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Mechanisms of Analgesic Action of Gln49-PLA_2 from *Gloydius ussurensis* Snake Venom

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Abstract Gln49-PLA_2 is a phospholipase A_2 isolated from the *Gloydius ussurensis* snake venom. In this paper, we studied its effect on the function of neural conduction. Electrophysiological studies demonstrated that Gln49-PLA_2 reduced the amplitude of the action potential and the velocity of nerve conduction on isolated mouse sciatic nerve. Patch clamp recordings confirmed that Gln49-PLA_2 significantly decreased neural excitability by the potentiation of sodium channels and the blockade of potassium channels in nerve terminal. In freshly isolated hippocampal pyramidal neurons, 54.25% of potassium current was inhibited by 20 μg/ml Gln49-PLA_2. However, sodium current was potentiated by 158.99% under the same condition. These findings demonstrate that the effect of Gln49-PLA_2 on ion channels is the main mechanism of analgesic action.

Keywords Analgesic action · Gln49 phospholipase A_2 · Neurotoxin · Patch clamp · Ion channel · *Gloydius ussurensis*

Abbreviations

PLA_2	phospholipase A_2
Gln49-PLA_2	phospholipase A_2 with Gln at position 49 from *Gloydius ussurensis*
HEPES	*N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid

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Introduction

Gloydius ussurensis (popular name, Manushi) is a member of the *Agkistrodon blomhoffii ussurensis* Emelianof (Crotalinae: Viperidae) that is distributed mainly over northeast China, Korea, and parts of Russia. PLA_2 from the venom of this species of snake constitute a puzzling group of molecules. Although these phospholipase A_2 have a similar three-dimensional structure and highly conserved molecular domains, they display a plethora of different pharmacological activities, such as myotoxic, neurotoxic, anticoagulant, hypotensive, hemolytic, platelet aggregation inhibiting, bactericidal, and pro-inflammatory [1], but only a few papers about PLA_2 neurotoxin character and mechanism of action were reported [2].

We have purified and characterized a basic PLA_2 homologue from *G. ussurensis* snake venom. Amino acid sequence analysis and molecular modeling showed that the protein was a new phospholipase A_2, named Gln49-PLA_2 with high homology to Asp49-PLA_2 such as PA21 AGKHA (similarity 93%) from *Agkistrodon halys blomhoffi* [3]. The toxic activity of G1n49-PLA_2 was weak, and the LD_{50} was 18.2 mg/kg. Hot-plate tests showed that its analgesic activity was dose-dependent [4]. In this paper, we characterized the mechanism of neurotoxity of G1n49-PLA_2 by analyzing its effects on mouse sciatic nerve preparation and on the modulation of voltage-dependent ion channel in rat hippocampal neuron.

Materials and Methods

Animals

Adult male Kunming white mice (28–35 g) and Sprague–Dawley rats aged 7 to 10 days were provided by the Animal House in the Department of Animal Care Center at Dalian Medical University. The animals were housed at 24 °C with access to food and water ad libitum. All experimental procedures were conducted in accordance with institutional guidelines for the care and use of laboratory animals in Dalian Medical University (Dalian, China) and the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85–23, revised 1985).

Purification of Gln49-PLA_2

Purification of Gln49-PLA_2 from *G. ussurensis* snake venom was carried out according to the methods described previously [3].

Measurement of the Action Potential and Nerve Conducting Velocity

A 5–7-cm-long sciatic nerve, including the proximal and distal regions, was dissected and mounted in a recording chamber as described by Paparounas et al. [5]. The action potential and nerve conduction velocity were measured every 10 min from 0 to 120 min. An untreated sciatic nerve was used as a control. All experiments were carried out with a biophysical signal collection and management system RM6420BD.

The Effect of Gln49-PLA_2 on Ion Channel in Hippocampal Neurons

Solutions for Patch Clamp

Incubation solution consisted of (in mM): NaCl 150, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2, $NaHCO_3$ 26, and glucose 10, pH 7.4. Intracellular solution A comprised (in mM) K-gluconate 100, $MgCl_2$ 2, EGTA 1, HEPES 5, and sucrose to 320 mOsm, pH 7.2, adjusted with KOH for recording K^+ currents, while intracellular solution B comprised (in mM) CsF 140, EGTA 1, NaCl 10, and HEPES 10, pH 7.3, adjusted with CsOH for Na^+ currents. External solution for measuring K^+ currents consisted of (mM): NaCl 140, KCl 2.5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 20, glucose 10, and sucrose 23.5, pH 7.2, adjusted with NaOH, while external solution for recoding sodium current consisted of (in mM): NaCl 43.3, KCl 7.5, $MgCl_2$ 2.12, $CaCl_2$ 2.1, HEPES 10, tetraethylammonium chloride 165.7, 4-aminopyridine 0.5, CsCl 168.4, and $CdCl_2$ 0.02, adjusted to pH 7.2 with NaOH. Each solution was filtered in membrane (0.22 μm), and intracellular solutions were stored at −20 °C. All experiments were performed at room temperature (20–25 °C).

Isolation of Hippocampal Neurons

Single hippocampal pyramidal neuron was isolated from the brain of Sprague–Dawley rats aged 7 to 10 days as described previously [6] with slight modification. In brief, after animals were killed by cervical dislocation, the brain was rapidly dissected out, and the hippocampus were removed and sliced manually with thickness of about 400–600 μm. The slices were incubated in incubation solution bubbled with 100% O_2 at 32 °C for 90 min, then treated with pronase E (1 mg/ml) for 30 min. The enzymes were washed out with the same solution. The hippocampal neurons were mechanically dissociated in external solution using a fire-polished Pasteur pipette with a tip of 100–400 μm in diameter.

Whole-Cell Patch Clamp Recordings

Voltage-clamp recordings were performed using whole-cell configuration of patch clamp technique. Patch-clamp recordings were made using electrodes with a resistance of 3–6 MΩ. All electrodes were fabricated from filamented borosilicate glass (GC150-F10, Clark Electromedical Instruments, Reading, UK) using a micropipette puller (Model P-97, Sutter Instrument, Novato, CA, USA) and were filled with intracellular solution. Electrophysiological recordings were made using an EPC10 amplifier (HEKA Electronik, Lambrecht, Germany), controlled by Pulse software (version 8.78 HEKA) running on a Lenovo computer. Drug-containing solutions were applied by pressure injection with a micro-injector (IM-5B, Narishige, Olympus) through a micropipette (with a tip of 30–50 μm in diameter) placed at a distance of 80–100 μm from the cell. Data were sampled between 4 and 24 kHz after being filtered at one third of sampling frequency. Terminal series resistance (14–30 MΩ) was monitored and compensated (69–95%) throughout the recordings. Data with unstable series resistance were excluded from subsequent analysis. Except indicated, the currents were leak-subtracted online using a p/4 subtraction routine. Data analysis was done using Pulsefit (version 8.05, HEKA), Igor (version 2.04, Wavemetrics), and Origin software. The data are presented as mean values, where n=number of cells.

Statistical Analysis

Paired Student's *t* test was used to compare agonist responses before and after treatment in the same preparation. A one-way analysis of variance was performed for multiple comparisons at the time points indicated. Data are expressed as mean±SEM with statistical significance whenever $p<0.05$.

Results

The Action Potential and Nerve Conduction Velocity

We studied the effect of G1n49-PLA_2 on action potentials and conduction velocity in mouse sciatic nerve. As shown in Fig. 1, 100 and 90 μg/ml Gln49-PLA2 caused a rapid reduction in the amplitude of the potentials and conduction velocity with a time constant of 40 min. A significant decrease in the amplitude was observed after 20 min ($n=5$, $p<0.05$ compared to the controls, Fig. 1a). At a lower concentration (50 μg/ml) of G1n49-PLA_2, a slight increase in action potential occurred between 10 to 40 min and then decreased after

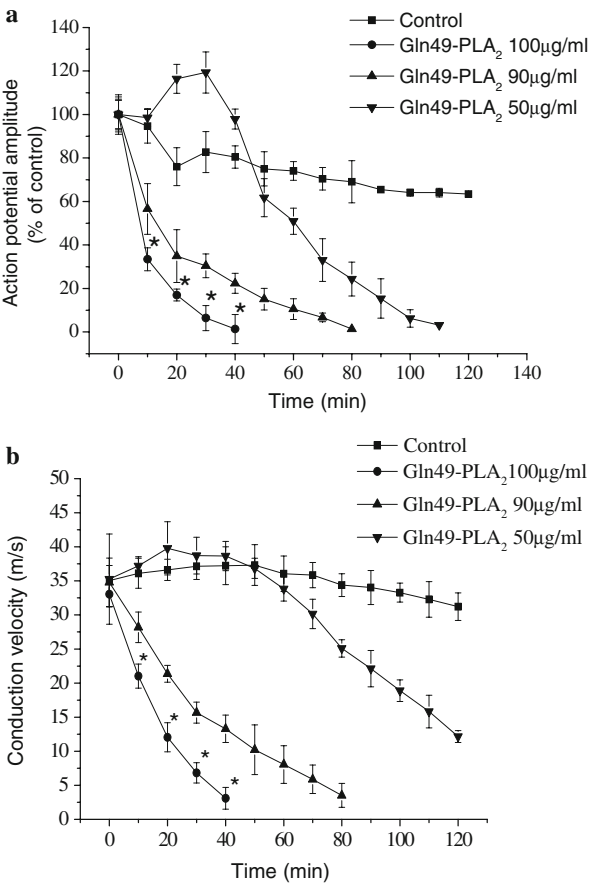

Fig. 1 Changes of action potentials and nerve conduction velocities in mouse sciatic nerve. **a** Changes of action potentials. **b** Changes of nerve conduction velocities. Data shows the mean±SEM of three experiments for each test. *$p<0.05$ for G1n49-PLA_2 (100 μg/ml) compared to time 0

40 min. The control produced no significant changes during the entire 120 min (Fig. 1a). Our data indicate that G1n49-PLA_2 caused concentration and time-dependent decrease in amplitude. Similar effect of G1n49-PLA_2 on sciatic-nerve conduction velocity was observed (Fig. 1b).

The Effect of G1n49-PLA_2 on Ion Channel

Figure 2 shows whole-cell potassium currents from hippocampal pyramidal neurons, recorded during voltage-clamp steps applied from a holding potential of −70 mV. The peak potassium currents were changed significantly by 20 μg/ml G1n49-PLA_2 blockade (Fig. 2A). The potassium currents with an amplitude of about 1–2 nA were observed (Fig. 2B) and were activated at potentials more positive than −40 mV (Fig. 2C). Using this protocol, G1n49-PLA_2 produced a concentration-independent blockade of 54.25% of the current at 20 μg/ml (Fig. 2C) during 5 min of application.

Figure 3a shows the whole-cell sodium currents from hippocampal neurons in the absence and presence of G1n49-PLA_2 (20 μg/ml, n=5), recorded during voltage-clamp

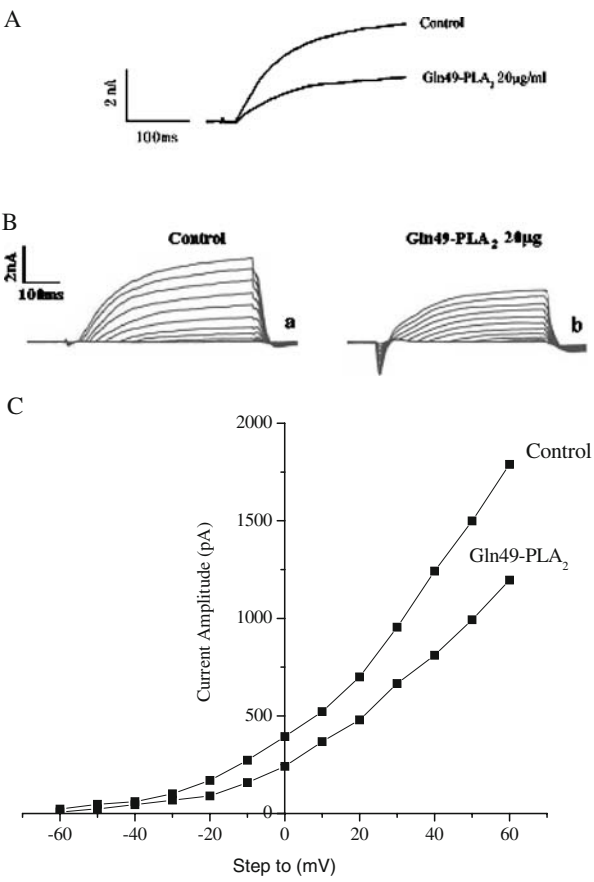

Fig. 2 Whole-cell potassium currents recorded from hippocampal pyramidal neurons. **A** Typical blockade of peak potassium currents by 20 μg/ml G1n49-PLA_2. **B** Typical family of potassium currents evoked by 10 mV depolarizations from −70 to 60 mV, *a* before, *b* 5 min after toxin applied. **C** The typical transfer of charge (I–V) in control preparations and after 5 min of exposure to G1n49-PLA_2 (20 μg/ml, n=5). *p<0.05 compared to the controls

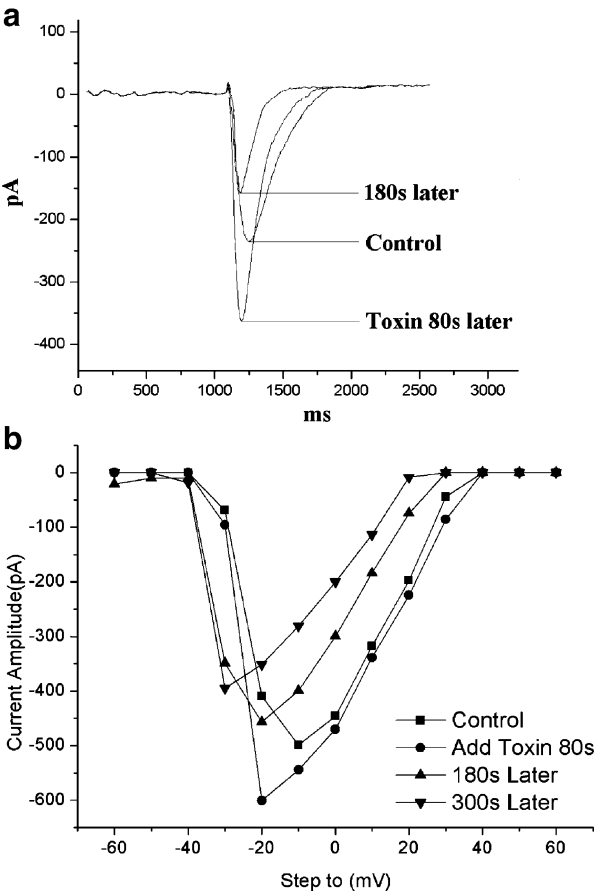

Fig. 3 G1n49-PLA_2 effect on the voltage-dependent Na^+ current of Hippocampal pyramidal neurons. **a** Typical increasing of peak sodium currents by 20 μg/ml G1n49-PLA_2 after 80 s but subsequently following a decreasing current 5 min later. **b** I–V curve of the sodium current was deviated from beginning that reflects the effect of toxin on voltage-dependent Na^+ channel's activity (20 μg/ml, $n=5$). *$p<0.05$ compared to the controls

steps applied from a holding potential of −70 mV. The current with amplitude of about 1.5–2.5 nA was observed at 60 mV from a holding potential of −70 mV. G1n49-PLA_2 significantly potentiated sodium current (an increase of 158.99%) within 2 min after application (Fig. 3a). However, with the decreasing potassium current, the sodium currents also decreased to 72.78% from initials. Figure 3b shows that I–V curves of sodium currents recorded in the presence of toxin shifted to more hyperpolarized voltage compared to that of the control, suggesting that G1n49-PLA_2 enhanced the excitability of sodium channel in the neurons.

Discussions

G1n49-PLA_2 from *G. ussurensis* is a monomeric toxin and new type II secretory PLA_2 ($sPLA_2$) that has high homology with Asp49-PLA_2 and Lys49-PLA_2. However, despite having greater sequence similarity to Asp49-PLA_2 than Lys49-PLA_2, Gln49-PLA2 is more similar to Lys49-PLA_2 by having no detectable hydrolytic activity. Remarkably, the biological activities of $sPLA_2$ are mediated by interaction with PLA_2 receptors that are dependent on a tandem CRD-like domain in the receptor [7]. Molecular analysis of

pancreatic $sPLA_2$ has shown that the calcium-binding loop and catalytic sites are critical regions for molecular recognition of these membrane receptors [8], and these sites are probably also important. However, G1n49-PLA_2 does not have the calcium-binding loop, and this may explain the low toxicity of G1n49-PLA_2 (LD_{50}, 18.2 mg/kg in 615 mice).

Neuromuscular blockade in vertebrate nerve–muscle preparations by PLA_2 might be associated with the blockade of certain types of nerve terminal channels [9, 10]. A direct effect of G1n49-PLA_2 on ion channels of hippocampal pyramidal neurons was demonstrated by its ability to reduce the currents of the K^+ channels while decreasing the currents of Na^+ channels. The blocking of nerve terminal K^+ channels would delay membrane repolarization after the nerve action potential. Instantaneously, excessive opening of Na^+ channels would perpetuate the depolarization that would exhaust neuron's excitability, and with time, a reduction of the outward potassium currents might favor the inactivation of sodium channels by prolonging the period of depolarization of the cell. This phenomenon causes a general decrease or disappears of neuronal excitability by increasing the percentage of the Na^+ channels in the inactivated state. As result, the neuromuscular transmission will gradually be blocked by the action of G1n49-PLA_2.

In conclusion, G1n49-PLA_2, a Gln49 basic PLA_2 from *G. ussurensis* venom markedly affected the function of voltage-dependent ion channels, blocking neuronal signal transduction by the potentiation of sodium channels and the blockade of potassium channels in nerve terminal. This is the main mechanism of analgesic action of Gln49-PLA_2. Further investigations are needed to determine the effects of G1n49-PLA_2 site-directed mutant on the function of ion channels and to reveal the relationship between enzymatic activity and neurotoxic activity of snake venom phospholipase A_2.

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