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Research Report

Involvement of batrachotoxin binding sites in ginsenoside-mediated voltage-gated Na^+ channel regulation

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ABSTRACT

Recently, we showed that the 20(S)-ginsenoside Rg_3 (Rg_3), an active ingredient of *Panax ginseng*, inhibits rat brain $Na_V1.2$ channel peak currents (I_{Na}). Batrachotoxin (BTX) is a steroidal alkaloid neurotoxin and activates Na_V channels through interacting with transmembrane domain-I-segment 6 (IS6) of channels. Recent report shows that ginsenoside inhibits BTX binding in rat brain membrane fractions. However, it needs to be confirmed whether biochemical mechanism is relevant physiologically and which residues of the BTX binding sites are important for ginsenoside regulations. Here, we demonstrate that mutations of BTX binding sites such as N418K and L421K of rat brain $Na_V1.2$ and L437K of mouse skeletal muscle $Na_V1.4$ channel reduce or abolish Rg_3 inhibition of I_{Na} and attenuate Rg_3-mediated depolarizing shift of the activation voltage and use-dependent inhibition. These results indicate that BTX binding sites play an important role in modifying Rg_3-mediated Na^+ channel properties.

1. Introduction

Na^+ channels are transmembrane proteins that consist of a pore-forming α subunit and auxiliary β1, β2 and β3 subunits (Goldin, 1995). The α subunit is composed 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 inward Na^+ current (I_{Na}) initiates axonal and somatic action potentials in nerve and muscle fibers, and may also be involved in axonal intraneuronal or interneuronal information transfer (Stuart and Sackmann, 1994). Na^+ channels are one of the targets of cardiac- and neuroprotective treatments against pathologic conditions including arrhythmia and brain ischemia. Site-directed mutagenesis has helped to reveal drug/toxin binding sites in the Na^+ channel α subunit (Wang and Wang, 1998). For example, batrachotoxin, which is a skin neurotoxin from the South American frog *Phyllobates terribilis*, activates brain $Na_V1.2$ and skeletal muscle $Na_V1.4$ channels. Further studies using site-directed mutagenesis demonstrated that I433, N434 and L437 residues of $Na_V1.4$ channel and their homologous residues such as I417, N418 and L421 of brain $Na_V1.2$ channel in domain-I segment 6 (IS6) play important roles as BTX interaction sites (Wang and Wang, 1998).

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 its effects are the ginsenosides, amphiphilic molecules comprising a hydrophobic aglycone backbone linked to monomeric, dimeric or tetrameric hydrophilic carbohydrate side chains (Fig. 1). Ginsenosides have

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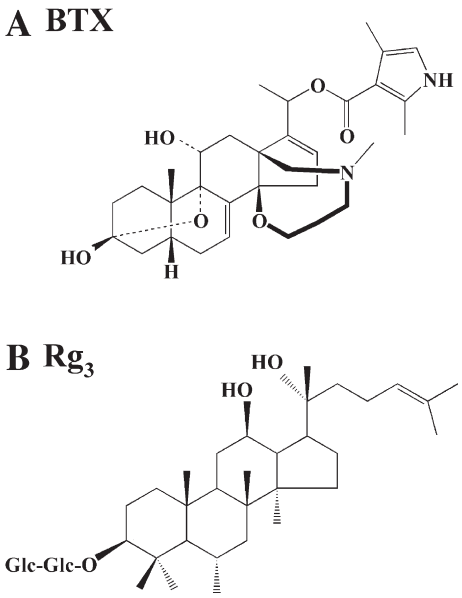

Fig. 1 – Structures of BTX and Rg_3. Glc, glucopyranoside. Subscripts indicate the carbons in the glucose rings that link the two carbohydrates.

neuroprotective actions *in vitro* and *in vivo* (Kim et al., 2005), but their molecular bases remain elusive. The 20(S) but not the 20(R) form of ginsenoside Rg_3 inhibits tonic I_{Na} expressed in *Xenopus* oocytes in the resting and open states, and induces a depolarizing shift in the activation voltage and use-dependent inhibition (Jeong et al., 2004; Lee et al., 2005). Ginsenoside Rh_2 inhibits [^{3}H]BTX-B binding in rat brain membrane fractions (Duan et al., 2006), suggesting that the ginsenoside-induced rat brain $Na_V1.2$ channel effects may involve the BTX binding sites. However, it is not clear whether the interference by ginsenoside with [^{3}H]BTX-B binding in rat brain membrane fractions is relevant to ginsenoside-induced Na^+ channel regulations, and which residues of the BTX binding sites are important for ginsenoside-induced Na^+ channel regulations. The aim of the present study was to investigate the relationships between ginsenoside-mediated interference with Na^+ channel function and the BTX binding sites using the *Xenopus* oocyte gene expression system and site-directed mutagenesis. For this, we used rat brain $Na_V1.2$ and mouse skeletal muscle $Na_V1.4$ channels and examined whether Rg_3 effects on both Na^+ channel isoforms are influenced with the same manner after mutations of BTX binding sites. We present evidence that BTX binding sites such as N418 and L421 of $Na_V1.2$ and L437 of $Na_V1.4$ in IS6 play important roles in modifying Rg_3-mediated Na^+ channel properties.

2. Results

2.1. *Point mutations of residues in the BTX binding sites of $Na_V1.2$ and $Na_V1.4$ channels shift the Rg_3 concentration response curve rightward and abolish Rg_3 inhibition*

We showed in a previous report that Rg_3 inhibits $Na_V1.2$ channel currents (I_{Na}) in a stereospecific manner (Jeong et al., 2004). Recently, Duan et al. (2006) reported that ginsenosides inhibit [^{3}H]BTX-B binding in rat brain membrane fractions, suggesting the existence of specific binding sites for Rg_3 and raising the possibility that ginsenoside affects $Na_V1.2$ channel activity via the BTX binding sites. We therefore tested whether substitutions of amino acid residues in the BTX binding sites alter Rg_3-mediated tonic inhibition of the channels.

Using the two-electrode voltage-clamp technique we recorded $Na_V1.2$ and $Na_V1.4$ channel currents from *Xenopus* oocytes, in which cRNA of $Na_V1.2$ α or $Na_V1.4$ α was co-injected with cRNA of β1 subunit. To elicit the currents we applied voltage steps (200-ms duration) to −10 mV at 5-s intervals from a holding potential of −100 mV. The currents evoked were transient inward Na^+ currents (I_{Na}) that decayed rapidly (Fig. 2) (Lee et al., 2005). As previously reported, Rg_3 (100 μM) resulted in an inhibition of I_{Na} in wild-type channels averaging 65.1±3.5 % for $Na_V1.2$ and 63.4±4.1 % for $Na_V1.4$. The Rg_3 effect was concentration-dependent (Figs. 2A and B), and the IC_{50} value and Hill coefficient for two Na^+ channels are given in Table 1.

Next, to test whether mutations of the BTX binding sites affect Rg_3 inhibition of the $Na_V1.2$ and $Na_V1.4$ channels, we constructed both channels with mutated BTX binding sites (I417K, N418K and L421K for $Na_V1.2$; I433K, N434K and L437K for $Na_V1.4$ channel) (Wang and Wang, 1998). Fig. 2A shows representative traces of peak I_{Na} in the absence or presence of Rg_3 in oocytes expressing wild-type and L421K of $Na_V1.2$ and wild-type and L437K of $Na_V1.4$ channel. Fig. 2B reproduces representative concentration–response curves for peak I_{Na} inhibition of the wild-type and mutant channels by Rg_3. For $Na_V1.2$, L421K abolished Rg_3 inhibition, I417K had no effect on inhibition and N418K caused an approximately eleven-fold increase in IC_{50} value from 29.4±3.2 to 329.9±37.6 μM. For $Na_V1.4$, I433K and N434K had no effect on Rg_3 inhibition but L437K caused an approximately two-fold increase in IC_{50} value from 58.5±6.3 to 121.6±20.6 μM (Fig. 2B and Table 1).

We assessed current–voltage relationships in the absence or presence of Rg_3 with voltage steps from −50 to +50 mV from a holding potential of −100 mV every 5 s. As shown in Fig. 2C, Rg_3 treatment caused a voltage-dependent reduction in peak I_{Na} in wild-type channels with more pronounced inhibition at the lower voltages. However, Rg_3 had no significant effect on the current–voltage relationship in oocytes expressing L421K for $Na_V1.2$ and L437K for $Na_V1.4$, indicating that BTX binding sites are important for Rg_3 effects on $Na_V1.2$ and $Na_V1.4$ channel regulation.

2.2. *Point mutations of the BTX binding sites abolish the Rg_3 effect on the steady-state activation of $Na_V1.2$ and $Na_V1.4$*

In our previous report we also showed that Rg_3 induces a depolarizing shift in the steady-state activation voltage but not in the inactivation voltage, in addition to inhibiting peak I_{Na}. However, the mutation of K859 to K859Q in voltage sensor segment domain II abolished the Rg_3-induced depolarizing shift in steady-state activation without affecting Rg_3-induced peak I_{Na} inhibition (Lee et al., 2005). This suggested that K859 residue is another residue that Rg_3 interacts with. We next examined whether mutations of the BTX binding sites alter the effect of Rg_3 on the voltage-dependence of Na^+ channel steady-state activation and inactivation. The data obtained are summarized in Table 2. There was a significant depolarizing shift of the half-maximal activation voltage in wild-type isoforms (Fig. 3A and