INTERACTION OF THE GINSENG COMPOUNDS 20(*S*)PROTOPANAXADIOL AND Rh_2 WITH VOLTAGE-GATED SODIUM CHANNELS IN MAMMALIAN BRAIN

by

Yin Duan
B.Sc., Sun Yat-Sen University, 2004

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Examining Committee:

Chair: Dr. B.J. Crespi, Professor

Dr. R.A. Nicholson, Associate Professor, Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. F.C.P. Law, Professor
Department of Biological Sciences, S.F.U.

Dr. C.J. Kennedy, Professor
Department of Biological Sciences, S.F.U.

Dr. G. Rintoul, Assistant Professor
Department of Biological Sciences, S.F.U.

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ABSTRACT

Bioactive components of ginseng have been found to be responsible for inducing multiple pharmacological responses, including changes to nervous system function. This investigation examined the interaction of 20(*S*)protopanaxadiol (PPD) and its monoglucoside Rh_2 with voltage-gated sodium channels. These compounds inhibited [^{3}H]batrachotoxinin A 20-α-benzoate binding to sodium channels, by targeting a locus that is allosterically coupled to neurotoxin binding site 2. Further studies on sodium channel-dependent functions revealed that these ginseng natural products also blocked veratridine-evoked depolarization of the nerve as measured by a voltage-sensitive fluoroprobe, and inhibited release of the neurotransmitters L-glutamate, GABA and L-aspartate from the nerve ending.

This research clarified the mechanism by which PPD and Rh_2 inhibit voltage-gated sodium channels from a biochemical standpoint. Reduced ability of the nerve to respond to a depolarizing stimulus and inhibition of neurotransmitter release may underly some of the depressant effects reported for ginsenosides on the nervous system.

Keywords: 20(*S*)protopanaxadiol; Rh_2; Central nervous system; Voltage-gated sodium channel; [^{3}H]batrachotoxinin A 20-α-benzoate; Membrane potential; Neurotransmitter release; Synaptosomes; Synaptoneurosomes.

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LIST OF ABBREVIATIONS

CFC	contextual fear conditioning
DMSO	dimethyl sulfoxide
FI	fluorescence intensity
GABA	γ-aminobutyric acid
GR	glucocorticoid receptor
GRE	glucocorticoid response elements
GTS	ginseng total saponin
[^{3}H]BTX-B	[^{3}H]batrachotoxinin A-20α-benzoate
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine (serotonin)
IC_{50}	concentration of an inhibitor that is required for 50% inhibition of its target
K_d	equilibrium dissociation constant
nAChR	nicotinic acetylcholine receptor
NTS	nucleus tractus solitarius
OPA	*o*-phthaldialdehyde reagent
PPD	20(*S*)protopanaxadiol
PPT	20(*S*)protopanaxatriol
S.E.M.	standard error of mean
[^{35}S]GTPγS	[^{35}S]guanosine-5'-(γ-thio)-triphosphate
SNARE	soluble NSF (N-ethylmaleimide sensitive factor) attachment receptor
TBARs	thiobarbituric acid reactive substances
TTX	tetrodotoxin
VGSC	voltage-gated sodium channel
VTD	veratridine

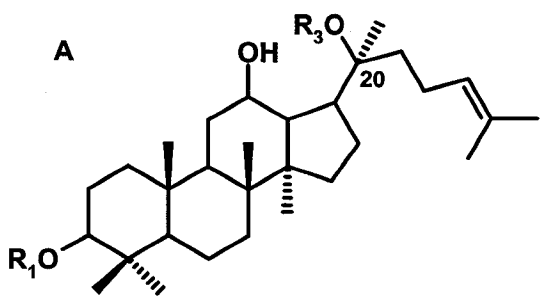
CHAPTER 1: GENERAL INTRODUCTION

1.1 Ginsenosides: their origin and properties

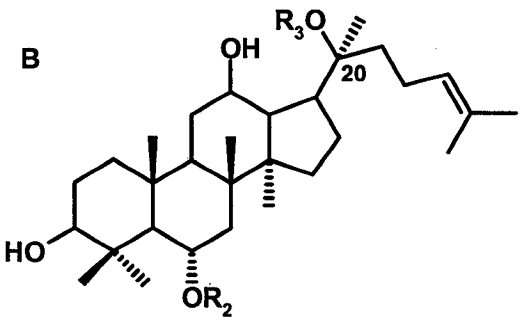
Commonly referred to as "Ginseng", this legendary herbal material is derived from the roots of several different species of plants, mainly *Panax ginseng* C A Meyer (Asian Ginseng), *Panax quinquefolius* (American Ginseng), *Panax pseudoginseng* (Himalaya Ginseng) and *Panax japonicus* (Japanese Ginseng) (Attele *et al.*, 1999). *Panax ginseng* C A Meyer has been used as a tonic and revitalizing medicine in South and East Asia specifically China, Korea and Japan, for more than two thousand years. At the present time *Panax quinquefolius* L is cultured on an industrial scale in both Canada and U.S.A. for various commercial applications.

Besides the protein and carbohydrate components, which are common in most herbs, ginseng contains a variety of other constituents, such as volatile oils, peptides, polysaccharides, fatty acids, vitamins, alcohols and a family of steroidal saponins, generally known as ginsenosides or ginseng saponins, which are major bioactive components of ginseng (Huang, 1999). Hydrolysis of each saponin may generate sapogenin and one or more sugar molecules. Based on their chemical structure, these compounds can be classified into four major groups: panaxadiols (e.g. Rb_1, Rb_2, Rb_3, Rc, Rd, Rh_2, notoginsenosides Fa and R_4), panaxatriols (e.g. Re, Rf, Rg_1, Rh_1, notoginsenosides R_1 and R_2), oleanane (e.g. Ro and Chikusetsusaponin 1Va) and ocotillol (e.g. pseudoginsenoside F_{11} and RT_4) (Fig. 1.1).

Figure 1.1. Chemical structures of major ginseng saponins found in the root of various species of ginseng. **A**: panaxadiol type; **B**: panaxatriol type; **C**: oleanane type; **D**: ocotillol type. glc, glucose; ara(p), arabinose in pyranose form; ara(f), arabinose in furanose form; xyl, xylose; rha, rhamnose; glcUA, glucuronic acid (Adapted from Attele *et al.*, 1999; Tohda *et al.*, 2002).



Panaxadiol-type

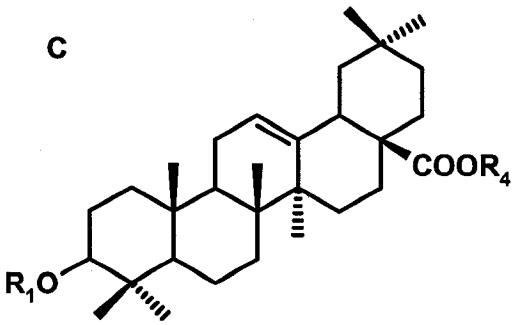


Panaxatriol-type

PANAXADIOL TYPE		
Compound name	**R_1**	**R_3**
Ginsenoside Rb_1	-glc(2-1)glc	-glc(6-1)glc
Ginsenoside Rb_2	-glc(2-1)glc	-glc(6-1)arap
Ginsenoside Rb_3	-glc(2-1)glc	-glc(6-1)xyl
Ginsenoside Rc	-glc(2-1)glc	-glc(6-1)araf
Ginsenoside Rd	-glc(2-1)glc	-glc
Ginsenoside Rg_3	-glc(2-1)glc	-H
Ginsenoside Rh_2	-glc	-H
Notoginsenoside Fa	-glc(2-1)glc	-glc(6-1)glc(6-1)xyl
Notoginsenoside R_4	-glc(2-1)glc(2-1)xyl	-glc(6-1)glc
20(*S*)Protopanaxadiol	-H	-H

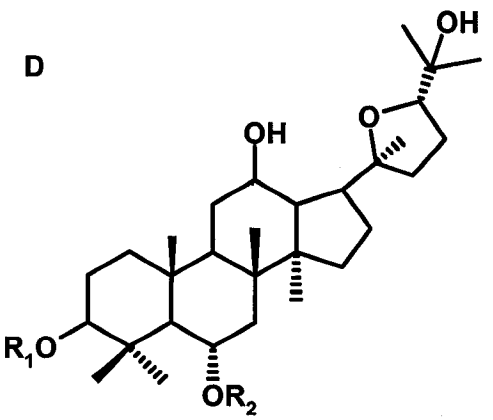
PANAXATRIOL TYPE		
Compound name	**R_2**	**R_3**
Ginsenoside Re	-glc(2-1)rha	-glc
Ginsenoside Rf	-glc(2-1)glc	-H
Ginsenoside Rg_1	-glc	-glc
Ginsenoside Rg_2	-glc(2-1)rha	-H
Ginsenoside Rh_1	-glc	-H
Notoginsenoside R_1	-glc(2-1)xyl	-glc
Notoginsenoside R_2	-glc(2-1)xyl	-H
20(*S*)Protopanaxatriol	-H	-H

C



Oleanane-type

D



Ocotillol-type

OLEANANE TYPE		
Compound name	**R_1**	**R_4**
Ginsenoside Ro	-glcUA(2-1)	-glc
Chikusetsusaponin 1Va	-glcUA	-glc

OCOTILLOL TYPE		
Compound name	**R_1**	**R_2**
Majonoside R_1	-H	-glc(2-1)xyl
Pseudoginsenoside RT_4	-H	-glc
Pseudoginsenoside F_{11}	-H	-glc(2-1)rha

The amount and ratios of the ginseng saponins content can vary widely depending on the species, culturing conditions, age and the part of the ginseng plant that is analysed (Huang, 1999). As illustrated in Fig. 1.1, the saponins represent a structurally diverse family of compounds. They differ from one another according to the type of sugar moieties (their number and their site of attachment), the number and site of attachment of hydroxyl groups as well as stereochemistry at C-20. Despite their different sugar moieties, ginseng saponins of one type share a common aglycone. For instance, 20(*S*)protopanaxadiol (PPD) is the aglycone backbone for all panaxadiol saponins and also a compound of interest in the present study (see Fig. 1.1).

Most ginsenosides (except for oleanane type saponins) consist of a lipophilic aglycone that adopts a four trans-ring rigid steroid skeleton, and varying numbers of hydrophilic sugar moieties, which enhance the solubility of ginsenosides. Sugar moieties usually include glucose, galactose, xylose or methylpentose, all of which are glycosidically connected to the steroidal backbone (Huang, 1999). Since they possess both hydrophobic and hydrophilic moieties, ginsenosides are also classified as amphipathic molecules that are able to lower the surface tension of a liquid as well as the interfacial tension between two liquids. Therefore, it is not surprising that high concentration of saponins have a lytic action on erythrocytes (Francis *et al.*, 2002). It has been proposed that this haemolytic action results from the formation of insoluble complexes by saponin aglycone moieties and sterols of plasma membrane (e.g. cholesterol), and subsequent increase of membrane permeability (Glauert *et al.*, 1962; Bangham & Horne, 1962).

1.2 Pharmacology of ginsenosides

In the last several decades, researchers have conducted numerous investigations, with the ultimate objective of exploiting the potential of ginseng and its active components for medicinal purposes, and a large number of reports have accumulated. In fact, by the early 1990s, ginseng had already attracted considerable interest and was thought to have huge promise as a therapeutic agent for a variety of conditions. Various claims have been made, for instance in treating fatigue and stress, in improving physical and mental performance, in modulating the cardiovascular system, as well as promoting general health and longevity (Huang, 1999). In this thesis, I review ginseng's effects on behavioral and cognitive performance, as well as its other effects on nervous system function.

1.2.1 The action of ginsenosides on behavioural and cognitive performance

A number of pharmacologically-based behavioral studies in animal models demonstrated that ginseng saponins have the ability to modulate brain and behavioral functions, including suppression of hyperactivity (Hao *et al.*, 2007), relief of stress (Yobimoto *et al.*, 2000), as well as improvement of learning (Qiao *et al.*, 2004) and cognitive performance (Kennedy *et al.*, 2004).

Ginseng and its active components have also been reported to ameliorate certain symptoms associated with narcotic abuse (Kim *et al.*, 1995; Guo *et al.*, 2004; Kim *et al.*, 2005; Hao *et al.*, 2007). Cocaine produces hyperactivity and acts as a stimulant on the central nervous system (Oka and Hosoya, 1977). In the conditioned place preference test, both single dose and repeated administration of ginseng total saponin (GTS; 100 and 200 mg/kg) inhibited cocaine-induced (2 mg/kg) climbing behavior (an index of

hyperactivity), suggesting that GTS may be useful in the therapy of certain stereotypic cocaine-mediated behaviors (Kim *et al.*, 1995). Morphine also induces hyperactivity and sensitization-related stereotype behavior in animals (Shuster *et al.*, 1975). In their experiments using cultured Chinese hamster ovary cells, Li and colleagues discovered that pseudoginsenoside F_{11}, an ocotillol type saponin from *Panax quinquefolium* L. inhibited the binding of diprenorphine (an opiate antagonist) to opioid receptors (IC_{50}: 6.1 μM) and significantly attenuated morphine-stimulated [^{35}S]GTPγS binding to cell membranes (a part of signaling pathway triggered by morphine) with IC_{50} of approximately 87.7 nM, indicating that F_{11} can initiate a direct inhibition of morphine-induced opioid receptor signaling (Li *et al.*, 2001). Another *in vivo* study revealed that daily oral administration of F_{11} (at a daily dose of 8 mg/kg for 7 days) reduced both morphine related symptoms such as behavioral sensitization and release of the excitatory amino acid neurotransmitter L-glutamate from medium prefrontal cortex of mouse (Hao *et al.*, 2007).

Learning plays a central role in human activity. Neurogenesis in the hippocampus has been shown to be necessary for a variety of learning tasks (Gould *et al.*, 1999). To investigate the effect of ginseng in neurogenesis, Qiao *et al.* (2004) administered dry *Panax ginseng* powder to rats at doses of 100 and 200 mg/kg per day for 5 consecutive days. Their results indicated an improved survival of newly generated hippocampal neurons and better performance in contextual fear conditioning (CFC) tests, implying that ginseng might enhance learning ability through inducing CFC-related neurogenesis. Learning impairment can arise from brain lesioning due to enhanced production of reactive oxygen species (reviewed by Calabrese *et al.*, 2007). After 30 days of

postoperative administration of crude ginseng extract (0, 40 and 80 mg/kg), strategic learning deficits occurring in rats because of medial prefrontal cortex lesions were significantly reduced in the ginseng-treated group, and this occurred in a dose-dependent manner (Zhao and McDaniel, 1998). The thiobarbituric acid reactive substances (TBARs) assay is widely employed to determine lipid peroxidation, an important indicator of oxidative stress. Oral pretreatment of mice with Vietnamese ginseng saponin (15-25 mg/kg) and its major component majonoside R_2 (1-10 mg/kg, injected intraperitoneally) significantly attenuated the psychological stress-induced increase of TBARS in the brain (Yobimoto *et al.*, 2000). Using the passive avoidance test on rats, intraperitoneal injection of red ginseng powder (0.6, 0.9 and 1.5 g/kg), crude ginseng saponin (50 or 100 mg/kg), and the ginsenoside Rb_1 (10 and 20 mg/kg) all prevented ischemia-induced decreases in response latency and rescued a significant number of ischemic hippocampal CA1 pyramidal neurons, while Rg_1 and Ro showed no effect (Wen *et al.*, 1996). These results suggest that certain ginseng compounds and extracts may have the capacity for reducing brain lesioning and therefore act as neuroprotectants.

Panax Ginseng extracts also demonstrated cognitive performance-enhancing effects in humans. Using an electroencephalograph recording method, Kennedy *et al.* (2003) studied a group of 15 healthy volunteers treated with a single dose of *P. ginseng* extract (200 mg). All subjects showed a significant shortening of the P300 neuronal evoked potential (its latency reflects the amount of time necessary to come to a decision about the stimulus (Sutton *et al.*, 1965)), and significant reductions in frontal 'eyes closed' θ and β activity, which is normally considered to be associated with an increase in alertness (Geßner *et al.*, 1985). Their findings suggested a role of ginseng extract in direct

modulation of cerebroelectrical activity (Kennedy *et al.*, 2003). In a double-blind, counterbalanced, placebo-controlled study carried out by the same group, 28 participants administered a single oral dose of 200 mg of *Panax ginseng* extracts exhibited improved performance in the speed of attention tests and memory-dependent tasks, as well as in serial subtraction tasks, supporting cognition-enhancing properties of ginseng (Kennedy *et al.*, 2004).

1.2.2 The action of ginsenosides on nervous system function

The discovery that ginseng bioactives can elicit behavioural and cognitive effects has prompted scientists to explore the physiological and cellular mechanism underlying these actions. It is now known that ginseng saponins and related derivatives play versatile roles in modulating synaptic transmission, through targeting ion channels (Kim *et al.*, 2005; Lee *et al.*, 2005), affecting neurotransmitter cycling (Kudo *et al.*, 1998; Tachikawa *et al.*, 2003; Tachikawa *et al.*, 2001; Yuan *et al.*, 1998) and reducing neuronal excitability and oxidative stress (Tian *et al.*, 2004; Lian *et al.*, 2005).

1.2.2.1 Effects on ion channels

There is considerable evidence in support of the idea that ginsenosides elicit potent actions at a number of ion channels. Voltage-gated Na^+ channels are membrane proteins that support axonal and somatic action potentials (Hille, 1984). Native Na^+ channels comprise one α subunit (responsible for pore formation) and subsidiary β subunits. Investigations using the whole-cell patch clamp technique demonstrated that an aqueous extract of American ginseng (*Panax quinquefolius*) (3 mg/ml) and the ginsenoside Rb_1 (150 μM) both tonically and reversibly blocked voltage-gated Na^+

channels in a concentration- and voltage-dependent manner (Liu *et al.*, 2001). Rg_3 has been observed to induce tonic and use-dependent inhibition of the Na^+ current in both resting and open states of rat brain wild-type Na^+ channels expressed in oocytes as measured by a two microeletrode voltage-clamp (Lee *et al.*, 2005). Moreover, in this preparation, Rg_3 produced a reversible inhibitory effect on the sodium current of Na^+ channels of low micromolar concentrations (IC_{50}: 32.2 ± 4.5 μM) (Kim *et al.*, 2005). It is worth pointing out that all reports above agree on the reversibility of the inhibition of Na^+ channels by ginsenosides, or put another way, these ginseng compounds do not suppress VGSCs persistently.

Ginsenosides can also interfere with Ca^{2+} channel function. Reports in the 1990s showed that ginseng root extract was able to inhibit voltage-dependent Ca^{2+} channels in sensory neurons. Ginsenoside Rf (IC_{50}: 40 μM) was found more potent than several other ginsenosides Rb_1, Rc, Re, and Rg_1 in inhibiting Ca^{2+} channels through binding to a pertussis toxin-sensitive G protein in cultured rat sensory neurons (Nah *et al.*, 1995). Rhim and associates used whole cell patch-clamp recording to examine the effect of ginseng total saponins and single ginsenoside on high voltage-activated Ca^{2+} channel currents in isolated rat dorsal root ganglion neurons. Application of ginseng total saponins (IC_{50}: 61.4 μg/ml) suppressed Ca^{2+} currents in a dose-dependent manner while Rg_3 (100 μM) inhibited Ca^{2+} channels (by 65.4%) although minor effects were observed with other ginsenosides (e.g. Rb_1, Rc, Rd, Re, Rf, Rg_1 and Rg_2) (Rhim *et al.*, 2002). Despite the fact that many investigations have examined whether ginsenosides are capable of modulating Ca^{2+} channels (Nah *et al.*, 1994; Kim *et al.*, 1998; Lee *et al.*,

2006), few studies have attempted to characterize the detail of the mechanisms underlying this action.

1.2.2.2 Effects on neurotransmitter dynamics

Neurotransmitters are a family of small molecules playing a crucial role in regulating signaling between neurons in the brain. It has been proposed that ginseng saponins affect brain function by interfering with neurotransmitter uptake, release and receptor binding.

In the study by Tsang *et al.* (1985), radiolabeled transmitter uptake experiments were carried out using synaptoneurosomes isolated from rat brain. It was found that in a total ginseng saponin extract, the Rd-enriched fraction (where Rd is approximately 84.5 μM) was most effective in reducing the uptake of γ-aminobutyric acid (GABA), noradrenaline, dopamine, glutamate, and serotonin (5-HT). In contrast, the uptake of 2-deoxy-D-glucose (a non-metabolizable analog of glucose) and leucine (representing a non-neurotransmitter amino acid) by synaptosomes was unaffected. The results indicated that Rd likely has the ability to increase the persistence of both excitatory and inhibitory neurotransmitters in the synaptic cleft, which would be expected to enhance stimulation of postsynaptic receptors. However, the transport of the glucose surrogate and the non-neurotransmitter amino acid appears to be unaffected, implying Rd has a selective effect on neurotransmitter dynamics in synaptic regions.

Several other investigations support the idea that catecholamine secretion in adrenal chromaffin cells can be reduced indirectly by ginseng saponins (Kudo *et al.*, 1998; Tachikawa *et al.*, 2003; Tachikawa *et al.*, 2001). Ginsenosides Rg_3 (1-100 μM), Rh_2 (10-100 μM), 20(*R*)- and 20(*S*)-Rg_2 (1-100 μM) were found to reduce acetylcholine-

evoked secretion of Ca^{2+} influx and catecholamine efflux from cultured bovine adrenal chromaffin cells. The mechanism proposed to explain this phenomenon was that ginsenosides blocked Na^{+} influx through nicotinic acetylcholine receptor-operated cation channels. Consequently the excitability of chromaffin cells was supressed, which lead to a decrease in calcium ion influx, and reduction of catecholamine secretion due to lack of Ca^{2+} signal (Kudo *et al.,* 1998). Thus from these experiments it can be inferred that inhibition of catecholamine release originates from a primary effect of ginsenosides on cation channels.

γ-Aminobutyric acid (GABA) is an important neurotransmitter which exerts inhibitory effects in the brain (reviewed by Mann and Paulsen, 2007). It functions by binding to the $GABA_A$, $GABA_B$ or $GABA_C$ receptors in the pre- or post-synaptic membrane (reviewed by Lujan *et al.*, 2005). While $GABA_B$ receptors are G protein-coupled receptors (reviewed by Bowery, 2006), $GABA_A$ and $GABA_C$ receptors are ligand-gated ion channels (reviewed by Chebib and Johnston, 2000). The spontaneous activity of two types of nucleus tractus solitarius (NTS) units could be inhibited by the $GABA_A$ receptor agonist muscimol, and this inhibition was antagonized by the selective $GABA_A$ receptor antagonist bicuculline (reviewed by Izquierdo and Medina, 1991). In experiments using caudal brainstem and cervical spinal cord preparation from neonatal rats, superfusion of brainstem compartment with *Panax quinquefolium* L. extracts (3.0 μg/ml) was shown to reduce the resting discharge rate of these NTS neurons (approximately 27% compared to the control level), but this reduction could not be reversed by bicuculline. On the other hand, the same treatment on the brainstem preparation significantly decreased NTS inhibitory effects when induced by the $GABA_A$

receptor agonist muscimol by between 33 to 51%. Taken together, Yuan and co-workers suggest that the regulation of GABAergic neurotransmission in brain may be an important action of *Panax quinquefolium* L. extracts (Yuan *et al.*, 1998). However, a depressant action on the NTS neurons possibly by blocking voltage-gated sodium channels may also explain these observations.

When acetylcholine binds to neuronal acetylcholine receptors, it invariably leads to excitatory actions. The effects of Rg_2 were examined on different types of human nicotinic acetylcholine receptors (nAChRs), both homomeric and heteromeric forms as expressed in *Xenopus* oocytes. The nAChRs are ionotropic receptors that form ligand gated ion channels in the plasma membrane. Rg_2 (100 μM) did not affect the acetylcholine-induced currents in α_7 human receptors, but reduced the peak current and also increased the rate of desensitization of heteromeric receptors $\alpha_3\beta_4$, $\alpha_3\beta_2$, $\alpha_4\beta_4$, and $\alpha_4\beta_2$, in a dose-dependent way (as examined in 1, 10 and 100 μM) (Sala *et al.*, 2002). These observations demonstrate the ability of Rg_2 to specifically interfere with heteromeric human nAChRs, very likely by interacting with the ionic channel part of the receptors and accelerating the desensitization of nAChRs.

1.2.2.3 Neuroprotective actions of ginsenosides

Neuroprotective properties of ginsenosides have been consistently demonstrated in a number of studies. Brain ischemia may be caused by interruption of blood supply to any part of the brain and if this interruption persists, it results in tissue death and loss of nerve function (Martin, 1990). During their experiments to investigate drugs which might protect against cerebral ischemia by ginsenosides, Lim *et al.* (1997) found that intracerebroventricular infusion of ginsenoside Rb_1 (1.25 mg/ml, 12.5 mg/ml) following

3.5 min or 3 min forebrain ischemia, dramatically reduced the ischemia-induced shortening of response latency and also rescued a significant number of hippocampal CA1 neurons from lethal damage. It was proposed that the protective mechanism in ischemic episodes could involve the scavenging of free radicals by Rb_1 since from a chemical perspective Rb_1 can react rapidly with these reactive substances, which are overproduced *in situ* after brain ischemia and reperfusion (reviewed by Bemeur *et al.*, 2007). By monitoring regional cerebral blood flow and measuring the activities of superoxide dismutase, glutathione-peroxidase, the respiratory control ratio and levels of malondialdehyde and ATP, Tian *et al.* (2004) observed that orally administrated Rg_3 (10 and 5 mg/kg) provides neuroprotection against cerebral ischemia-induced injury in the MCAO rat brain by reducing lipid peroxides, by scavenging free radicals and by boosting energy metabolism. Other studies on American ginseng saponins Rb_1, Rb_3, and Rd showed that pretreatment with these compounds significantly reduced 3-nitropropionic acid-induced motor impairment and cell loss in the striatum, and furthermore completely prevented any mortality (Lian *et al.*, 2005). Since ginsenosides inhibit sodium channels (Liu *et al.*, 2001; Lee *et al.*, 2005; Duan *et al.*, 2006) there is much support for the idea that ginsenosides may protect brain neurons against ischemic damage like other neuroprotective sodium channel blocking drugs which reduce excitation such as lifazirine (Brown *et al.*, 1994). In their study using the whole-cell patch clamp technique, Liu *et al.* (2001) report a tonic blockade of sodium currents on the tsA201 cells with α subunits of the $brain_{2a}$ Na^+ channel by American ginseng extract (3 mg/ml) and ginsenoside Rb_1 (150 μM). This indicated that both the crude mixture and the pure ginsenoside may have

important neuroprotective potential during ischemic episodes in the brain through their effects on Na^+ channels.

The ability of neurons to extend/regrow is crucial for recovery and repair of neuronal networks damaged by neurodegenerative diseases such as dementia. In the studies of Tohda *et al.* (2002), methanol extracts of ginseng (root of *Panax. ginseng*), notoginseng (root of *Panax. notoginseng*) and ye-sanchi (rhizome of a relative to *Panax. vietnamensis*) (all at 50 μg/ml) were found to stimulate neurite outgrowth in SK-N-SH cells. Furthermore, protopanaxadiol-type saponins, ginsenosides Rb_1 and Rb_3, and notoginsenosides R_4 and Fa isolated from extract of ye-sanchi stimulated neurite extension at 100 μM, while protopanaxatriol-, ocotillol- and oleanane-type saponins had much weaker effects on the neurite outgrowth. In a subsequent investigation using a mouse model of Alzheimer's disease involving intracerebral ventricle injection of amyloid beta peptide, ginsenoside Rb_1 (10 μmol/kg) and M1 (10 μmol/kg) (a metabolite of protopanaxadiol-type saponin), were found to bring about a recovery of spatial memory deficits and increase levels of phosphorylated H-type neurofilaments and synaptophysin in amyloid peptide-treated rats. Significantly, in a later study, M1-induced extension of axons following neurite atrophy occurred as readily in the presence of Rb_1 as in the control situation (Tohda *et al.*, 2004). Both these studies offered the exciting possibility that ginsenoside Rb_1 and possibly its metabolite have the ability to initiate neuronal extension and repairs, but clearly the connection between memory loss and ginsenoside stimulated neuronal regrowth requires much further exploration.

1.2.3 Cellular mechanisms underlying the actions of ginsenosides

It is generally believed that the steroidal skeleton makes the most important contribution to the pharmacological promiscuity of ginsenosides. Firstly, the steroid-like backbone provides the molecule with capability to intercalate with lipids arrays in membranes and seek out cholesterol-rich domains. This can then result in membrane fluidity changes (Zhou *et al.*, 2006), and interference with various functions of the lipid membrane itself and in turn those of integral protein signaling macromolecules (Attele *et al.*, 1999). While some membrane proteins (e.g. acetylcholine receptors) were found to selectively locate in and favour the cholesterol-enriched regions (Schroeder *et al.*, 1991), the activity of many ATPases are often compromised in these areas (Bastiaanse *et al.*, 1997). In contrast, certain ginsenosides e.g. Rb_1 enhanced the activity of the ATP-dependent Na^+/Ca^{2+} exchange in canine cardiac sarcolemmal vesicles (Yamasaki *et al.*, 1987) and the activity of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase activity in neurons (Jiang *et al.*, 1996). Therefore, it was proposed that the ginsenosides displace membrane cholesterol from the surface environment of ATPases (Attele *et al.*, 1999). Because the fluidity of the membrane increased after displacement of cholesterol, it was thought that ginsenosides might trigger conformational changes to the ATPases during their active transport cycle (Jiang *et al.*, 1996; Attele *et al.*, 1999).

In addition, other work suggests that ginsenosides might target membrane proteins such as receptors and ion channels. Several ginsenosides (Rb_1, Rb_2, Rc, Re, Rf, and Rg_1) were found capable of modulating the binding of the $GABA_A$ agonist muscimol in nerve membrane preparations, while total ginseng extract and Rc reduced the affinity of binding of the $GABA_B$ agonist baclofen (Kimura *et al.*, 1994). Research carried out by Kim *et al.* (1998) and Rhim *et al.* (2002) suggested that ginsenosides also have the ability

to block high-threshold voltage-gated Ca^{2+} channels in both chromaffin cells and sensory neurons. However, as mentioned before, due to the amphipathic character, ginseng saponins may affect the function of membrane proteins by integrating themselves the lipid bilayer and modifying lipid microenvironment of these proteins (Lundbæk *et al.*, 2005). In spite of many investigations on this specific question, it is still difficult to determine whether the effects of ginsenosides on membrane receptors and ionic channels are dependent on their direct interaction with the proteins themselves, or an indirect effect via relaxation or stiffening of the lipid microenvironment of the receptor or ion channel complex or both. Furthermore, the steroid moieties of ginsenosides enable them to traverse cellular membranes quite freely, which open up various opportunities for interaction with intracellular receptors, enzymes and other macromolecules. Several lines of evidence suggest that, like hormones and some steroidal drugs, ginsenosides may target nuclear steroid receptors. Nuclear steroid receptors are where ligand:receptor complexes are formed to act as a transcription factor and modulate transcription of mRNA and subsequent protein synthesis. For instance, when the τ_1 transactivation domain of glucocorticoid receptors (GRs) is occupied by ligand, the DNA binding domain of the GR is activated and associates with specific DNA sequences known as glucocorticoid response elements (GREs), stimulating the transcription of target genes (McEwan *et al.*, 1994). Investigations of Lee *et al.* (1997) showed that ginsenoside Rg1 is a functional but low potency ligand of GRs. Rg_1 (1-100 μM) was found to displace the binding of the synthetic glucocorticoid dexamethasone from GRs with equilibrium dissociation constant (K_d) value of 1-10 μM and also activate GRE-containing reporter plasmids in a concentration-dependent manner.

1.3 Synaptic transmission

Amongst all the pharmacological properties noted so far, the ability of ginsenosides to interact with the voltage-gated sodium channel (one of the most important channels mediating excitation in the central nervous system), is a major experimental focus in this thesis. Accordingly, the following sections are devoted to reviews of voltage-gated sodium channels and the critical role they play in the propagation of the action potential and neuronal signaling.

1.3.1 Voltage-gated sodium channels (VGSCs)

Neurons require rapidly acting and precise mechanisms in place both to generate ionic gradients and to control the flow of various ions, this latter function performed predominantly by a variety of ion channel proteins. These pore-forming, proteinaceous macromolecules generally respond to voltage change and / or the binding of ligand (e.g. neurotransmitters), and when activated undergo conformational modification, resulting in either opening or closing of the ion-selective transporting pathway.

Sodium channels (also known as voltage-gated sodium channels or voltage-sensitive sodium channels) are integral membrane proteins that conduct sodium ions through the neuronal plasma membrane when activated. In the majority of excitable cells such as neurons, myocytes and endocrine cells, sodium channels generate and propagate electrical signals as well as trigger cellular responses by facilitating a rapid increase in membrane Na^+ conductance which brings about the rapidly depolarizing phase of the action potential.

Voltage-gated sodium channels (VGSCs) belong to a family of plasma membrane proteins identified by an abrupt voltage-dependence of opening, a phenomenon termed

"activation" (Hille, 1992). VGSCs respond to changes in membrane potential by rapid conformational change which allows water molecules to associate with the pore of the complex and, in turn, permit sodium ions to diffuse passively down their electrochemical and concentration gradient into the neuron. In common with other voltage-gated ion channels, the VGSC has three distinctive properties that enable it to function as a neuronal electrical signaling complex: selective ion (Na^+) conductance, voltage-dependent activation which opens the Na^+-conductive pore and finally inactivation which terminate Na^+ conductance (reviewed by Yu and Catterall, 2003).

In mammalian brain, VGSCs form a heterotrimeric complex composed of one α subunit, and varying numbers of different subtypes of β subunits. When expressed by in an excitable cell, the α subunit, which forms the core of the channel, is able to conduct Na^+ in a voltage-dependent way without β subunits, but with strong reduction in the amplitude of peak Na^+ current, as well as slower activation and inactivation (Isom *et al.*, 1992; Patton *et al.*, 1994). β subunits modulate voltage-dependence and regulate expression level of sodium channels (reviewed by Isom, 2001). The α-subunit has four repeat domains (I to IV), each containing six trans-membrane segments, labeled S_1 through S_6 (reviewed by Catterall, 1986) (see Fig. 1.2).

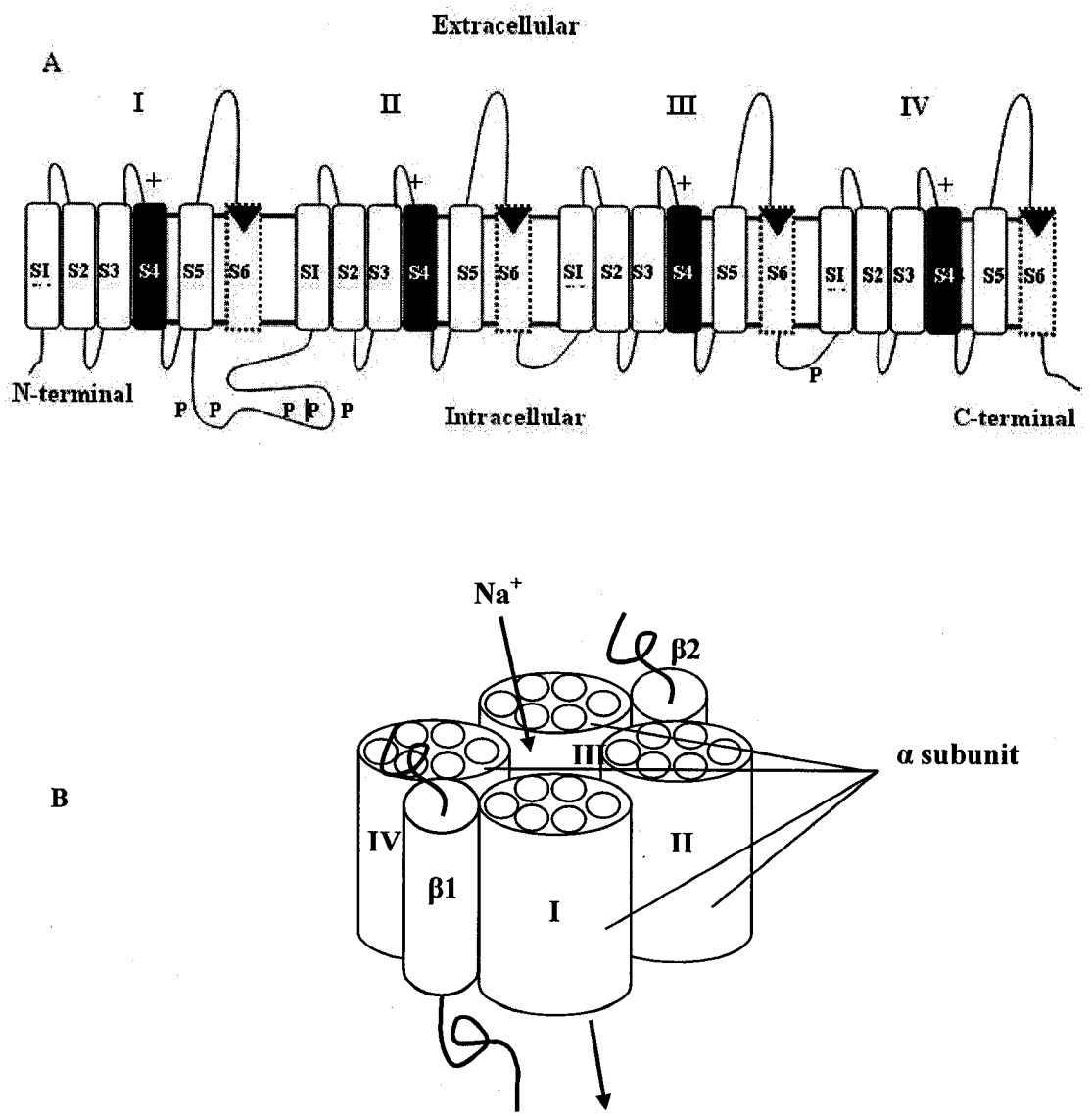


Figure 1.2. General protein structure of voltage-gated sodium channels. **A**: Schematic representation of the transmembrane arrangement of the α subunit of voltage-gated sodium channel, which has a general structure of four homologous repeated domains (I-IV) forming a central ion pore together. Each of these domains consists of six putative transmembrane α helical segments (S_1-S_6). P: phosphorylation site; Positive charge (+) in segment 4 is important for transmembrane voltage sensing (adapted from Gutman and Lazarovic, 1997). **B**: The α and β subunits of an assembled sodium channel complex (adapted from Isom, 2001).

The pore of the sodium channel is lined with negatively charged amino acid residues, which attract positive sodium ions and exclude negatively charged ions such as chloride. A more constricted part of the pore, which is approximately 0.3 by 0.5 nm wide, excludes potassium ions, however, this region is large enough to allow passage of a single sodium ion with an associated water molecule (Favre *et al.*, 1996).

Being a critical element in the excitation process, it is hardly surprising that some animal and plants have developed the ability to produce various neurotoxins which target a number of distinct binding sites on the VGSC. For example, tetrodotoxin is a potent neurotoxin that carried by several species like pufferfish, porcupinefish and triggerfish (Bagnis *et al.*, 1970; Brett, 2003); batrachotoxins are extremely poisonous alkaloid toxins found in the poison dart frogs (e.g. *Phyllobates terribilis*) (Daly and Witkop, 1971), Melyridae beetles and certain types of birds; scorpion venoms are a class of protein toxins and usually neurotoxic in nature (reviewed Cury and Picolo, 2006), used by most scorpion species for hunting and defending. The similarity shared by these neurotoxins is that they target sodium channels with high affinity and specificity. As a result they can alter the normal activity of VGSCs, interfering with the electrical signaling, resulting in convulsions or paralysis and eventually death. For the past few decades, these neurotoxins have attracted considerable attention because of the increasing poisoning incidents and their economical impact (Bagnis *et al.*, 1970; Hughes and Merson, 1976; Brett, 2003). An important consequence of these investigations is that these neurotoxins are exploited by scientists as highly specific biochemical pharmacological tools for investigating into the mechanism of action of other chemicals that affect sodium channel

function as well as studies which can reveal novel aspects of sodium channel structure and functions (reviewed by Catterall, 1980; 1986).

At least six distinct classes of neurotoxins which target VGSCs have been identified on the basis of their toxicology, physiological activity and competitive binding studies (reviewed by Catterall, 1980; 1986; reviewed by Barchi, 1988, see Fig. 1.3 and Table 1.1). Neurotoxins that exhibit simple competition with one another (e.g. batrachotoxin, veratridine, aconitine) have been assigned to the same class (i.e. alkaloid/neurotoxins), although members of one class do not necessarily bind to precisely the same binding domain (Gordon *et al.*, 1992; 1996a; 1996b). Accordingly, six neurotoxin binding sites have been assigned to each of the known neurotoxin classes. However, the actual binding locus for several toxins like *Goniopora* coral toxin, *Conus striatus* toxin remained unidentified (see Table 1.1).

Significantly, the development of specific radioligands for many of the known binding sites enabled and facilitated the detection of conformational changes in the channel protein that occur when many toxins binds to the sodium channel, promoting allosteric modification of other toxin binding sites in many cases (see Table 1.1).

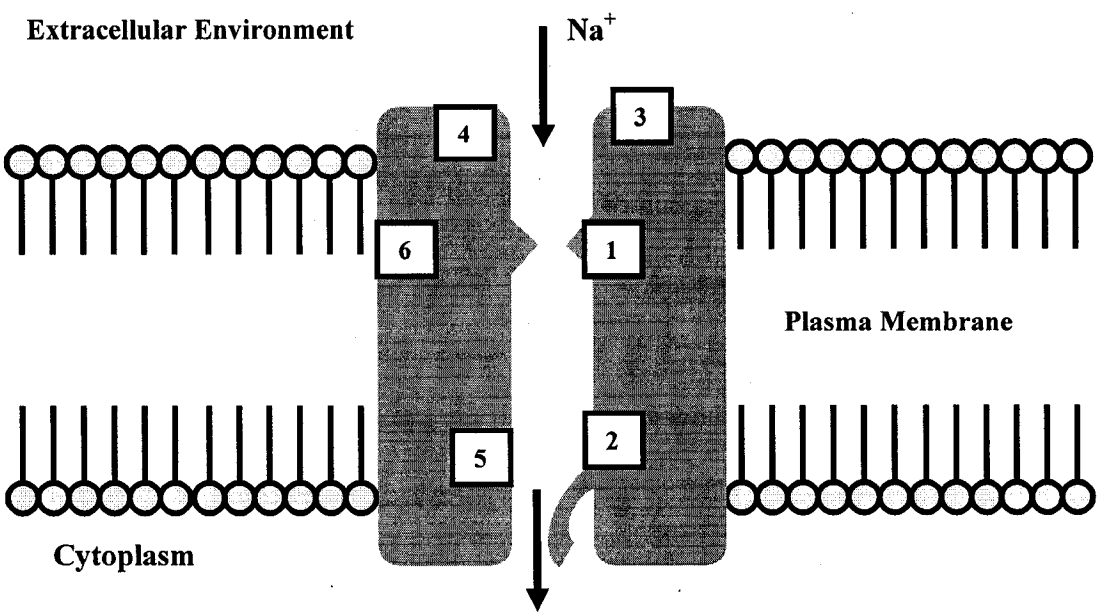


Figure 1.3. Diagram illustrating neurotoxin binding sites (1-6) on the voltage-gated sodium channel α subunit protein. (Adapted from Gutman and Lazarovic, 1997).

Table 1.1 Sodium channel-directed neuotoxicants act by binding to sites 1 to 6, giving rise to various effects on vertebrate sodium channels including allosteric modification of other sites (Adapted from Gutman and Lazarovic, 1997). Allosteric modulation produced by occupancy of a site can induce a positive or negative effect on the binding behavior of other toxins. Positive modulation donates an increase of binding of other toxins at their indicated target locus, and / or stimulation of Na^+ influx, while negative modulation refers to an inhibitory interaction.

Site	**Class Typical Toxin**	**Effect**	**Allosteric modulation**	
			Positive	*Negative*
1	Tetrodotoxin Saxitoxin	Inhibition of ion conductance	3,5	2
2	Batrachotoxin Veratridine Aconitine	Persistent activation	3	6
3	α-Scorpion toxins Sea anemone toxin II	Inhibit inactivation; enhance persistent activation	2	
4	β-Scorpion toxins	Shift voltage-dependence of activation		
5	Brevetoxins Ciguatoxins	Shift voltage-dependence of activation	2,4	3
6	δ-Conotoxins	Inhibit inactivation		
Unidentified sites	*Goniopora* coral toxin *Conus striatus* toxin	Inhibit inactivation		

1.3.2 Action potential and VGSC-dependent depolarization

Voltage-gated sodium channels play an important role in the generation and propagation of neuronal action potentials. An action potential is an impulse that carries an electrical signal across and along the plasma membrane. The ability to support action potentials is a fundamental property of neurons, facilitating electrical signaling within the nervous system of all animals. In the nervous system, action potentials are extensively used for communication between neurons, and for transmitting signals from neurons to other body tissues such as muscles and glands. The characteristics of action potentials vary in different cell types and at different locations of one cell. This thesis is exclusively concerned with the action potential of neurons.

When there are no incoming action potentials or other changes of the membrane potential, neurons remain at resting potential, which is approximately -70 mV (Purve *et al.*, 2001). The resting potential is determined by the ion concentrations in the fluids on both sides of the cell membrane, binding of ions to proteins in neurons, the ion transport proteins in the cell membrane and most importantly, the permeability of membrane to K^+ (Hille, 1984).

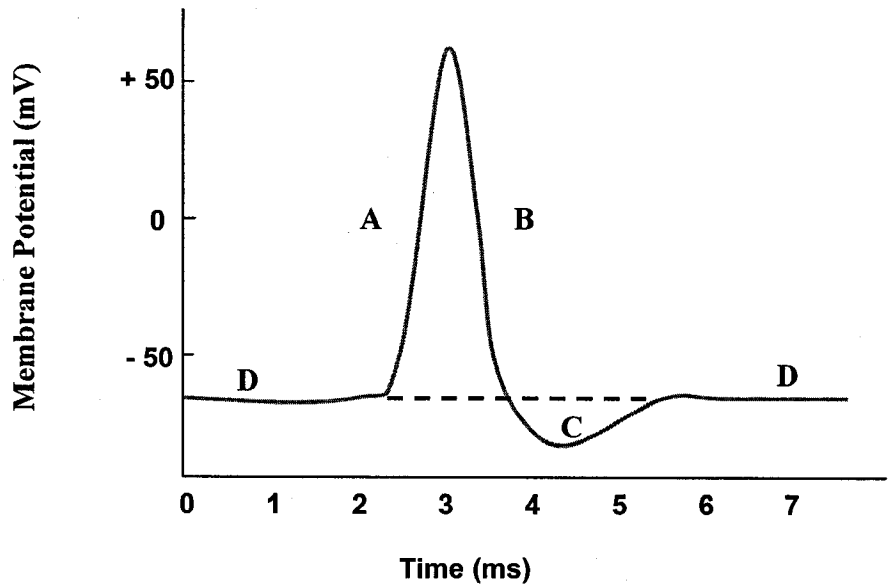


Figure 1.4. Schematic view of an idealized neuronal action potential including various phases. **A**: Depolarization; **B**: Repolarization; **C**: Refractory period; **D**: Resting potential.

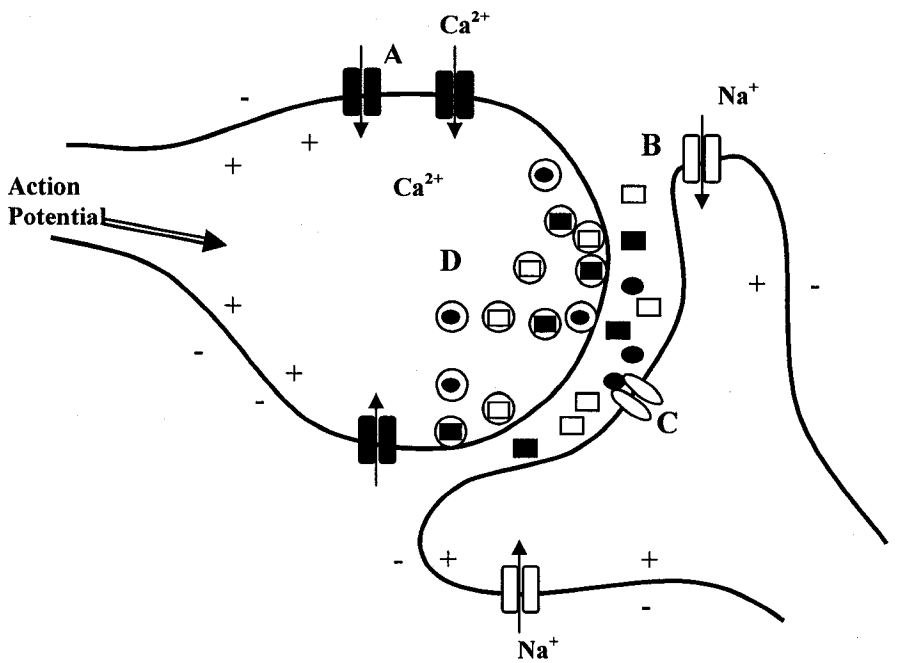


Figure 1.5. Generalized illustration of release of neurotransmitters in the central nervous system. •: L-glutamate; □: GABA; ■: L-aspartate; **A**: voltage-gated calcium channel; **B**: voltage-gated sodium channels; **C**: L-glutamate receptor; **D**: synaptic vesicles.

A typical action potential can be conveniently divided into 3 phases (see Fig. 1.4), the rising phase (depolarization), the falling phase (repolarization) and the refractory period (Hodgkin and Huxley 1952a, 1952b; Hodgkin *et al.*, 1952). The normal sequence of events is as follows: firstly, upon stimulation, a localized depolarization (often initiated by an excitatory stimulus) causes a small number of VGSCs in the neuronal membrane to open and sodium ions diffuse in through the channels along their electrochemical gradient (Clay, 1998). As sodium ions enter, the extent of depolarization increases and this triggers the opening of more sodium channels, resulting in increasing influx of sodium ions and an increasingly positive potential inside the neuron (Purve *et al.*, 2001; Bear *et al.*, 2001). Secondly, when the action potential approaches its peak, the voltage-dependence of sodium channel opening dictates that these channels close (Hille, 1984), however, K^+ channels open at these reduced potentials leading to rapid efflux of positively charged potassium ions from the nerve (Hille, 1984; Purve *et al.*, 2001). These events drive repolarization and typically, this falling phase of an action potential leads to a brief hyperpolarizing overshoot i.e. a potential that is more negative than the resting potential of the cell (reviewed by Bear *et al.*, 2001). This is mainly because after the membrane has completely repolarized the efflux of potassium ions continues via delayed rectifier potassium channels (Shibasaki, 1987). Lastly, during the process of re-establishing the initial ion distribution at resting potential, neurons experience a refractory period immediately following repolarization (Purve *et al.*, 2001). During the refractory period, neither Na^+ nor K^+ channels can be opened by an electrical stimulus (Bramwell, 1911), while the Na^+/ K^+ ATPase actively pumps Na^+ out and K^+ into the neuron.

1.3.3 VGSC-dependent release of neurotransmitters

Neurotransmitters are a large family of low weight signaling molecules that regulate and amplify electrical signals between neurons or a neuron and another cell. They are synthesized endogenously, stored in vesicles within the presynaptic nerve terminals, then released into the synaptic cleft where they diffuse rapidly to receptors on the postsynaptic neuron, and elicit their physiological effects (Purve *et al.*, 2001) (see Fig. 1.5).

Neurotransmitters are normally categorized into the following chemical classes:

1) Acetylcholine.

2) Monoamines (norepinephrine, dopamine and serotonin).

3) Amino acid-based transmitters (primarily L-glutamate, GABA, L-aspartate and glycine).

Synaptic transmission normally involves an important trafficking organelle, the synaptic vesicle. On the membrane of synaptic vesicles exist transporter proteins (e.g. vesicular GABA transporter, vesicular glutamate transporter, vesicular acetylcholine transporter etc), which are responsible for moving neurotransmitter into inner vesicular space (reviewed by Iversen, 2001). The functioning of these proteins requires energy from proton efflux generated by the vacuolar H^+-ATPase on the vesicular membrane (reviewed by Inoue *et al.*, 2003).

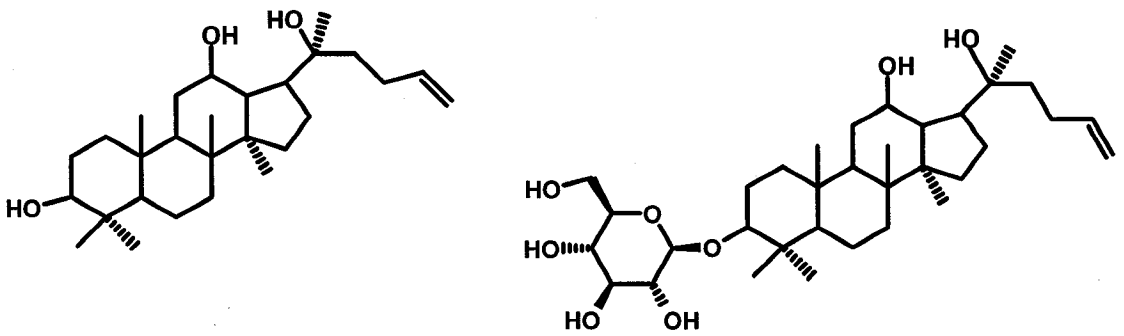
The release of neurotransmitters from synaptic vesicles is a process of exocytosis initiated by an action potential arriving at the presynaptic nerve ending. Prior to this synaptic vesicles are mobilized toward the active zones in the presynaptic membrane and become fusion competent with the help of SNARE proteins such as syntaxin, SNAP-25 and synaptobrevin (Chen and Scheller 2001). Through opening voltage-sensitive calcium

channels, action potentials trigger Ca^{2+} influx and increase intracellular Ca^{2+} concentration. Ca^{2+}-sensor protein synaptotagmin on the surface of synaptic vesicles detects this ionic signal (Nonet *et al.*, 1993), and forms SNARE complexes with syntaxin, SNAP-25 and synaptobrevin, which enable vesicles to fuse with the presynaptic membrane (Hanson *et al.*, 1997), and discharge their vesicular contents into the synaptic cleft. Neurotransmitters then diffuse rapidly across the synaptic cleft and bind to their specific receptors sites on the postsynaptic membrane (reviewed by Salinas, 2005), selectively altering the ionic permeability of the postsynaptic membrane, and causing either excitatory postsynaptic potentials (reviewed by Takagi, 2000) or inhibitory postsynaptic potentials (reviewed by Mott and Lewis, 1994). The former, normally induced by the excitatory transmitter L-glutamate, causes a local depolarization to propagate the electrical signal, whereas the latter, mostly triggered by GABA and glycine, reduces the possibility for an action potential to be generated since the postsynaptic neuron is held in a more depolarized state. Most neurotransmitters in the synaptic cleft are either broken down by the enzyme and / or taken up into the nerve ending primarily via clathrin-mediated endocytosis (De Camilli and Takei, 1996).

1.4 Objectives of the present study

Since initial studies in Dr. Nicholson's lab revealed that the ginseng aglycone, 20(*S*)protopanaxadiol (PPD) and its monoglucoside (Rh_2) (see Fig. 1.6) have the ability to inhibit [^{3}H]batrachotoxinin A-20α-benzoate binding to nerve sodium channels, the aim of this research was to examine the pharmacological action of these compounds in more depth both on VGSCs and on sodium channel-dependent nerve functions in mammalian brain. The investigation was divided into two phases. Firstly, a [^{3}H]batrachotoxinin A-

20α-benzoate binding assay was performed to delineate the interaction of each ginseng compound with VGSCs using synaptic preparations from mouse brain. Afterwards, functional assays were performed to study the effects of these compounds on the membrane potential and release of neurotransmitters from isolated nerve endings (synaptosomes). The underlying objectives of the binding experiment were for the first instance to establish concentration:response relationships for the study compounds and then use equilibrium binding and kinetic approaches to provide insights into the mechanism by which these substances target VGSCs. Following this functional assays were conducted. Initially the result of membrane potential measurements allowed me to establish that the binding of both ginseng bioactives to VGSCs does indeed lead to inhibition of the channel complex. Lastly I examined the effects of the study compounds on voltage-gated sodium channel-dependent release of neurotransmitters (L-glutamate, GABA and L-aspartate).



20(*S*)protopanaxadiol (PPD) **Rh_2**

Figure 1.6. 20(*S*)protopanaxadiol (PPD) and Rh_2.

CHAPTER 2:
20(*S*)PROTOPANAXADIOL AND Rh_2 INHIBIT [^{3}H]BATRACHOTOXININ A 20-α-BENZOATE BINDING TO SODIUM CHANNELS IN MAMMALIAN BRAIN*

2.1 Introduction

The finding that *in vivo* injection of extracts of ginseng reduces central nervous system activity in mice (Takagi *et al.*, 1972) has prompted widespread interest in the pharmacological properties of this plant. Numerous reports have subsequently appeared which describe a variety of effects in mammalian brain attributable to extractives of ginseng (Attele *et al.*, 1999). A large proportion of studies on brain identify responses to ginseng bioactives, which are intrinsically depressant in nature.

Ginseng root components inhibit sensory neurons in rat trigeminal and dorsal root ganglia by a pertussis toxin-sensitive, opioid-like mechanism, and this effect is thought to contribute to the antinociceptive effects of ginseng (Nah and McCleskey, 1994; Rhim *et al.*, 2002). Other studies have shown that oral administration of the ginsenoside Rb_1 prior to forebrain ischemia, or intracerebroventricular infusion of Rb_1 after ischemic insult, reduces damage to CA1 neurons in the hippocampus (Wen *et al.*, 1995; Lim *et al.*, 1997). Free-radical scavenging clearly contributes to this neuroprotective effect (Lim *et al.*, 1997); however, ischemia typically produces sustained neural excitation, and the extensive inhibitory effects ginseng bioactives have on other brain processes are thought

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* Reproduced from European Journal of Pharmacology, 530, 9-14, 2006, Y. Duan, J. Zheng, V Law and R. A. Nicholson, Natural products from ginseng inhibit [^{3}H]batrachotoxinin-20α-benzoate binding to sodium channels in mammalian brain © 2005 Elsevier B. V. with permission.

to play a critical role. For example, ginseng (*Panax quinquefolium* L.) extracts reduce the discharge of nucleus tractus solitarius neurons in a similar way to the inhibitory neurotransmitter GABA (Yuan *et al.*, 1998), and a number of ginsenosides including Rf and Rb_1 enhance peak currents induced by the inhibitory neurotransmitter glycine at human glycine a1 receptors (Noh *et al.*, 2003). Ginsenosides, particularly Rg_2 and Rg_3, have also been found to inhibit other ligand-gated ion channels such as NMDA receptors (Kim *et al.*, 2002), 5-HT_{3A} receptors (Choi *et al.*, 2003) and nicotinic acetylcholine receptors (Choi *et al.*, 2002; Sala *et al.*, 2002).

Voltage-gated ion channels are also clearly affected by ginseng bioactives. Patch clamp experiments using the bovine adrenal chromaffin cell model of sympathetic catecholaminergic neurons have demonstrated that ginsenosides inhibit calcium currents carried by N-, P-, and putative Q-type calcium channels (Choi *et al.*, 2001), an observation corroborated in studies with rat dissociated dorsal root ganglion neurons using ginseng total saponins and Rg_3 (Rhim *et al.*, 2002).

Electrophysiological studies have also identified voltage-gated sodium channels in brain as a sensitive target of certain ginsenosides (Liu *et al.*, 2001; Kim *et al.*, 2005). To date, six separate toxin-binding sites on voltage-gated sodium channels have been described (Cestele and Catterall, 2000). Alkaloid neurotoxins such as batrachotoxin bind with high affinity to site 2 which is associated with the S6 membrane spanning segment of domain I of the α subunit (Trainer *et al.*, 1996). Binding studies using [^{3}H]batrachotoxinin-20α-benzoate ([^{3}H]BTX-B) have emerged as an important tool for providing valuable perspectives on the actions of depressant drugs at sodium channels

(Willow and Catterall, 1982; Postma and Catterall, 1984; Ratnakumari and Hemmings, 1996).

Preliminary experiments in our laboratory found that the ginsenoside Rh_2 and the aglycones 20(*S*)protopanaxadiol and 20(*S*)protopanaxatriol have the ability to displace the binding of [^{3}H]BTX-B in mouse brain synaptoneurosomal preparations. The present investigation was conducted to characterize further this pharmacological property of ginseng compounds on the basis of sodium channel sensitivity and mechanism.

2.1 Materials and methods

2.1.1 Chemicals and radioligand

20(*S*)protopanaxadiol, 20(*S*)protopanaxatriol and Rh_2 (all > 95% purity by high-performance liquid chromatography) were purchased from the Canfo Chemical Company Ltd., Chengdu, P.R. China via CNCC Sichuan Imp/Exp Company Ltd., Chengdu, P.R. China. The structures of the study compounds are provided in Fig. 2.1. [benzoyl-2,5-^{3}H] Batrachotoxinin A 20-α-benzoate ([^{3}H]BTX-B) was obtained at a specific activity of 48 Ci/mmol from PerkinElmer Life Sciences Inc., Boston, MA, USA. Veratridine and scorpion (*Leiurus quinquestriatus*) venom were from Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada. Other chemicals including saline components were purchased from either from Sigma-Aldrich Canada Ltd. or EM Science, Gibbstown, NJ, USA.

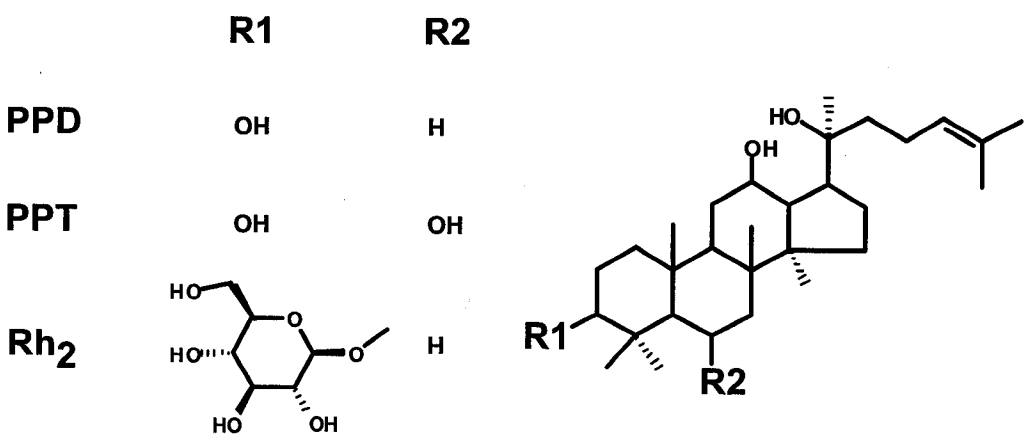


Figure 2.1. Chemical structures of 20(*S*)protopanaxadiol (PPD), 20(*S*)protopanaxatriol (PPT) and Rh_2.

2.2.2 Isolation of synaptoneurosomes from mouse brain

The isolation of synaptoneurosomes from male CD1 mice (20–25 g) was performed between 1 and 4 °C and based on methods described by Creveling *et al.* (1980) and Harris and Allen (1985). All experimental procedures involving animals had approval from the Simon Fraser University Animal Care Committee and complied with Canadian Council on Animal Care guidelines. Mice were killed by cervical dislocation followed by decapitation. The whole brain from a single mouse was cut into small fragments and homogenized in 5 ml ice-cold Na^+-free buffer (130 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 5.5 mM glucose and 50 mM Hepes adjusted to pH 7.4 with Tris base). After dilution with 15 ml Na^+-free buffer, the homogenate was passed through 100-μm nylon mesh and centrifuged (1000×*g*; 15 min). The pellet was resuspended in 10 ml Na^+-free buffer (10 ml) and centrifuged again to provide the washed synaptoneurosomal pellet which was then gently dispersed in 0.9 ml Na^+-free buffer and placed on ice.

2.2.3 Binding studies using [^{3}H]BTX-B

Binding assays with [^{3}H]BTX-B were carried out at 37 °C using published procedures (Catterall *et al.*, 1981; Creveling *et al.*, 1983) with modifications. Standard binding reactions were initiated by adding synaptoneurosomes (20 μl; 250 μg protein) to 140 μl Na^+-free buffer containing bovine serum albumin (1 mg/ml), [^{3}H]BTX-B (6 nM for IC_{50}, kinetic and reversibility measurements; 2–160 nM for Scatchard experiments), scorpion (*L. quinquestriatus*) venom (17 μg) and study compounds or solvent control (dimethylsulfoxide), as appropriate. After 60 min, the binding reaction was terminated by adding 3 ml of ice-cold wash buffer (163 mM choline chloride, 0.8 mM $MgSO_4 \cdot 7H_2O$,

1.8 mM $CaCl_2 \cdot 2H_2O$ and 5 mM Hepes adjusted to pH 7.4 with Tris base) to the incubation followed by vacuum filtration (Hoefer FH 225V) using Whatman GF/C filters. Filter-trapped synaptoneurosomes were immediately washed with three 3-ml volumes of ice-cold wash buffer. Membrane-associated radioactivity was solubilized (10% sodium dodecyl sulfate) and then quantitated by liquid scintillation counting. [^{3}H]BTX-B binding not displaced from synaptoneurosomes by 300 μM veratridine was taken as the non-specific binding signal.

To determine effects of study compounds on the kinetics of association of [^{3}H]BTX-B with site 2 on voltage-gated sodium channels, the increase in specific binding of radioligand was monitored for 10 min. For dissociation experiments, synaptoneurosomes were equilibrated with [^{3}H]BTX-B for 60 min, then either a saturating concentration of veratridine (with solvent control) or veratridine with study compound was added and specific binding monitored over a 25-min time course. For reversibility experiments, synaptoneurosomes preincubated with dimethylsulfoxide, veratridine (300 μM) or study compound dissolved in Na^+-free buffer containing bovine serum albumin were either (a) directly mixed with buffer containing [^{3}H]BTX-B and scorpion venom to initiate the binding reaction or (b) subjected to 1, 2 or 3 washes by centrifugation and resuspension in Na^+-free buffer (no study compound present) before proceeding with the binding assay in the absence of added study compound.

All IC_{50} experiments, Scatchard analyses, association and reversibility assays were conducted in triplicate. Dissociation experiments were carried out in duplicate. Protein estimations were carried out using the method of Peterson (1977).

2.2.4 Analysis and presentation of data

Linear and non-linear regression analyses, determination of IC_{50}s, binding and kinetic parameters were carried out with Prism 3 software (GraphPad Software Inc., San Diego, CA). All data points represent means ± S.E.M. of at least three experiments using different mouse brain fractions.

2.3 Results

The effects of the study compounds on the binding of [^{3}H]BTX-B to synaptoneurosomal membranes are shown in Fig. 2.2. PPD, PPT and Rh_2 inhibited binding in a concentration-dependent fashion with IC_{50} values of 41.8 μM (95% confidence limits 32.7 to 53.4 μM), 78.6 μM (95% confidence limits 74.4 to 83.0 μM) and 161.8 μM ((95% confidence limits 140.1 to 187.0 μM), respectively. Both 20(*S*)protopanaxadiol and 20(*S*)protopanaxatriol were capable of causing full (ca. 90%) inhibition of [^{3}H]BTX-B binding; however, solubility constraints in our assay precluded investigation of the inhibitory effects of Rh_2 above 500 μM. Vanessa Law performed the experiments on 20(*S*)protopanaxatriol under my guidance.

We then performed saturation and kinetic experiments with [^{3}H]BTX-B to help clarify the mechanism(s) underlying the interaction of 20(*S*)protopanaxadiol and Rh_2 with alkaloid neurotoxin site 2 on the sodium channel. The way in which 20(*S*)protopanaxadiol and Rh_2 close to their IC_{50} levels affect the concentration dependence of [^{3}H]BTX-B binding are shown by the Scatchard plots of Fig. 2.3. Both ginseng bioactives reduced the B_{max} of [^{3}H]BTX-B binding from the control value of 1395 fmol mg^{-1} to 928 fmol mg^{-1} (20(*S*)protopanaxadiol) and to 1021 fmol mg^{-1} (Rh_2). Rh_2 increased the K_d of [^{3}H]BTX-B binding from a control value of 29.6 nM to 52.6 nM;

however, 20(*S*)protopanaxadiol did not affect the affinity of radioligand for site 2. Even at high concentrations, the study compounds were unable to reduce the initial rate of association of [^{3}H]BTX-B with alkaloid neurotoxin site 2 (Table 2.1). In marked contrast, both 20(*S*)protopanaxadiol and Rh_2 concentration-dependently increased the rate of dissociation of [^{3}H]BTX-B above that produced by a saturating concentration of the site 2-specific alkaloid veratridine (Fig. 2.4).

When synaptosomal membranes were preequilibrated with approximate IC_{50} concentrations of 20(*S*)protopanaxadiol or Rh_2 and then subjected to 1–3 washes followed by binding assay under study compound-free conditions, the ability of [^{3}H]BTX-B to bind to site 2 recovered by approximately 50% (20(*S*)protopanaxadiol) and 30% (Rh_2) (Fig. 2.5).

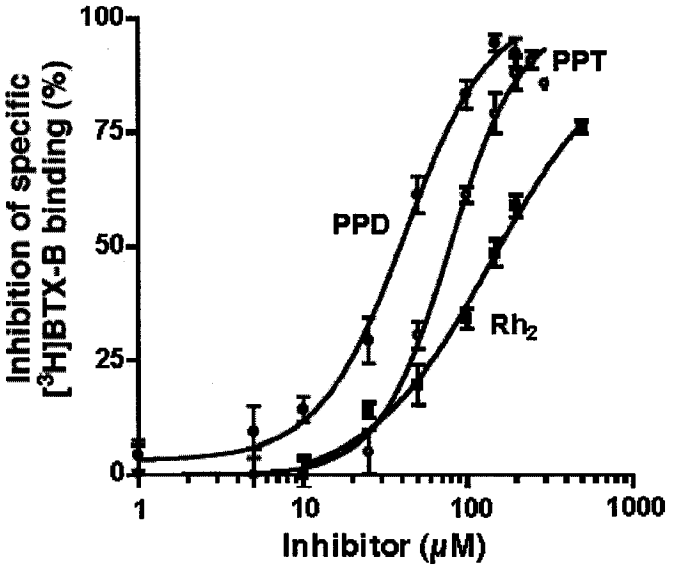


Figure 2.2. Inhibition by 20(*S*)protopanaxadiol (PPD), 20(*S*)protopanaxatriol (PPT) and Rh_2 of the specific binding of [^{3}H]BTX-B to mouse brain sodium channels. All data points represent means ± S.E.M. of three independent experiments and assays within each experiment were performed in triplicate.

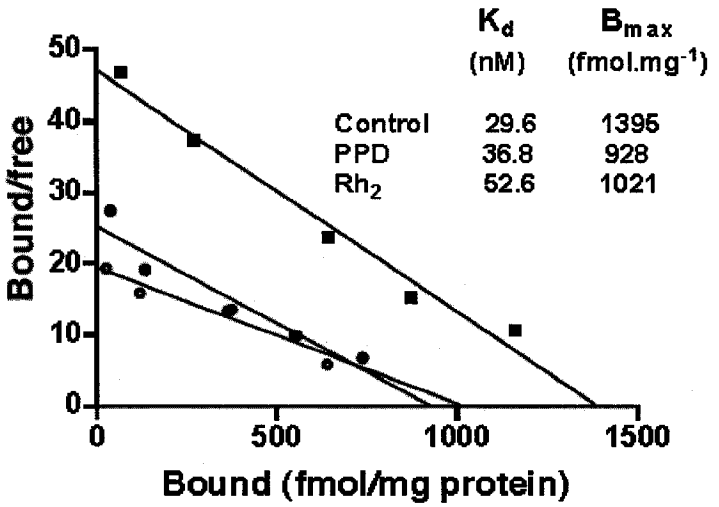


Figure 2.3. Scatchard analysis of [^{3}H]BTX-B binding to sodium channels in the absence (■; $r^2 = 0.98$) and presence of 40 μM PPD (●; $r^2 = 0.92$) and 200 μM Rh_2 (○; $r^2 = 0.94$). Values as means ± S.E.M. of three independent experiments, each performed in triplicate.

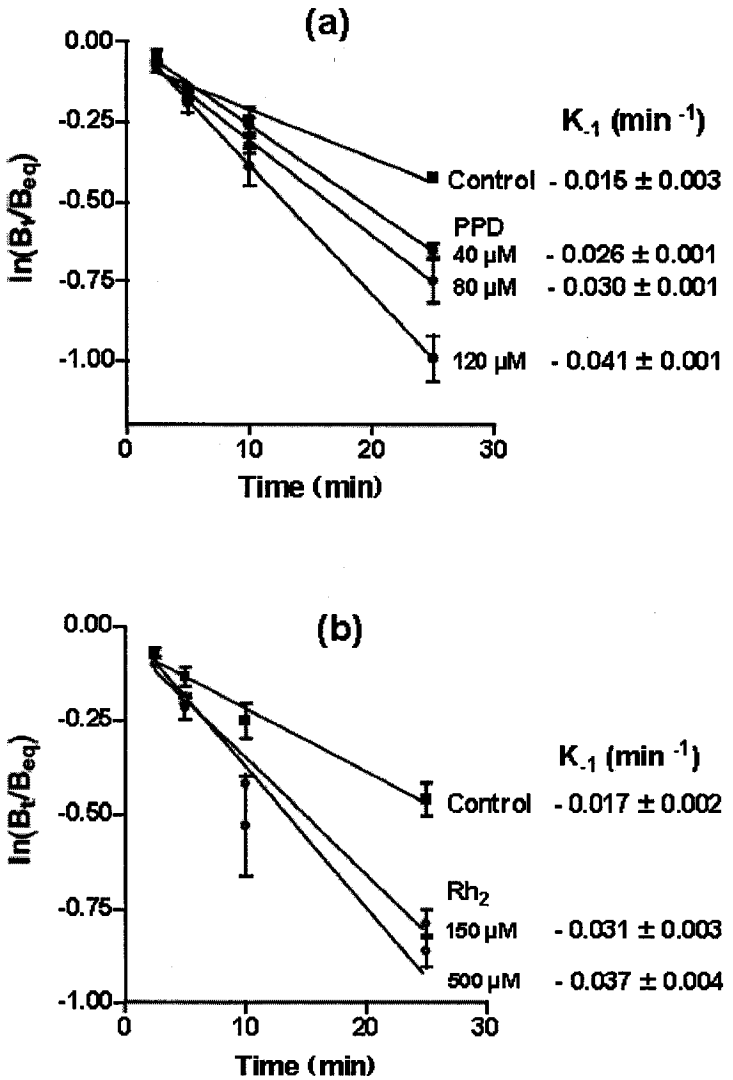


Figure 2.4. Dissociation of the [^{3}H]BTX-B:sodium channel complex (initiated by 300 µM veratridine) in the absence (■) and presence of (A) 20(*S*)protopanaxadiol (PPD; ●) or (B) Rh_2 (○). For the PPD series of experiments, K_{-1} values were control − 0.152 ± 0.003; 40 µM − 0.026 ± 0.001; 80 µM − 0.298 ± 0.001 and 120 µM − 0.4067 ± 0.001. For the Rh_2 series of experiments K_{-1} values were control 0.016 ± 0.002; 150 µM − 0.031 ± 0.003; 500 µM − 0.037 ± 0.003. Values for r^2 ranged from 0.93 to 0.99. All data points represent means ± S.E.M. of three independent experiments, each performed in duplicate.

Table 2.1. Lack of effect of PPD and Rh_2 on the initial rate of association of [^{3}H]BTX-B with alkaloid neurotoxin site 2 on sodium channels. Values represent mean ± S.E.M. of 3–6 independent experiments each conducted in triplicate.

	Concentration (μM)	K_{obs} (min^{-1})
Control		0.13 ± 0.01
20(*S*)protopanaxadiol	40	0.16 ± 0.01
	120	0.15 ± 0.02
Rh_2	150	0.13 ± 0.01
	500	0.16 ± 0.02

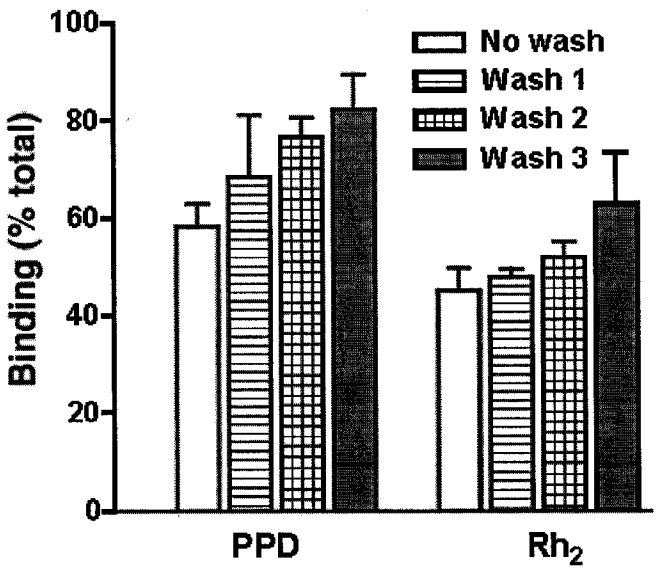


Figure 2.5. The reversibility of the inhibition of [^{3}H]BTX-B binding by PPD and Rh_2. Membrane preparations preequilibrated with study compounds received either no wash or up to three sequential washes (see 2.2 Materials and methods). Columns show mean ± S.E.M. of three independent experiments, each of which was performed in triplicate.

2.4 Discussion

The present investigation provides biochemical evidence that the ginseng natural products 20(*S*)protopanaxadiol, 20(*S*)protopanaxatriol and Rh_2 have the ability to modify the properties of the [^{3}H]BTX-B recognition site (site 2) on voltage-gated sodium channels in mammalian brain. This finding is in general agreement with previous electrophysiological findings describing sodium channel inhibitory activity of the ginsenosides Rb_1 (Liu *et al.*, 2001) and Rg_3 (Kim *et al.*, 2005) in the low to mid-micromolar range. 20(*S*)Protopanaxadiol, 20(*S*)protopanaxatriol and Rh_2 affect the binding of [^{3}H]BTX-B to sodium channels in a similar way to anticonvulsant, antiarrhythmic, local anesthetic and neuroprotective drugs (Catterall, 1981; Willow and Catterall, 1982; Postma and Catterall, 1984; MacKinnon *et al.*, 1996) as well as some sodium channel-blocking insecticides (Deecher *et al.*, 1991; Leong *et al.*, 2001). Our concentration–response data demonstrate that 20(*S*)protopanaxadiol, 20(*S*)protopanaxatriol and the ginsenoside Rh_2 inhibit [^{3}H]BTX-B binding with mean IC_{50} values of 42, 79 and 162 μM, respectively. These inhibitory potencies compare favorably with lidocaine and other local anesthetics (Postma and Catterall, 1984) and anticonvulsants such as carbamazepine and diphenylhydantoin (Willow and Catterall, 1982), but are lower compared to the neuroprotectant lifarizine (MacKinnon *et al.*, 1996). It should also be noted that based on IC_{50}s, 3 of the 14 local anesthetics investigated by Creveling *et al.* (1983) were > 10-fold more potent than 20(*S*)protopanaxadiol in inhibiting [^{3}H]BTX-B binding to brain vesicular preparations. In this study, cocaine exhibited a similar activity to 20(*S*)protopanaxadiol, while compounds including procaine, piperocaine methiodide and benzocaine were of lower inhibitory potency.

Since 20(*S*)protopanaxadiol and Rh_2 represent an example of an aglycone:glucoside pair, differing by a single glucose moiety attached to carbon 3 of the steroidal skeleton (Rh_2), we selected these compounds for a more detailed saturation analysis and kinetic investigation. 20(*S*)protopanaxadiol decreased the number of sites available for [^{3}H]BTX-B binding (B_{max}) with minimal influence on radioligand affinity (K_d). By contrast, Rh_2 caused a reduction in both binding site density and K_d of radioligand. The lowering of binding maxima suggests that 20(*S*)protopanaxadiol and Rh_2 are non-competitive inhibitors of [^{3}H]BTX-B binding. Since Rh_2 has the capacity to reduce the affinity of [^{3}H]BTX-B for site 2 and dissociation experiments suggest that 20(*S*)protopanaxadiol and Rh_2 each form a ternary ([^{3}H]BTX-B:sodium channel:displacer) complex, we conclude that both compounds reduce [^{3}H]BTX-B binding site density through an indirect allosteric mechanism and do not bind directly to the BTX-B recognition site.

Kinetic analysis of [^{3}H]BTX-B binding can furnish useful biochemical perspectives on the state dependence of drug binding to sodium channels. A compound which allosterically reduces the velocity of formation of the [^{3}H]BTX-B:sodium channel complex has been interpreted as reflecting an interaction with non-activated states of the channel. However, since [^{3}H]BTX-B selectively binds to and stabilizes activated states of the sodium channel (Catterall, 1977), drugs such as lidocaine, which stimulate dissociation of the [^{3}H]BTX-B:brain sodium channel complex (Postma and Catterall, 1984), likely bind to BTX-B-activated configurations. At concentrations close to those causing maximum displacement of [^{3}H]BTX-B binding, 20(*S*)propanaxadiol and Rh_2 were unable to reduce the rate of radioligand association. Therefore, in addition to

demonstrating that the study compounds cannot interact directly in a simple competitive manner with the BTX-B recognition site, this result implies that 20(*S*)propanaxadiol and Rh_2 have negligible affinity for channels to which [^{3}H]BTX-B is not already bound (i.e., non-conducting states). In clear contrast, both compounds (at IC_{50} and above) were found to accelerate the dissociation of [^{3}H]BTX-B when initiated by a saturating concentration of the competitive alkaloid displacer veratridine. As well as indicating that 20(*S*)propanaxadiol and Rh_2 interact with a region that is distinct from, but coupled to, the radioligand binding site, these results suggest that it is a BTX-B-activated form of the channel that is sensitive to the study compounds. While there is evidence to suggest that the BTX-B-activated state may not be the same as that produced by voltage manipulation (Huang *et al.*, 1979), it is important to note that in experiments on sodium channel α-subunits expressed in tsA201 cells, Liu *et al.* (2001) have concluded that although components of American ginseng extract and the ginsenoside Rb_1 may bind to the open state, they interact predominantly with an inactive state of the channel. In this regard, it is possible that inhibition of [^{3}H]BTX-B binding by 20(*S*)propanaxadiol and Rh_2 may allosterically destabilize the BTX-B-activated channel in favor of an inactivated state of the sodium channel which is similar to the electrophysiologically generated inactivated state.

Our data confirm that the inhibitory effects of 20(*S*)propanaxadiol and Rh_2 on [^{3}H]BTX-B binding progressively decline as a result of three wash–resuspension cycles in Na^+-free buffer without study compound present. The results indicate that washout of both compounds is relatively slow with 20(*S*)propanaxadiol's inhibitory effect on [^{3}H]BTX-B binding declining almost twice as fast as that of Rh_2. Thus,

20(*S*)propanaxadiol and Rh_2 can be classified as slowly reversible drugs. These results contrast sharply with the local anesthetic analog procaine isothiocyanate which irreversibly inhibits [^{3}H]BTX-B binding (Creveling *et al.*, 1990).

The binding results with [^{3}H]BTX-B clearly demonstrate that 20(*S*)propanaxadiol, 20(*S*)propanaxatriol and Rh_2 have the capacity to indirectly modify the nature of the alkaloid neurotoxin site 2 of voltage-gated sodium channels. In our quasi-cellular synaptoneurosomal system, we cannot rule out the possibility that a primary target remote from the sodium channel itself, such as a protein kinase, may be involved. We also cannot exclude a mechanism along the lines recently proposed by Lundbaek *et al.* (2005), which emphasizes an effect on bilayer elasticity as the basis for the inhibitory effects of vanilloid receptor modulators and amphipathic drugs in general at voltage-gated sodium channels.

Using sodium channels expressed in *Xenopus* oocytes, Kim *et al.* (2005) found that chemical modifications to the terminal glucose moiety of the ginsenoside Rg_3 (20(*S*)protopanaxadiol-3-β-d-glucopyranosyl-β-d-glucopyranoside) abolished sodium channel inhibitory activity and observed no I_{Na} inhibitory activity for the aglycone 20(*S*)protopanaxadiol, leading to the conclusion that the carbohydrate portion of Rg_3 is important in sodium channel inhibition. Our results, demonstrating a weaker inhibition of [^{3}H]BTX-B binding to native sodium channels by Rh_2 (which is structurally identical to Rg_3 except that it has one glucose moiety at carbon 3) compared to 20(*S*)protopanaxadiol, suggests that the ability to modify the site 2 region of sodium channels is still retained (albeit at a somewhat lower level compared to the aglycone) when a single glucose moiety is present at carbon 3. Assuming these ginseng components, which have

amphipathic character, are indirectly restricting the freedom of movement of sodium channels through modification of lipid bilayer elasticity, this may account for apparent discrepancies between the results of our study and those of Kim *et al.* (2005), since we would expect differences in the lipid microenvironment of oocyte-expressed and our native sodium channels.

In conclusion, our binding studies offer a novel biochemical perspective on the interaction of representative aglycones 20(*S*)propanaxadiol, 20(*S*)propanaxatriol and the ginsenoside Rh_2 with the alkaloid neurotoxin binding site 2 of voltage-gated sodium channels. It remains to be established whether the concentrations of our study compounds needed to influence sodium channels overlap with the plasma levels attainable during regular consumption of ginseng.

CHAPTER 3:
PROTOPANAXADIOL AND Rh_2 INHIBIT SODIUM CHANNEL-DEPENDENT DEPOLARIZATION AND RELEASE OF AMINO ACID NEUROTRANSMITTERS IN SYNAPTIC PREPARATIONS ISOLATED FROM MAMMALIAN BRAIN

3.1 Introduction

Several investigations describe the ability of ginseng saponins to modify the properties of ion transporting membrane proteins, including ligand-gated channel-linked receptors such as 5-HT receptors (2007), NMDA receptors (Kim *et al.,* 2007; Lee *et al.,* 2006) and nicotinic acetylcholine receptors (Tachikawa *et al.,* 2003). These natural products also interfere with the operation of voltage-gated ion channels for example sodium channels (Liu *et al.*, 2001; Kim *et al.*, 2005; Lee *et al.*, 2005) and calcium channels (Lee *et al.,* 2006). Results from my binding studies have shown that 20(*S*)protopanaxadiol (PPD) and its monoglucoside Rh_2 inhibit the binding of [^{3}H]BTX-B to neuronal voltage-gated sodium channels in a concentration-related manner (Duan *et al.,* 2006). Specifically, these equilibrium binding and kinetic experiments have demonstrated that PPD and Rh_2 bind to a site on the sodium channel complex that is allosterically coupled to neurotoxin binding site 2. This research therefore extended the findings of previous electrophysiological studies by providing biochemical / pharmacological insights into the mechanism of action of ginseng components at sodium channels. However, while my investigations showed that the inhibitory effect of these ginseng compounds on [^{3}H]BTX-B binding had parallels with certain depressant drugs

that act as sodium channel blockers, my experiments did not provide any information on their ability to modify sodium channel function *per se*.

In this phase of my research I used *in vitro* synaptic preparations to investigate potential effects that PPD and Rh_2 might have on downstream physiological events occurring subsequent to their binding to sodium channels. I first used the rhodamine 6G membrane potential assay, configured for synaptoneurosomes with veratridine as the activator, since this offers a very useful means to explore potential inhibitory effects of study compounds on sodium channel-dependent depolarization of the neuronal plasma membrane (Verdon *et al.*, 2000; Duan *et al.*, 2007). I then examined the effects of PPD and Rh_2 on depolarization-evoked release of endogenously stored neurotransmitters from synaptosomes using high performance liquid chromatography (HPLC). Together my data provide biochemical evidence for functional inhibition of voltage-gated sodium channels by micromolar concentrations of PPD and Rh_2.

3.2 Experimental methods and procedures

3.2.1 Effects of ginseng bioactives on veratridine-evoked depolarization of synaptoneurosomes

Synaptoneurosomes were freshly prepared from the whole brains of male CD1 mice (20-25 g) according to the procedure of Harris and Allen (1985). Membrane potential measurements were conducted with a Perkin-Elmer LS50 fluorescence spectrophotometer using excitation and emission wavelengths set at 520 nm and 550 nm, respectively. The membrane potential assays were started by adding synaptoneurosomes (30 µl, approximately 0.75 mg protein) into a quartz cuvette containing 3 ml saline (NaCl 128 mM; KCl 5 mM; $CaCl_2 \cdot 2H_2O$ 0.8 mM; $MgCl_2 \cdot 7H_2O$ 1.2 mM; glucose 14.3 mM;

HEPES 20 mM buffered to pH 7.4 with Tris base) and 20(*S*)protopanaxadiol, Rh_2 (both in 5 µl DMSO) or DMSO control, as appropriate. The curvette contents were maintained at 35°C and stirred continuously during the experiment. The voltage-sensitive fluoroprobe rhodamine 6G (0.03 µM final concentration) was rapidly injected in 1 µl DMSO at 200 seconds. This was then followed at 900 seconds by the sodium channel activator veratridine (5 µM final concentration; also injected in 1 µl DMSO). The recording was terminated at 1400 seconds and the increase in fluorescence intensity (FI) was calculated by subtracting the FI immediately before veratridine addition from the maximum FI occurring after the introduction of VTD. Calibration was performed using salines with varying concentrations of K^+ which also had the $[Na^+]$ adjusted to maintain osmolarity. The membrane potential of synaptoneurosomes in resting and depolarized states was estimated according to the reduced Goldman equation as previously described (Duan *et al.*, 2007). In some assays, KCl (final concentration 53.6 mM) was added to at 1200 seconds after the VTD peak had stabilized to examine whether PPD and Rh_2 had any depolarizing effect on synaptoneursomes. All experimental procedures using animals had approval from the Animal Care Committee of Simon Fraser University and complied with Canadian Council on Animal Care guidelines. In all cases, animals were euthanized by rapid cervical dislocation.

3.2.2 Determination of neurotransmitter release from synaptosomes exposed to 20(*S*)protopanaxadiol and Rh_2

The whole brains of male CD1 mice (20–25 g) were quickly removed and homogenized, followed by purification using a Percoll step gradient (Dunkley *et al.*, 1986). Fractions 3 and 4 (the layers containing most of the synaptosomal material) were

washed, pelleted, and resuspended in physiological saline (NaCl 128 mM, KCl 5 mM, $MgCl_2.7H_2O$ 1.2 mM, $NaHCO_3$ 5 mM, $CaCl_2.2H_2O$ 0.8 mM, glucose 14.3 mM, HEPES 20 mM, sodium pyruvate 4 mM buffered to pH 7.4 with Tris base). Synaptosomal suspensions (7 mg protein/ml) were stored on ice before use and subject to a preincubation at 30°C for 5 min prior to assays.

Synaptosomal suspensions (50 µl) were added to saline (100 µl) containing either the DMSO vehicle, PPD or Rh_2 (final DMSO concentration 1.33%) or TTX (added in 1 µl H_2O). This mixture was then vortexed gently and incubated for 4 min at 30 °C. To trigger sodium channel activation-dependent release of transmitters, veratridine [final concentration 2 µM; dissolved in DMSO:H_2O (1:3)] was introduced, followed by gentle vortexing. After another incubation (5 min), samples were subjected to rapid centrifugation (Beckman Microfuge E; 40 seconds), and 130 µl of the supernatant was then removed from each sample and acidified (6 M perchloric acid; 3 µl).

The acidified perchloric acid extracts were centrifuged and 50 µl aliquots of each supernatant were removed and mixed with borate buffer (200 µl; 0.1 M) and *o*-phthaldialdehyde reagent (OPA; 50 µl; prepared in borate buffer, final concentration 0.04 M) in preparation for HPLC analysis. HPLC was performed using a Hewlett Packard 1050 series chromatograph and a C18 column (15 cm×4.6 mm ID; particle size 5 µm). Two buffers: A (80% 0.05 M sodium phosphate buffer + 20% methanol; pH 5.7) and B (20% 0.05 M sodium phosphate buffer + 80% methanol; pH 5.7) were used to form the mobile phase. Chromatography was started by injection of the samples (40 µl) onto the column. Buffer A was reduced linearly from 85% (Buffer B: 15%) to 14% (B: 86%) over

a 14-min elution. Column clean-up and re-equilibration was carried out by following this with a 3-min elution with buffers A and B mixed in their original proportions. Effluent from the column was passed directly to an HP 1046A programmable fluorescence detector with excitation and emission wavelength set at 330 nm and 450 nm respectively. Peak areas were quantitated and traces recorded with an HP 3396 Series II integrator. OPA-derivatives of endogenous L-aspartate, L-glutamate and GABA in synaptosomal extracts were matched through comparisons with authentic OPA standards. Complete separation of OPA derivatives of these amino acid neurotransmitters from other OPA-amino acids present in supernatants was achieved. OPA-derivatized L-aspartate, L-glutamate and GABA standards produced linear fluorescence response over a wide concentration range.

3.2.3 Data analysis

Prism 4 (Graphpad Software Inc. San Diego, CA, USA) was used to estimate IC_{50}s and to carry out curve fitting using non-linear regression analysis. Data are expressed as means and variability as S.E.M. (standard error of mean). Statistical comparisons used Student's *t* test and a value of < 0.05 was considered to indicate statistical significance.

3.3 Results

3.3.1 The effect of 20(*S*)protopanaxadiol and Rh_2 on VGSC-dependent depolarization of synaptoneurosomes

The resting potential of synaptoneurosomes averaged -80 ± 1 mV as measured with the voltage-sensitive fluoroprobe rhodamine 6G. Exposure to 5 μM veratridine caused rapid depolarization and increased the potential of synaptoneurosomes to -22 ± 2 mV. The increases in fluorescence intensity triggered by this sodium channel activator were inhibited strongly by PPD and Rh_2 within the 10-200 μM range (see Fig. 3.1). Estimated IC_{50} value were 48 μM for PPD and 100 μM for Rh_2. 3 μM tetrodotoxin (TTX) almost completely eliminated the increase in potential induced by VTD (Fig. 3.2), implying that the inhibitory effect of PPD and Rh_2 on the depolarization are closely linked to blockade of sodium ion influx through sodium channels. The fluorescence increases in the presence of PPD and Rh_2 after K^+ challenge demonstrated that neither study compound depolarized synaptoneurosomes.

3.3.2 The effect of 20(*S*)protopanaxadiol and Rh_2 on VGSC-dependent release of L-glutamate, GABA and L-aspartate from synaptosomes

We next examined the effect of PPD and Rh_2 on sodium channel-dependent release of the amino acid-based transmitters L-aspartate, L-glutamate and GABA from synaptosomes. At micromolar concentrations, both of these ginseng compounds blocked veratridine-evoked transmitter release in a concentration-dependent manner (Figs. 3.3, 3.4 and 3.5). IC_{50} values were as follows: PPD (L-aspartate 13 μM; L-glutamate 33 μM; GABA 36 μM) and Rh_2 (L-aspartate 29 μM; L-glutamate approximately 2-300 μM; GABA 36 μM). The two ginseng components can be viewed as being of similar efficacy in inhibiting the release of L-aspartate (Fig. 3.3) whereas for L-glutamate and GABA,

PPD showed a significantly stronger suppressive capacity ($P < 0.05$, GABA, Fig. 3.4); ($P < 0.01$, L-glutamate, Fig. 3.5). In related series of experiments, I confirmed that 2 μM tetrodotoxin fully blocked veratridine-induced release of each transmitter (Fig. 3.6). Increased amounts of extra-synaptosomal L-glutamate and GABA (33.6 and 23.2% of the quantities released by veratridine) were detected when synaptosomes were exposed to 300 μM Rh_2 a concentration capable of causing maximum possible inhibition of veratridine-induced release of these neurotransmitters (Table 3.1). In marked contrast, very weak release of L-glutamate and GABA (5.4 and 3.4% respectively) was observed with a concentration of PPD that caused an identical level of inhibition of VTD-induced release.

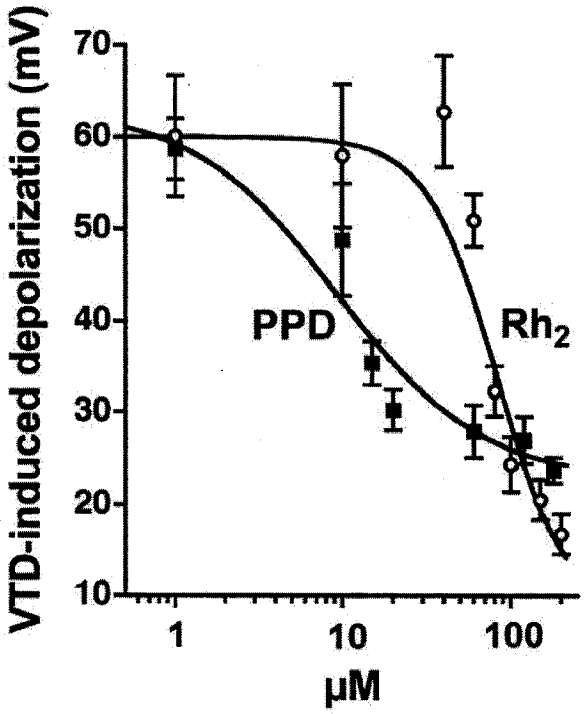


Figure 3.1. Inhibition of VTD-induced depolarization by 20(*S*)protopanaxadiol (PPD) and Rh_2. The membrane potential of synaptoneurosomes was determined by the rhodamine 6G method. Data points represent means ± S.E.M.s of 3-4 independent experiments. PPD and Rh_2 curves are significantly different ($P < 0.01$).

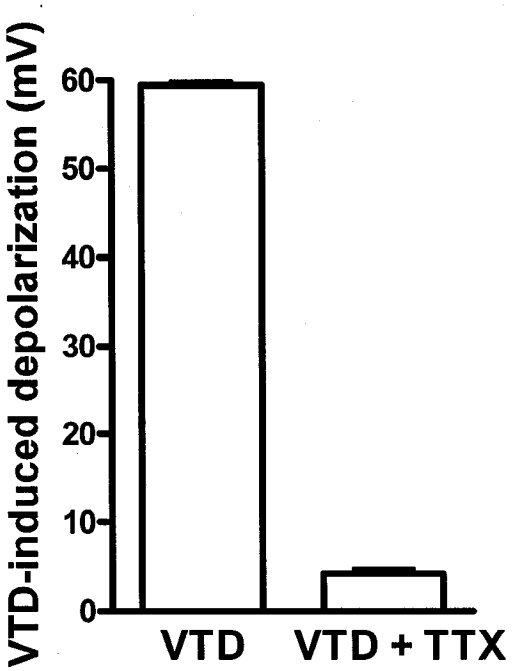


Figure 3.2. Depolarization initiated by 5 μM veratridine (VTD) in the absence and presence of tetrodotoxin (TTX). VTD alone caused a membrane potential increase of 59.5 ± 0.4 mV, which was reduced by TTX (3 μM) to approximately 4.2 ± 0.5 mV. Columns represent means and error bars the S.E.M.s from 3 - 6 independent experiments.

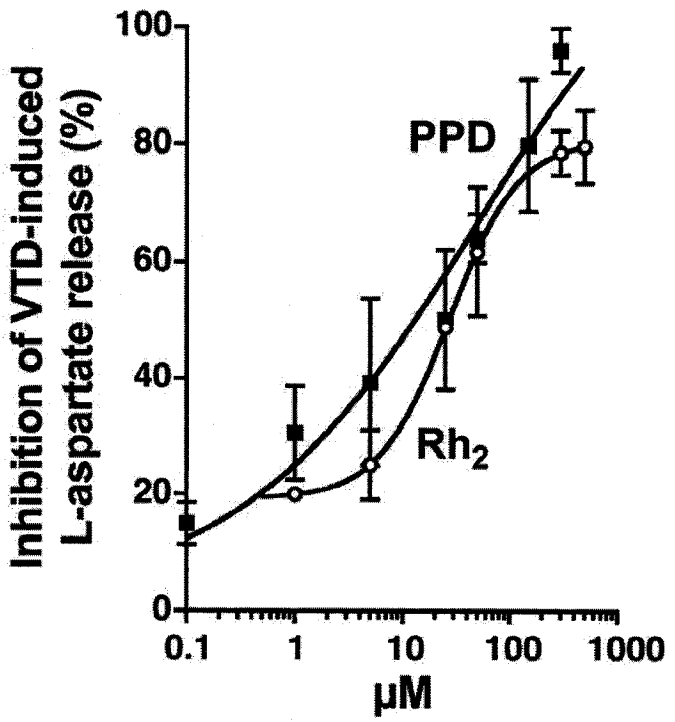


Figure 3.3. Inhibitory effects of 20(*S*)protopanaxadiol (PPD) and Rh_2 on veratridine (2 μM)-evoked release of L-aspartate. Data points represent means ± S.E.M.s of at least 3 determinations. There was no significant difference between the inhibitory effects produced by PPD and Rh_2 over their full concentration ranges.

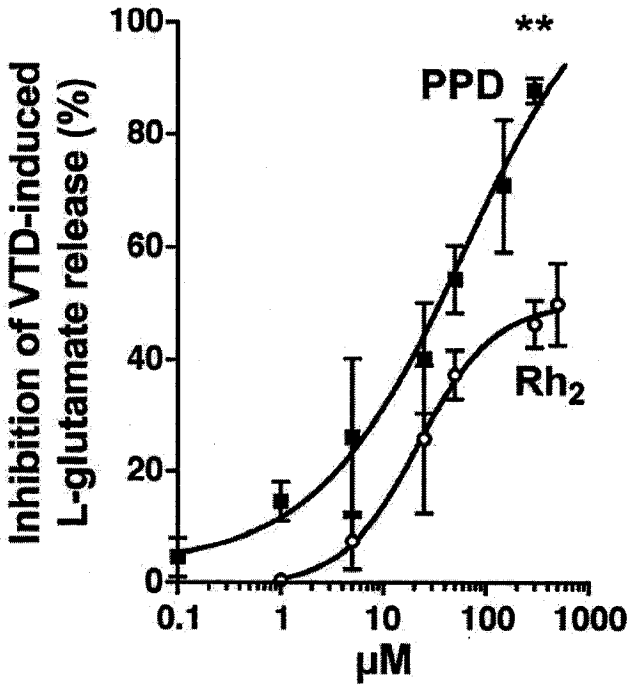


Figure 3.4 Inhibitory effects of 20(*S*)protopanaxadiol (PPD) and Rh_2 on veratridine (2 μM)-evoked release of L-glutamate. Data points represent means ± S.E.M.s of at least 3 determinations. There was no significant difference between the inhibitory effects observed for PPD and Rh_2 except at maximum effect concentration ($P < 0.01$), as indicated by asterisks.

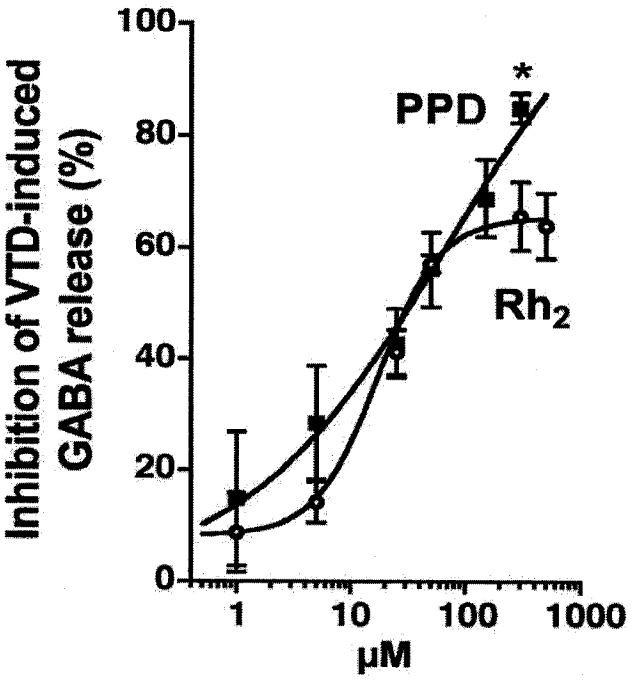


Figure 3.5. Inhibitory effects of 20(*S*)protopanaxadiol (PPD) and Rh_2 on veratridine (2 μM)-evoked release of GABA. Data points represent means ± S.E.M.s of at least 3 determinations. There was no significant difference between the inhibitory effects observed for PPD and Rh_2 except at maximum effect concentration ($P < 0.05$), as indicated by asterisk.

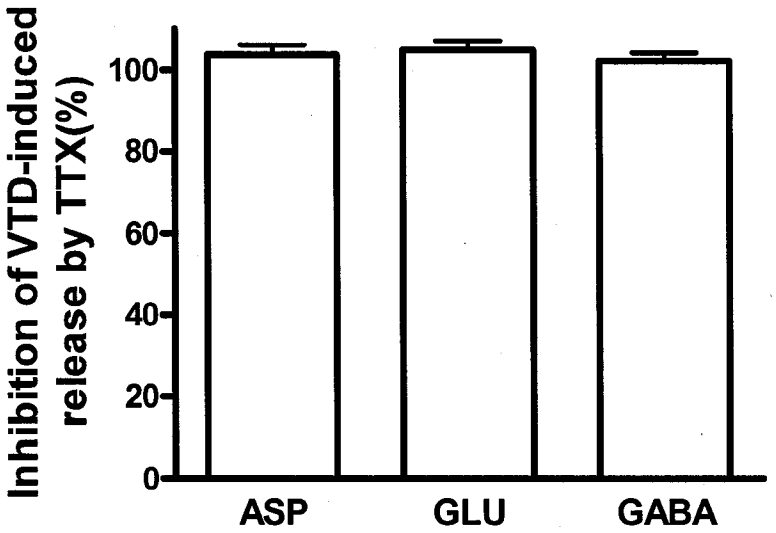


Figure 3.6. Complete inhibition by TTX (2 μM) of VTD (2 μM)-induced release of L-aspartate (ASP), L-glutamate (GLU) and GABA from synaptosomes. Columns represent means and error bars the S.E.M.s from 9 independent experiments.

Table 3.1. Increase in extra-synaptosomal neurotransmitters occurring after synaptosomes were challenged with Rh_2 alone at 300 μM (a concentration which causes maximal inhibition of VTD-induced L-glutamate and GABA release) compared to a concentration of PPD (150 μM) which causes an identical level of inhibition of VTD-induced transmitter release. Values represent the amount of each transmitter as a percentage of that released by 2 μM veratridine ± S.E.M.s from 3 independent experiments.

Ginseng compounds	**Neurotransmitter release (%)**	
	L-glutamate	GABA
Rh_2 (300 μM)	33.6 ± 5.9	23.2 ± 5.7
PPD (150 μM)	5.4 ± 6.3	3.4 ± 1.7

3.4 Discussion

In the previous chapter I reported that PPD and Rh_2 inhibit the binding of [^{3}H]BTX-B to neurotoxin binding site 2 on voltage-gated sodium channels (VGSCs) by an allosteric mechanism. The research described here specifically identifies functional perturbations that PPD and Rh_2 elicit in *in vitro* synaptic preparations as a result of their binding to VGSCs.

Veratridine selectively targets neurotoxin site 2 on VGSCs, causing persistent activation of sodium channels and Na^+ entry (reviewed by Gutman and Lazarovic, 1997). When exposed to VTD, sodium channels open at the resting potential and this rapidly depolarizes synaptoneurosomes and synaptosomes. Previous studies have demonstrated that the fluorescence profile of the voltage-sensitive dye rhodamine 6G closely reflects changes in the electrical potential of the plasma membrane, and therefore this probe represents a very useful tool for monitoring changes in membrane potential (Aiuchi *et al.*, 1982; Mandala *et al.*, 1999). In the present study [as our laboratory has reported previously (Nicholson *et al.*, 2003; Duan *et al.*, 2007)], the increase in fluorescence intensity due to VTD was almost entirely eliminated by the sodium channel blocker tetrodotoxin. This observation suggested strongly that the fluorescence response to veratridine reflected a virtually complete sodium channel-dependent alteration in neuronal membrane potential. The strong blockade of the VTD-evoked depolarization of synaptoneurosomes by the two study compounds provided my first piece of *in vitro* evidence that these ginseng bioactives really do have the ability to modify VGSC-dependent function in synaptic regions. Similar to my findings in the [^{3}H]BTX-B binding assay, PPD was the stronger inhibitor compared with Rh_2. This observation supports the

idea that the interaction of these study compounds with VGSCs as revealed by binding experiments is likely relevant to the direct functional blockade of the sodium channel complex.

The concentration-response curves demonstrate that PPD and Rh_2 inhibit the VTD-triggered VGSC opening with calculated IC_{50} values of 48 and 100 μM respectively. These results are generally in good agreement with our binding data although our IC_{50}s are somewhat higher than that observed for the protopanaxadiol type ginsenoside Rg_3. In experiments on wild-type Na^+ channels expressed in oocytes, Rg_3 induced voltage-, dose- and use-dependent inhibition of peak sodium current with an IC_{50} value of 32 ± 6 μM. Rg_3 treatment also produced a 11.2 ± 3.5 mV depolarizing shift in the activation voltage, but did not alter the steady-state inactivation voltage (Lee *et al.*, 2005).

The second piece of evidence supporting the notion that PPD and Rh_2 interfere with VGSC function came from experiments designed to investigate the effects of these compounds on chemically-evoked release of amino acid neurotransmitters (L-aspartate, L-glutamate and GABA) from mouse brain synaptosomes. My results demonstrate that at generally low micromolar concentrations, PPD and its glucoside Rh_2 readily inhibit VTD-evoked (TTX-suppressible) release of amino acid neurotransmitters from nerve terminal preparations in a concentration-related fashion. However, Rh_2 (in contrast to PPD), was unable to achieve full suppression of veratridine-evoked release of GABA and L-glutamate at high concentrations. In experiments designed to follow this up, a maximal inhibitory concentration of Rh_2 (300 μM) was found to also induce significant "release" of transmitters from synaptosomes. While this may represent true Rh_2-induced efflux

elicited via either a physiological or non-physiological (e.g. membrane disrupting) mechanism, it is also possible that this ginseng glycoside increases extracellular accumulation of transmitters by inhibiting amino acid neurotransmitter reuptake (Tsang *et al.*, 1985) or endocytosis. Further studies would be required to clarify the mechanism. However, my data suggests the apparent inability of high micromolar concentrations of Rh_2 to fully inhibit VTD-evoked release of amino acid neurotransmitters from synaptosomes may be because at these concentrations, Rh_2 stimulates efflux or blocks influx of L-glutamate and GABA in addition to its sodium channel-blocking effects. It is noteworthy that certain ginsenosides have been reported to elevate the release of excitatory amino acid transmitters in the brain. According to Hao *et al.* (2007), daily oral administration of pseudoginsenoside F_{11} (at 4 and 8 mg/kg per day for 7 days) antagonized the behavioral sensitization caused by morphine and significantly reversed the morphine-induced decrease in release of L-glutamate from mouse medial prefrontal cortex.

The actions of ginseng saponins and their metabolites on neurotransmitter receptors, for instance at NMDA receptors (Jang *et al.*, 2004; Kim *et al.*, 2002), 5-HT_{3A} receptors (Choi *et al.*, 2003; Lee *et al.*, 2007), $GABA_A$ receptors (Jang *et al.*, 2004; Yuan *et al.*, 1998) and nicotinic acetylcholine receptors (Tachikawa *et al.*, 2003, 2001), represent mechanisms by which these natural products potentially elicit depressant effects on the nervous system. However, my experimental data suggest that PPD and Rh_2 are certainly capable of exerting significant depressant effects in the nervous system through inhibition of sodium channels and by attenuating sodium channel-dependent release of neurotransmitters. Voltage-gated sodium channels are arguably one of the more

important ion channels mediating excitation in the brain. The pharmacological profile I observed with PPD and Rh_2 in the [^{3}H]BTX-B binding experiments and transmitter release assays parallels those of certain anesthetic and anticonvulsant drugs such as propofol and lamotrigine which target Na^+ channels and inhibit veratridine-induced release of amino acid neurotransmitters (Ratnakumari and Hemmings, 1997; Leach *et al.*, 1986). It is unlikely that the concentrations of PPD and Rh_2 needed to block sodium channels are achieved in CNS tissues after normal consumption of herbal preparations of ginseng. However, for a number of anticonvulsant drugs that are known to inhibit sodium channels, the clinically effective concentrations fall in the low to moderate micromolar range. For example, the therapeutic plasma concentrations of diphenylhydantoin and carbamazepine are 40-80 μM and 13-130 μM respectively (Lang *et al.*, 1993) whereas the concentrations of lamotrigine required for seizure suppression range from 1 to 40 μM Frizelle *et al.* (1999). Clearly, PPD which inhibits [^{3}H]BTX-B binding with an IC_{50} of 42 μM and, depending on the assay/neurotransmitter involved, achieves functional inhibition of sodium channels at IC_{50}s of 17-48 μM, may represent a useful prototype for the design of a novel therapeutic drug which depress sodium channel function. However, such an opportunity may not apply to Rh_2, which generally exhibits lower potency or efficacy in its interaction with sodium channels in these assays. Moreover, since I found Rh_2 stimulates release of L-glutamate and GABA at high concentrations, glucosides of this type may promote unacceptable side effects such as excitotoxic amino acid neurotransmitter release.

CHAPTER 4: CONCLUSION

My Master's research has shed light on the mechanism underlying the pharmacological interaction of PPD and its glucoside Rh_2 with voltage-gated sodium channels as well as clarified the influence of PPD and Rh_2 on downstream events occuring as a result of sodium channel activation. Specifically, my work demonstrates that PPD and Rh_2 act as effective inhibitors of [^{3}H]BTX-B binding to brain voltage-gated sodium channels through allosteric modification to neurotoxin binding site 2 and also that they reduce neural excitability and transmitter secretion from the nerve ending as a direct result of sodium channel inhibition. It is conceivable that these actions could be a common feature of other compounds present in ginseng that bear a close structural resemblance to PPD and Rh_2, and this would represent a useful area for future exploration. Moreover, given the close parallels in the effects on [^{3}H]BTX-B binding and inhibition of VGSC-dependent function between the study compounds and clinically effective anticonvulsant, anesthetic and neuroprotective agents, my findings could inspire the design of a new series of CNS depressant and neuroprotective drugs based on the PPD structure.

4.1 The interaction of ginseng bioactives with sodium channels

Apart from the insights offered by my [^{3}H]BTX-B binding experiments, there remain significant gaps in our knowledge regarding the way in which ginseng saponins access the sodium channel and then modify its function. A report has shown that dansyl

M1, a fluorescent analog of a metabolite of ginsenoside metabolite M1, accumulates in the cytosol of B16-BL6 mouse melanoma cells after a 15-min incubation (Wakabayashi *et al.*, 1998), indicating that M1 and possibly other ginsenoside metabolites can penetrate the plasma membrane and target the cytosolic side of channel proteins. A potential concern is that since the dansyl moiety is hydrophobic and adds significantly to the bulk of the transport properties of the fluorescent ginsenoside may be different from that of the natural compounds. However, another study suggests that compounds with amphipathic character (ginseng saponins are an eminent example of this), readily insert themselves into the lipid bilayer of the plasma membrane, where they can modify the elasticity of the lipid microenvironment surrounding the sodium channel protein and therefore affect the conformations this complex can adopt (Lundbæk *et al.*, 2005). In this context it is worth emphasizing that the allosteric mechanism I propose in this thesis for ginseng compounds reducing the binding of [^{3}H]BTX-B to site 2 could arise from two broad mechanisms. Firstly, the study compounds could bind to a distinct receptor region on the sodium channel complex, triggering a conformational change-encompassing site 2. Alternatively, the study compounds may intercalate with lipids in the immediate microenvironment of the sodium channel complex, changing lipid elasticity and causing a conformational change to envelop site 2. Either mechanism would be ultimately lead to a reduction in the number of binding sites available to [^{3}H]BTX-B a fundamental conclusion of my studies. Support for ginseng compounds binding directly to the sodium channel has been provided by the work of Lee and colleagues (2005). Using a two-microelectrode voltage-clamp technique, these researchers investigated the effect of Rg_3 on Na^+ currents in wild-type rat brain sodium channels expressed in the membrane of *Xenopus laevis* oocytes.

Their results showed that among different loci such as the channel entrance, the pore region, the tetrodotoxin binding site (neurotoxin binding site 1), the S4 voltage sensor, and the Ile-Phe-Met inactivation cluster, Rg_3 demonstrated specific interactions with the S4 voltage-sensor segment of domain II. However, it was acknowledged that the structural variation among different ginsenosides may lead to diversity in their binding loci. In the binding studies I report here, Rh_2 reduced the affinity of radioligand for site 2 whereas PPD was ineffective. This result suggests there is some difference in the mode of interaction of the study compounds with sodium channels which could possibly be explained by each compounds targeting non-identical (overlapping or separate) binding regions.

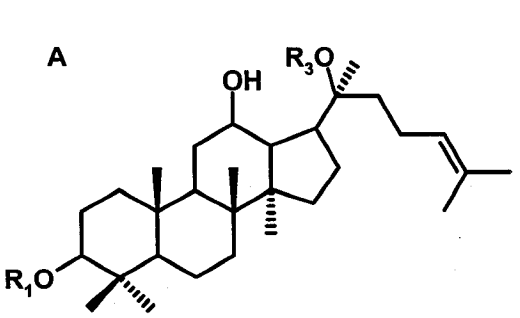
4.2 Potency differences between PPD and Rh_2

Another topic worth critical investigation is that the inhibitory potency on sodium channels between ginsenosides and their metabolites. Both in the [^{3}H]BTX-B binding assays (Duan *et al.,* 2006) and functional studies on the sodium channel-dependent depolarization, we observed a higher potency of PPD over Rh_2, (the glucoside derivative of PPD). Nevertheless, according to reports of Kim *et al.* (2005), Rg_3 posed a stronger inhibition on the inward sodium peak current than its aglycone, protopanaxadiol in experiments performed on the oocytes-expressed rat brain sodium channels. They suggested the importance of the carbohydrate portion in the inhibition of Na^+ channels by ginsenosides.

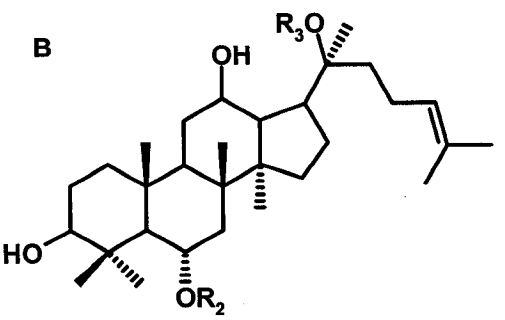
A possible explanation on this discrepancy could be that the lipid microenvironment of oocyte-expressed and native synaptoneurosomes sodium channels lead to sensitivity difference observed. The lipid-rich yolk mass of oocyte immediately

under the vitelline membrane could be preferentially sequestering lipophilic substances (Grissmer *et al.*, 1994). As a result, the proportion of protopanaxadiol available to the Na^+ channel target could be reduced compared to the more water soluble Rh_2.

Studies carried out by Hasegawa *et al.* (1996) and Karikura *et al.* (1991) indicated that the sugar moieties of the ginsenosides are subject extensive hydrolysis by gastric acid in the stomach and by the enzymes of bacteria in the large intestine following oral administration of ginseng. Another study revealed that the metabolites of ginseng saponin M1, M2, M3, M5, M12 (see Fig. 4.1) have stronger inhibitory effects on the ACh-evoked secretion of catecholamines from bovine chromaffin cells (IC_{50} from 15 to 38 μM) than their corresponding ginsenosides Rb_1, Rb_2, Rb_3, Rc, Rd and Rs (IC_{50}s > 100 μM) (Tachikawa *et al.,* 2003). These results are in good agreement with my [^{3}H]BTX-B binding and rhodamine 6G membrane potential assays. Ginseng saponins could possibly act as prodrugs (Tachikawa *et al.,* 2003) and their metabolites, which have less or no sugar moieties, function as the major active components of ginseng in modulating the nervous system, especially under circumstances of oral administration. The inhibitory effect of ginsenoside metabolites may therefore be important in mediating the pharmacological effects of ginseng saponins in the central nervous system.



Panaxadiol-type



Panaxatriol-type

	R_1	R_3
Ginsenosides		
Rb_1	-glc(2-1)glc	-glc(6-1)glc
Rb_2	-glc(2-1)glc	-glc(6-1)arap
Rb_3	-glc(2-1)glc	-glc(6-1)xyl
Rc	-glc(2-1)glc	-glc(6-1)araf
Rs	-glc(2-1)glc	-H
Rh_2	-glc	-H
Metabolites		
M1	-H	-glu
M2	-H	-glc(6-1)arap
M3	-H	-glc(6-1)araf
M5	-glu	-glu
M12	-H	-H

	R_1	R_3
Ginsenosides		
Re	-glc(2-1)rha	-glu
Rf	-glc(2-1)glc	-H
Metabolites		
M4	-H	-H
M11	-H	-glc

Figure 4.1. Comparison of ginsenosides and their related metabolites. **A**: panaxadiol type; **B**: panaxatriol type. glc, glucose; ara(p), arabinose in pyranose form; ara(f), arabinose in furanose form; xyl, xylose; rha, rhamnose. M12: 20(S)protopanaxadiol; M11: 20(S)protopanaxatriol. (Adapted from Tachikawa *et al.*, 2003).

4.3 Individual and collective actions of ginsenosides

Ginseng saponins are a large family of structurally related compounds. The diversity of ginseng natural products may help explain the multiple actions of ginseng, which includes modifying human behavior and congition, as well as regulating various other aspects of nervous system and immune function.

Many of the ginseng saponins have been shown to produce individual effects, while a single component may also synergize or antagonize the actions of others. Therefore it is prudent that future experimental designs account for potential effects of both individual constituents and mixtures.

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