and backbone of Rg_3 could interact with the Kv1.4 channel protein.

Ginsenoside Rg_3 regulates voltage-gated Na^+ (Nav1.2 and Nav1.4) channels through interactions with amino acids in batachotoxin (BTX) binding sites

There are three subtypes of Na^+ channels in excitable organs: neuronal (Nav1.2)-, heart (Nav1.5)- and muscle (Nav1.4)-type Na^+ channels. These channels are further divided into at least nine different subtypes.[31] Previous reports have shown that Rg_3 inhibits peak current in all voltage-dependent neuronal, heart and muscle Na^+ channels.[5,32,33] Na^+ channels are transmembrane proteins consisting of a pore-forming α subunit and auxiliary $\beta1$, $\beta2$ and $\beta3$ subunits.[31] The α subunit is made up of four homologous domains (I–IV), each composed of six α-helical transmembrane segments (S1–S6), and is responsible for voltage-dependent increases in Na^+-selective permeability. The transient inward Na^+ current (I_{Na}) initiates axonal and somatic action potentials in nerve and muscle fibers and may also be involved in intraneuronal axonal or interneuronal information transfer.[34] Na^+ channels are one of the primary targets in cardiac and neuroprotective treatments of pathologic conditions including arrhythmia and brain ischemia. Currently, many pharmaceutical companies and research institutes are searching for novel Na^+ channel regulatory agents for clinical applications and therapies for diseases of the brain and heart.

As mentioned above, the Na^+ channel α subunit is the primary domain forming the channel pore and voltage-sensor, and site-directed mutagenesis has been applied to identify drug/toxin binding sites in this region.[35] For example, tetrodotoxin (TTX), a neurotoxin from pufferfish (*fugu*), is a very strong neuronal Na^+ channel blocker, and incorrect cooking of this fish still causes the deaths of many people. Using site-directed mutagenesis, investigators identified the TTX binding site, containing several amino acid residues such as phe385 and phe387.[36, 37] As a second example, the local anesthetic lidocaine exhibits its anesthetic effects by inhibiting Na^+ channel currents. Also using site-directed mutagenesis, the interaction sites for lidocaine were identified at Phe1764 and/or Tyr1771 of the Na^+ channel.[38, 39] In addition to these two agents, there are many kinds of drugs and toxins that affect Na^+ currents. These are classified into several categories, depending on their interaction sites.[35]

Two main characteristics of Rg_3-mediated regulation of wild-type Na^+ channels have been identified. One is that Rg_3 treatment causes a depolarizing shift in the activation voltage step in wild-type Na^+ channels, indicating that Rg_3 binding to the Na^+ channel does not allow the Na^+ channel to easily open at a given voltage step, requiring greater depolarizing stimulation compared to without Rg_3. The other characteristic is that Rg_3 induces use-dependent inhibition, meaning that the channel pore blocking actions of Rg_3 are enhanced by rapid, repeated stimulation over a very short time period, indicating that Rg_3 might be a kind of open channel blocker. The idea that Rg_3 is an open channel blocker of Na^+ channel is supported by experiments using inactivation-deficient Na^+ channel mutants, in which the inactivation gate has been deleted and transient inward currents are converted into long-lasting inward currents.[32] Thus, Rg_3 more potently inhibited the plateau than peak I_{Na} and facilitated channel closing in inactivation deficient channel mutants. Interestingly, mutations of one amino acid (lys859 to glu859) in the

voltage-sensor domain in the S4 helix abolished the Rg_3-mediated depolarizing shift without affecting Rg_3-mediated peak current (I_{Na}) inhibition. This indicates that Rg_3 might act through interactions with the Na^+ channel, and conformational changes of the Na^+ channel through mutations in specific amino acid residues affect Rg_3 action. These results did not identify, however, the exact interaction sites in the Na^+ channel proteins.

Since Rg_3 regulates 5-HT_3 receptors and Kv1.4 channels at common interaction sites shared with other drugs, it was examined whether Rg_3 also inhibits I_{Na} at common interaction sites shared with representative Na^+ channel blockers such as TTX and lidocaine. It appears, however, that Rg_3 still exerts its inhibitory effects on mutant channels in which the lidocaine and TTX binding sites were disrupted, indicating that Rg_3 inhibition of I_{Na} is not achieved via the lidocaine or TTX interaction sites. Batrachotoxin (BTX) is another neurotoxin that acts on Na^+ channels; it is found in the skin of South American frog *Phyllobates terribilis* and persistently activates brain $Na_V1.2$ and skeletal muscle $Na_V1.4$ channels rather than inhibiting I_{Na} as lidocaine and TTX do.[35] Interestingly, BTX is a kind of steroidal alkaloid toxin, and the backbone structure of BTX is similar to that of Rg_3. It is known that BTX interaction sites are located at the I433, N434 and L437 residues of the $Na_V1.4$ channel and equivalent residues such as I417, N418 and L421 of brain $Na_V1.2$ channel in domain-I segment 6 (IS6).[35] Channel mutations in BTX binding sites, such as N418K and L421K in the rat brain $Na_V1.2$ and L437K in the mouse skeletal muscle $Na_V1.4$ channel, attenuate or abolish Rg_3 inhibition of I_{Na}. In addition, channel mutants in BTX binding also greatly attenuate the Rg_3-mediated depolarizing shift of the activation voltage, which is observed in wild-type channels. Moreover, Rg_3-mediated use-dependent inhibition was also almost abolished in these mutant channels. These results indicate that the characteristics of Rg_3 action on Na^+ channels, such as the Rg_3-mediated depolarizing shift in the activation voltage and Rg_3-mediated use-dependent inhibition, are achieved through BTX binding sites and that BTX binding sites play an important role in modifying Rg_3-mediated Na^+ channel properties. Finally, BTX, but not lidocaine and TTX, binding sites in brain- and muscle-type Na^+ channels play important roles in Rg_3-mediated Na^+ channel regulation.

DISCUSSION

Since it has been known that ginsenosides are the primary active ingredients in *Panax* ginseng, mediating its pharmacological and physiological effects, numerous reports have been published using ginsenosides to explain the efficacy of ginseng. Until recently, however, the scientific rationale behind the actions of ginsenoside in explaining the efficacy of ginseng was not well established. This is partially due to the fact that they are plant-derived saponins, which usually non-specifically penetrate or perturb the plasma membrane and induce cell lysis. Accumulating evidence, however, shows that Rg_3 does not follow the usual saponin-like actions in its regulation of ion channels and receptors. Rather, Rg_3 has functional groups, shows stereospecificity and has specific, identified interaction sites on proteins in the plasma membrane through which it can achieve its pharmacological and physiological effects. Thus, Rg_3 shows a different mode of action from the usual saponins. These data suggest a new paradigm for ginseng pharmacology; i.e., Rg_3 is a novel ginseng-derived glycosidic ligand. These results further suggest the possibility that other plant-derived saponins with pharmacological effects besides ginsenosides might also act through as yet unidentified interaction sites.

When Rg_3 interaction sites on receptor and ion channels were identified, a number of findings were quite surprising. First, although the affinity of Rg_3 (IC_{50} values are in the μM range) for inhibition of receptors or ion channels is lower than that of specific drugs or toxins, single amino acid substitutions in the ion channel or receptor proteins can abolish or greatly attenuate the actions of Rg_3. Second, the Rg_3 interaction sites are not dependent on specific amino acid properties, which can be hydrophilic, hydrophobic or neutral; thus, Rg_3 did not show amino acid residue selectivity. For example, Rg_3 interacts with valine, isoleucine and phenylalanine in regulating the 5-HT_3 receptor; lysine for regulation of the Kv1.4 channel; and glutamine and lysine for Na^+ channel regulation. Third, Rg_3 shares interaction sites with subsets of other well-known drugs or toxins such as alcohol, BTX and TEA. These results suggest that the identified Rg_3 interaction sites overlap with those of other well-known drugs or toxins, although we cannot exclude the possibility that Rg_3 also interacts with other as yet unidentified amino acid residues in receptors and ion channels. Finally, sharing Rg_3 interaction sites with other drugs or toxins could explain why Rg_3 has multiple but not unique targets in various *in vitro* or *in vivo* systems, even though Rg_3 interaction sites differ in different receptors or ion channels. It seems that ginsenoside selectivity and specificity in the regulation of receptors and ion channels is different from those of other

drugs and toxins that show the property of one specific agent acting on one specific target. Ginsenoside, on the other hand, is a ligand with multiple targets but specific interaction sites in each target.

It is interesting to speculate about why Rg_3 acts in a different manner from specific drugs or toxins to regulate receptors and ion channels. This might be due to the structure of Rg_3. As shown in a previous report, Rg_3 consists of three main portions: an aliphatic side chain, a carbohydrate domain, and a hydrophobic backbone.[32] Previous reports have shown that the hydroxyl groups in the second carbohydrate portion of Rg_3 form hydrogen bonds with amino acid residues on ion channel proteins.[32] In ion channel mutants, however, the number of hydrogen bonds is reduced, and Rg_3-mediated regulation of ion channel activity is greatly attenuated or abolished. These results suggest that the carbohydrate domain of Rg_3 might play a role as a primary recognition site of amino acid side chains in receptors and ion channels. Mutations in receptors or ion channels, however, might cause conformational changes in ion channels or receptors without changes in native ion channel or receptor properties, providing a less hospitale environment for hydrogen bond formation. If the carbohydrate portion of Rg_3 plays a key role in binding and recognition by forming hydrogen bonds with ion channels and receptors, it might be possible to explain why Rg_3 interacts differently with ion channels and receptors compared to other agents. Again, the key element seems to be the carbohydrate of Rg_3. Carbohydrates attached to ginsenosides including Rg_3 are not unique, but are a rather common and universal component found in all biological systems, unlike other specific drugs or toxins with unique chemical structures or unique properties. Therefore, if plasma membrane proteins provide proper or suitable environments for interactions with Rg_3, the carbohydrate portion of Rg_3 could easily form hydrogen bonds with neighboring amino acids. This glycosidic property of Rg_3 might be the basis for why Rg_3 has multiple targets and shares binding sites with other drugs and toxins.

It might be questioned how the carbohydrate portion of Rg_3 has the opportunity to form hydrogen bonds with ion channels and receptors. For argument's sake, we can imagine two situations: one is an artificial lipid bilayer model and the other is a real plasma membrane. The backbone structure of Rg_3 is similar to that of hydrophobic steroid hormones. It is well-known that the pharmacological and physiological effects of most steroid hormones are achieved through binding to cytosolic steroid hormone receptors, indicating that most steroid hormones must penetrate the plasma membrane to exert their effects. In the case of Rg_3, uncharged hydrophilic carbohydrates are coupled to carbon-3 of the hydrophobic backbone, making Rg_3 weakly amphiphilic. The hydrophobic portion with the aliphatic side chain of Rg_3 could intercalate or penetrate the lipid bilayer, leaving the carbohydrate portion behind. Rg_3 might not penetrate the plasma membrane completely, however, because the hydrophilic carbohydrate might remain on the hydrophilic surface of the solution and might block further penetration of the hydrophobic backbone into the lipid bilayer. The degree of intercalation or penetration of the hydrophobic backbone into the lipid bilayer might dependent on the number of carbohydrates attached to the backbone structure. In the real plasma membrane, the situation would be changed, as the plasma membrane contains various proteins including ion channels, receptors and other functional proteins. Rg_3 would not be able to intercalate or penetrate into the plasma membrane as it does the lipid bilayer. Instead, Rg_3 might have a chance to interact with plasma membrane proteins a different manner compared to in a lipid bilayer. Plasma membrane proteins (e.g., receptors and ion channels) have extracellular, transmembrane and cytosolic domains, and the extracellular domains usually contain binding sites for ligands and drugs. Besides ligand binding sites, the extracellular domains of ion channels and receptors might also provide binding sites for Rg_3. These might be partially or entirely shared with other drugs or toxins. In the interaction of Rg_3 with plasma membrane proteins, it is unlikely that the hydrophobic backbone of Rg_3 is non-specifically binding to all plasma proteins, since extracellular plasma proteins consist of various amino acids with different chemical properties. Only subsets of receptors and ion channels that are regulated by Rg_3 and contain the complementary to the three-dimensional structure of Rg_3 could allow Rg_3 to bind and form hydrogen bonds, as we showed using homology modeling.[33]

The aliphatic side chain of Rg_3 has also been shown to participate in Rg_3-mediated ion channel regulation.[5] Interestingly, the role of the aliphatic side chain in Na^+ channel regulation seems to be different from that of Rg_3 epimers or the carbohydrate portion of Rg_3. Modifications in the aliphatic side chain exhibit differential effects on Na^+ channel current inhibition. A reduction of the double bond in the aliphatic side chain significantly potentiates Rg_3-induced Na^+ current inhibition by reducing IC_{50} values by ~3-fold; on the other hand, hydroxylation of the

dimethyl group and oxygenation or deletion of the aliphatic side chain attenuates or eliminates the effects of Rg_3. The enhancement of Na^+ current inhibition by reducing the aliphatic side chain might be due to following: a single rather than a double bond in the aliphatic side chain might be less rigid for geometrically better alignment in the interaction between Rg_3 and Na^+ channel proteins, reducing the steric hindrance caused by the double bond. In addition, since deletion of the aliphatic side chain removes the site for Rg_3 interaction with Na^+ channel proteins, Rg_3-C6 has no effects on Na^+ channel activity. Thus, the aliphatic side chain as well as the carbohydrate portions and the position of hydroxyl group on carbon-20 of Rg_3 are all involved in Na^+ channel regulation. These results show that the aliphatic side chain of Rg_3 plays a role as a functional group in ion channel regulation.

Future perspectives

Two main findings can be observed in Rg_3-mediated regulation of ion channels and receptors other than the identification of Rg_3 interaction sites. One is that Rg_3 could be a kind of non-specific open channel blocker of ion channels. Its potency, however, was much lower than other open channel blockers. The other is that the actions of Rg_3 are achieved through binding or interaction sites shared with other subsets of drugs or toxins. A previous report showed that simple modifications of the aliphatic side chain of Rg_3 showed differential regulation of Na^+ channels.[19] Thus, reduction of the aliphatic side chain enhances the action of Rg_3, while removal of the aliphatic side chain abolishes the action of Rg_3. These results indicate that the aliphatic side chain of Rg_3 is the primary functional group. In future studies, if more extensive modifications of this aliphatic side chain are used in *in vitro* or *in vivo* screening, it might be possible to create novel high-affinity Rg_3 agonists or antagonists. On the other hand, Rg_3 interacts with various ion channels and receptors without much selectivity, perhaps due to properties of the carbohydrate portion of Rg_3. This carbohydrate portion may also need to be modified in order to obtain Rg_3 derivatives with greater selectivity to individual ion channels and receptors. Finally, if Rg_3 derivatives can be successfully created with high affinity and selectivity and with few side effects in *in vitro* and *in vivo* systems, they should be highly efficacious and advance the study of ginseng.

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