ELECTROPHYSIOLOGICAL STUDIES OF *NEPHILA CLAVATA* VENOM AND TOXIN INFLUENCE ON HIPPOCAMPAL NEURONAL MEMBRANES AFTER PRETREATMENT WITH *ASPERGILLUS ORYZAE* PROTEASES

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**Background.** The use of Arthropodae toxins for electrophysiological experiments is very important, but experimental data in this regard are scarce. The aim of this study was to obtain experimental data about the influence of *Nephila clavata* spider venom and its main active component – toxin JSTX-3 on the glutamate channel-receptor complex.

**Methods.** Kainate was used as agonist of glutamate channel-receptor complex because it initiated non-inactivated transmembrane electric currents in rat hippocampal membranes in electrophysiological experiments were used for the study. All chemicals were applied to perfused hippocampal pyramidal neuronal membranes using 'concentration-clamp' technique and voltage-clamp recording.

**Results and discussion.** The studied substances – integral venom and toxin JSTX-3 – demonstrated the properties of glutamate channel-receptor complex antagonists. The amplitudes of electric transmembrane currents activated by glutamate, kainate, and quisqualate decreased (sometimes to zero) after the application of glutamate channel-receptor complex antagonists to the rat hippocampal membrane under the voltage-clamp conditions. The kinetics of activation and desensitization (in case of glutamate and quisqualate) of transmembrane electric currents were not affected by these antagonists.

The effects of *Nephila clavata* integral venom were studied in concentrations of $10^{-8} - 10^{-4}$ units/µL, the effects of JSTX-3 – in the concentrations of $10^{-6} - 10^{-5}$ mol/L. Integral venom did not block the studied currents completely, but it reduced their amplitudes to a certain level. Integral venom blocked glutamate-activated currents up to 36±15 % of the initial values, kainate-activated – up to 34±16 %. In contrast, JSTX-3 almost completely blocked ion currents activated by these agonists at the holding potential of -100 mV:
the amplitudes of kainate-activated currents under the action of this blocker decreased to 6±3 % of the initial values. Integral venom blocking effects were irreversible in contrast to partially reversible JSTX-3 action. The differences between antagonists were also revealed in the quantitative characteristics of blocking action.

The following effects were studied under the antagonists’ influence: the degree of currents suppression and their removal by “washing”, “dose–effect” dependency, the antagonists’ influence on activated and inactivated receptors; kinetics of the antagonists’ action and removal, dissociation constants for blockers with reversible action.

**Conclusions** about the mechanisms of the antagonists’ influence on the glutamate channel-receptor complex, the physiological role of integral venom and JSTX-3 as well as comparison of the caused effects were made. The influence of *Aspergillus oryzae* proteases pretreatment during hippocampal neurons preparation for experiments, and the creation of a physical model of the molecular system “glutamate channel-receptor complex – glutamate – antagonist – proteases” are discussed.

**Keywords:** Araneidae, venom, toxin, glutamate receptor antagonists, transmembrane electric current

**INTRODUCTION**

The excitatory action of glutamate in the mammalian brain and spinal cord has been known since the 1950s (Curtis and Watkins 1960, Hayashi 1952). However, only since late 1970s, has glutamate become widely recognized as the principal excitatory transmitter within the vertebrate nervous system. It was proposed that glutamate acts postsynaptically on three families of ionotropic receptors named after their preferred agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate. Therefore, the complete deciphering of all mechanisms of glutamatergic transmission has both theoretical and practical importance.

Pharmacological modifying of glutamate receptors by the arthropods’ toxins is an important direction of studying glutamate neurotransmission mechanisms [22]. It is known that Arthropods’ venoms contain a mixture of various biologically active substances including toxins that are their physiologically important components [6, 22, 35]. These toxins are used successfully in the contemporary electrophysiology [4, 6, 7, 9, 15, 26, 27–35].

In this article, the data of electrophysiological studies of glutamate receptors’ antagonists from *Nephila clavata* spiders (Joro spiders) – JSTX-V (integral venom) and toxin JSTX-3 (its main active component) are presented. JSTX-3 is a phenol derivative with the polyamine chain and its chemical structure was defined in [22]. In some publications, the effects of Araneidae toxins have been described [1–3, 5, 9, 12, 13, 16]. At present, arthropod toxins are used in pharmacology [8, 15, 18], agriculture [10], and for environmental monitoring [21]. Some of them are used in newly developed methods [20, 21, 23]. It is essential to study glutamate channel-receptor complex (gCRC) antagonists of *N. clavata* because the glutamatergic type of signals transmission in the nervous system is widely spread; it was discovered in many organisms, even phylogenetically distant ones. Irreversibility of JSTX-V action is a characteristic feature of this venom. This effect is biologically important for spiders-hunters who have to immobilize insects (their victims) quickly, acting specifically on gCRC in the central nervous system, neuro-muscular and other systems. The idea of using Araneidae toxins as “universal
gCRC markers in different species attracted attention of many researchers [22]. Spider toxin JSTX-3 from *N. clavata* played the leading role in search of a “universal” gCRC marker [1, 22].

We elaborated effective methods, for example, we used glutamate receptors’ agonist kainate (KK, sodium salt of kainic acid) that initiated steady-state inward ion transmembrane electric currents in rat hippocampal membranes for our experiments. At the background of KK-activated currents, the kinetics of *N. clavata* antagonists’ actions was well visualized [1]. Another original method consisted in using *A. oryzae* proteases for neuronal membranes enzymatic pretreatment [20, 23, 24]. This method allowed us to amplify chemo-activated electrical currents through gCRC to decrease the noises in the experimental electrical system so that successful registration of antagonists’ effects could be possible. The patents were obtained for some of these methods and results [20, 23, 24]. Finally, the developed process of enzymatic pretreatment allowed elaborating a physical model – the molecular system “gCRC – glutamate – antagonist – proteases” that provided for successful revealing of gCRC-antagonists’ effects. In this article, we have characterized some electrophysiological effects caused by *N. clavata* venom and JSTX-3 toxin.

**MATERIALS AND METHODS**

The study of the venom from *Nephila clavata* and its main active component toxin JSTX-3, as well as their influences on chemo-activated transmembrane currents was carried out using the electrophysiological and linked methods [1, 19, 21, 23]. The development of these methods was based on the methods previously elaborated by teams of scientists under the guidance of academicians P. G. Kostyuk and O.O. Krishtal, and by the research group of Drs. A.Ya. Tsyndrenko, M.I. Kiskin, O.M. Klyuchko.

**Object preparation, solutions and reagents.** Experiments were performed on internally perfused rat hippocampal pyramidal neurons of newborn as well as up to 3-weeks-old rats. All manipulations with animals were carried out in accordance with the International Convention for the protection of animals and the Law of Ukraine “On protection of animals from cruelty”, Protocol N 2 (October 20, 2016) of the meeting of the Bioethics Committee of the Educational and Scientific Centre “Institute of Biology and Medicine”, Taras Shevchenko National University of Kyiv. Hippocampal slices were treated enzymatically with a subsequent mechanical isolation of single pyramidal neurons [20, 24]. The cell culture was prepared from the dissociated pyramidal neurons of newborn rats. Microphotographs of the isolated pyramidal neurons and these cells in culture condition (4–5 days of survival) are shown in Fig. 1A, B. Experiments and object preparation procedures were carried out using the solutions described below (all concentrations are given in mmol/L).

**Solution A.** KF = 100, Tris-Cl = 30, pH = 7.2. Solution A was used as an intracellular one; its composition was not changed during the experiments.

**Solution B.** NaCl = 156, MgCl$_2$ = 1.1, Hepes-NaOH = 20, CaCl$_2$ = 2.6, pH = 7.4.

Solution B was used as an extracellular one during the experiments. The following substances were added to it in the course of the experiments: a) L-glutamate (Glu), kainate (KK), quisqualate (Ql), glycine, γ-aminobutyric acid (GABA); b) integral venom JSTX-V, its active component toxin JSTX-3; c) other substances.

Traditionally, JSTX-V quantities have been measured in units of action. One unit corresponded to the average amount of venom isolated from one spider gland. In our
experiments, 10 units of JSTX-V were used. This amount of venom was dissolved to 0.5 units/µL; further the solutions with the necessary concentrations were prepared. JSTX-3 concentrations were expressed in moles per liter (mol/L). According to the estimations, one unit/L of venom corresponds to one mol/L of the purified toxin JSTX-3. This result is similar in its order to those obtained on the basis of our experimental results (see below). Both gCRC antagonists – the integral venom JSTX-V and its main active component toxin JSTX-3 were kindly presented by Prof. N. Akaike (Tokyo University, Japan) for the collaborative use.

In addition to the above solutions, other ones of the following composition were used in the preparation of the object for the experiment. For rat hippocampal neurons dissociation the following solutions were used:

**Solution C.** NaCl = 150, KCl = 4, Hapes-NaOH = 20, Glucose = 10, pH = 7.4.

**Solution D.** NaCl = 150, KCl = 4, NaHCO$_3$ = 26, CaCl$_2$ = 0.9, EGTA = 1.0, Glucose = 10. In solution D, the concentration of free calcium was 5·10$^{-7}$ mmol/L. This solution was saturated with carbogen (5% CO$_2$, 95% O$_2$) to pH = 7.4. Dry media MEM and DMEM (Serva) and poly-L-lysine (Sigma) were used for the cell culture. For enzymatic tissue processing, the complex of proteolytic enzymes from *Aspergillus oryzae* produced by “Chemreactive”, Olaine (Latvia) was used as well as enzymes trypsin PM-14 (Serva), pronase E (Serva), collagenase type IV (Sigma) [20, 24].

**Fig. 1.** Micrographs of isolated rat hippocampal neurons (A) and hippocampal neurons in culture conditions (B, 4–5 days culture) (10 µm in 1 cm)

Рис. 1. Мікрофотографії ізольованих нейронів гіпокампа щура (A) та нейрони гіпокампа в умовах культиру (B, культура 4–5 днів) (в 1 см – 10 мкм)

*Modified procedures of the enzymatic treatment and subsequent preparation of hippocampal neurons for the electrophysiological experiment.* Mammalian brain cells, as well as hippocampal pyramidal neurons, are very “delicate” and can be easily damaged. They are very sensitive to temperature changes, saturation of extracellular solutions by oxygen and other gases, even to slight deviations in ion composition in solutions, etc. Using the existing methods of cells isolation and dissociation [1], we have developed new methods aimed to cause the least possible damage to non-NMDA receptors [20, 23, 24]. Three main factors in extracellular solutions defined the development of the enzymatic treatment procedure: enzymes and bivalent cations Ca$^{2+}$ and Mg$^{2+}$.

After rat decapitation, hippocampus was taken away and moved to solution C where cross slices of hippocampus from 300 to 400 µm thick were made and placed into the solution of enzymes prepared on the basis of solution D. The traditional optimal enzyme complex for these purposes – a complex of pronase (0.3%) with collagenase (0.1%) (prototype method) was substituted by the complex of proteolytic enzymes from
Aspergillus oryzae (0.1% – 0.8% solution). The enzymatic treatment was carried out at 37 °C for 1–2 h. Carbogen was passed constantly through the solution. Further, the enzymes were removed using a specific procedure and enzymes residues were inactivated. After that, the isolated neurons were obtained under the microscopic control by repeatedly passing the obtained brain slices in the solution with adding of 1.25 mmol/L CaCl₂ and 0.55 mmol/L MgCl₂ through the glass micropipette with a pore diameter of about 100 µm. The obtained suspension of neurons was added to the Eagle minimal medium (MEM), supplemented with of 5% bovine serum (other types of sera were used as well). The concentration of bivalent cations Ca²⁺ and Mg²⁺ in extracellular solutions was gradually increased to the normal level. Some stages of these methods are supported by the patents [20, 23, 24]. After dissociation, living neurons could remain for 3–4 h without noticeable changes in their morphological characteristics. Some of these slices were left in the solution to remove the enzymes by “washing” with a constant passage of carbogen. These slices could be used to obtain isolated cells for 6–8 h. The dissociated living pyramidal neurons in the extracellular solution had a characteristic pyramidal shape: elongated soma with preserved apical and basal dendrites of the second and third orders. The diameter of such cells was about 15–20 µm, and the length was about 20–40 µm (Fig. 1A). Neurons obtained using such procedures survived in culture conditions for 42–45 days (Fig. 1B). The experiments on cultured neurons were used for control studies.

Peculiarities of the enzymatic pre-treatment. The enzymatic treatment by proteases selected from Aspergillus oryzae demonstrated the best results. In the electrophysiological experiments, the amplitudes of Glu- and KK-activated ionic currents were 11.8 times higher after the influence of A. oryzae proteases than after pronase and collagenase pretreatment [20, 24]. Other standard methods of the enzymatic treatment demonstrated worse results. It is known that proteases are present in spiders' venoms in large quantities [6, 22, 35]. During the bite, along with its toxins a spider introduces a digestive secret with proteases and a significant number of free amino acids, mainly glutamate, into the victim’s body [22]. These substances were also represented in our experiments with the registration of Glu-, KK-activated ionic currents, forming molecular system “gCRC – glutamate – antagonist – proteases”. The role of this molecular system in our experiments will be discussed below.

Electrophysiological experiments. Experimental studies of Glu- and KK-activated currents in voltage-camp conditions were previously discussed [1, 19, 23]. Isolated neurons or a glass with cultured neurons were placed into the experimental chamber of electrophysiological setup. Experiments were carried out using the solutions described below as solutions A, B, C, D. All experiments were performed at room temperature (21–23 °C). All chemicals were applied to internally perfused hippocampal pyramidal neurons using a ’concentration-clamp’ technique [1] by which an external solution can be changed within a few ms to 20 ms in a step-wise manner [1, 19, 23]. An electrical circuit was used for single-electrode voltage-clamp recording [1]. Both the current and voltage were monitored and the data were stored in a computer for a detailed off-line analysis; all experiments were carried out under computer control. The scheme of the experimental setup that was used for electrophysiological study of transmembrane ion currents in voltage-clamp mode was previously described [1, 19, 23]. When L-glutamate was applied to an isolated pyramidal neuron, internally perfused and clamped to
-100 mV, a transient inward current was elicited (Fig. 2). Standard package MATLAB was used for mathematic processing of the obtained experimental data. The following effects were studied under the influence of the antagonists: degrees of currents suppression and blockers removal by “washing”, “dose–effect” dependencies, antagonists’ influences on the activated and inactivated receptors; the kinetics of the antagonists’ action and removal as well as the analysis of dissociation constants (for blockers with reversible action) and Hill plot, etc. The details of the described methods are supported by the patents [20, 23, 24].

RESULTS AND DISCUSSION

The influence of the integral venom JSTX-V and toxin JSTX-3 from Nephila clavata on chemo-activated transmembrane electric currents in rat hippocampal membrane. Chemo-activated transmembrane electric currents in rat hippocampal neuronal membranes and the influence exerted on them by the antagonists from N. clavata (the integral venom JSTX-3 and its main active component toxin JSTX-3) were studied in the electrophysiological experiments. JSTX-3 is the derivative of phenol coupled with a polyamine group [22]. Approximately 300 electrically excitable neurons were studied in our experiments. Transmembrane ionic currents – inward TTX-sensitive sodium currents and outward potassium currents – were initiated by membrane depolarizing from -100 mV to -30 mV. Chemosensitive ionic currents were activated at holding potentials from -100 mV to +20 mV by application of such agonists as L-glutamate (Glu), kainate (KK) and quisqualate (QL) (Fig. 2). These agonists activated inward ionic electrical currents with two different kinetics: desensitized Glu- and QL-activated currents and steady-state non-desensitized KK-activated currents. After the application of Glu and QL followed by a rapid (about 10 ms) activation of currents to the maximum, a decline in the amplitudes was registered due to the desensitization of the receptors. KK application initiated inward non-desensitized currents; their amplitudes increased to the stationary levels, which were maintained. It was convenient to register effects of receptor antagonists’ action at the background of such stationary currents [1, 21]. According to the obtained data, all these agonists (Glu, KK, QL) activated the same membrane receptor system [1, 19]. The prepared hippocampal neurons (freshly dissociated and taken from the culture) were sensitive to the inhibitory mediators – glycine and γ-butyric acid (GABA). Neither of the tested gCRC blockers (JSTX-V, JSTX-3) initiated chemoactivated currents by themselves or changed the characteristics of electrically excitable sodium input and potassium output currents [1, 19].

Integral JSTX-V venom and JSTX-3 toxin – irreversible antagonists of gCRC of non-NMDA type. Integral JSTX-V venom and its main active component JSTX-3 toxin – gCRC antagonists from the spiders Nephila clavata were studied in as described below. Substances for these experiments were used from both natural resources and synthetic JSTX-3. The studied substances from N. clavata decreased the amplitudes of Glu-, KK- and QL-activated ion currents (Fig. 2). Effects of JSTX-V and JSTX-3 were studied at $10^{-8}$–$10^{-4}$ units/µL and $10^{-6}$–$10^{-5}$ mol/L concentrations, respectively. The application of higher concentrations of the antagonists caused cell death. After the application of lower concentrations of the antagonists the blocking process slowed down and nearly stopped. These natural limits have determined the working concentration of antagonists used in the experiments.
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Both integral venoms, JSTX-V and JSTX-3, blocked Glu-, KK- and QL-activated currents. The degree of currents blocking was different for each substance. JSTX-V never blocked currents completely, but reduced their amplitude to a new level. It blocked the glutamate responses up to 36±15 % of the initial values (the number of cells in the experiment was n = 7), and KK-activated ones – up to 34±16 % (n = 25) (Figs 2–5). In contrast, JSTX-3 blocked ion currents activated by these agonists almost completely at the holding potential of -100 mV. Under the action of this blocker, the amplitude of KK-activated current decreased to 6±3 % of the initial value (n = 9) (Fig. 3)

The JSTX-V blocking effects were irreversible. Even prolonged up to 20–30 min, the removal of the venom (“washing” of the venom) with both a normal extracellular...
solution (Fig. 3) and one with the changed value of pH in interval (5.0–9.0) did not restore the decreased amplitudes of electrical responses. The irreversibility of blocking was also evidenced by the experimental results presented in Figs. 4A, B. After the blocking of KK-activated currents with JSTX-V in the concentration of $2.5 \times 10^{-5}$ units/µL, the amplitudes of the currents were not restored after 5 min of “washing” with a normal extracellular solution. The subsequent application of JSTX-V in a higher concentration of $2.5 \times 10^{-4}$ units/µL did not cause any additional decrease of KK-activated currents’ amplitudes. The maximal blockage of chemo-activated currents was achieved after the application of JSTX-V at minimal concentrations. Further increase of venom concentrations increased the “noise” of the stationary currents.

Like JSTX-V, JSTX-3 can be “washed” with a normal extracellular solution. In that case, the amplitudes of the Glu-, KK- and QL-activated ion currents were partially recovered, reaching on average about $39 \pm 17\%$ ($n = 9$) of the original value in the case of

**Fig. 3.** Different types of blocking of KK-activated currents by venom JSTX-V and toxin JSTX-3: A – JSTX-V reduced the amplitude of the control response by about 2/3 of the initial value; the amplitude was not restored after 6 min of “washing” with Ringer’s solution; B – JSTX-3 blocked the response almost completely. The response amplitude was partially restored after 15 secs of toxin “washing” with Ringer’s solution (2) to a new level (3). Time calibration 1, 2, 3 is different. Concentration: KK – 1 mmol/L; JSTX-V – $2.5 \times 10^{-4}$ units/µL; JSTX-3 – $5.0 \times 10^{-5}$ mol/L. $V_{\text{hold}} = -100$ mV. Records A and B were obtained on two different neurons. Here and in the following figures, the arrows show the moments of beginning of blockers “washing”. **Comment:** KK – kainate

Рис. 3. Різний характер блокування KK-активованих струмів отрутою JSTX-V і токсином JSTX-3: A – JSTX-V зменшував амплітуду контрольної відповіді приблизно на 2/3 початкової величини, і амплітуда не відновлювалася після 6 хв відмивання розчином Рінгера; B – JSTX-3 блокував відповідь практично повністю, і амплітуда відновлювалася приблизно після 15 с відмивання токсину розчином Рінгера (2) до нового рівня (3). Калібрування часу 1, 2, 3 різне. Концентрації: KK – 1 ммоль/л; JSTX-V – $2.5 \times 10^{-4}$ од./мкл; JSTX-3 – $5.0 \times 10^{-5}$ моль/л. $V_{\text{hold}} = -100$ мВ. Записи A і B отримані на двох різних нейронах. Тут і на наступних рисунках стрілками показані моменти початку відмивання блокаторів. **Примітка:** KK – каїнат

Unlike JSTX-V, JSTX-3 can be “washed” with a normal extracellular solution. In that case, the amplitudes of the Glu-, KK- and QL-activated ion currents were partially recovered, reaching on average about $39 \pm 17\%$ ($n = 9$) of the original value in the case of
Fig. 4. Reversibility of the action of JSTX-V venom and JSTX-3 toxin: A, B – JSTX-V irreversibly blocked stationary KK-activated current in the concentration of $2.5 \times 10^{-5}$ units/µL (A) and a repeated application of a higher concentration of the venom of $2.5 \times 10^{-4}$ units/µL did not cause further blocking (B). Concentration: KK – 1 mmol/L, $V_{\text{hold}} = -100$ mV. The recordings were made on one cell with different amplifications; C, D – JSTX-3 effects were partially reversible. The amplitude of KK-activated current was partially restored by JSTX-3 "washing" with Ringer’s solution (A). The response could have been re-blocked by JSTX-3 (B) (see text). Concentration: KK – 1 mmol/L, JSTX-3 – $10^{-4}$ mol/L, $V_{\text{hold}} = -100$ mV.

Comment: KK – kainate
KK-activated currents (Fig. 3). In addition, JSTX-3 demonstrated a significantly irreversible effect, although its course slightly differed from that of JSTX-V. Figs. 4C, D demonstrate that with a nearly complete disappearance of KK-activated current under the influence of the antagonist after “washing” with a normal extracellular solution for 19 min the response was partially restored to approximately 39% of the original value. The reapplication of JSTX-3 after the “washing” caused almost complete blocking of KK-activated responses. After the second “washing” for 25 min (see Figs. 4C, D), the KK-activated response was not fully restored and constituted 30–50% of the value of the response that followed the first “washing”. After several consecutive applications, it was not possible to wash the toxin longer. In the process of venom or toxin removal, the degree of “washing” and its nature did not depend on whether this removal was carried out in Ringer’s solution or in the presence of the agonists (Glu, KK).

JSTX-V did not influence the kinetics of desensitization of Glu- and QL-activated ion currents.

Characteristics of JSTX-V and JSTX-3 action: potential dependence of action, binding to activated and non-activated form of receptor. The type of JSTX-V and JSTX-3 blocking depended on the holding transmembrane potential.

When JSTX-V influenced the neuronal membrane at the holding potential of -30 mV, the kinetics of KK-activated currents blocking decelerated. The degree of blocking decreased significantly in comparison with similar experiments at the holding potential of -100 mV (Fig. 5). The amplitudes of currents’ component that was not blocked by JSTX-V increased and constituted 50–80% of the initial values.

At the holding transmembrane potential equal to 0, in a number of cases (17%), a tendency to restore the Glu- and KK-activated responses after the JSTX-V action was registered. A slight recovery occurred when the JSTX-V was washed with a normal extracellular solution for 3-10 min.

In similar experiments with JSTX-3, the potential-dependence of KK-activated current was particularly noticeable: almost complete blocking at the holding potential of -100 mV became incomplete when the potential increased to -30 mV (Fig. 5C, D). The current amplitude after the “washing” of JSTX-3 at the holding potential of 0 mV also exceeded the amplitude of responses restored during the “washing” at -100 mV. Apparently, this phenomenon reflects a decrease in the effectiveness of venom and toxin action during membrane depolarization.

The obtained results indicate that both JSTX-V and JSTX-3 interact with both activated and non-activated forms of receptors. In Fig. 5C, D, one can see that after receiving the control KK-activated response and a subsequent “washing” of KK, 2.5·10^{-4} units/µL JSTX-V was applied to the cell membrane. A repeated KK application activated a current that corresponded to 34±16% of the control value, i.e. a component that had not undergone JSTX-V blocking. A subsequent application of JSTX-V at the same concentration did not cause an additional decrease of current amplitude.

Thus, JSTX-V irreversibly influenced receptors before their activation. In comparison, the results in Fig. 3, 4A, B, demonstrate that JSTX-V venom acted on the activated receptors, since blocking was at the background of KK action.

The study of the effects of JSTX-3 on glutamate receptors in activated and non-activated forms showed similar results to those described above. JSTX-3 blocked both open and closed KK-activated channels. The results of these experiments are shown in Fig. 2A,B, 4C, D.
Fig. 5. Potential-dependence of the action of JSTX-V venom and JSTX-3 toxin: 

(A) Blocking of KK-activated current slowed down and the degree of blocking decreased at the holding potential of -30 mV in comparison with similar experiments at -100 mV (B). Experiments A and B were done on two different neurons. Concentration: KK – 1 mmol/L; JSTX-V – 5.0 \times 10^{-5} \text{ units/µL}; C, D – Potential-dependence of JSTX-3 action. The velocity of toxin blocking action decreased at the holding potential of -30 mV (A) in comparison with a similar experiment at -100 mV (B). Recordings were made sequentially on one neuron, the amplification of (B) was changed in comparison with (A). Concentration: KK – 1 mmol/L; JSTX-3 – 10^{-4} \text{ mol/L}. 

Comment: KK – kainate

Kinetics of JSTX-V and JSTX-3 interaction with channel-receptor complexes. The use of KK, the gCRC agonist that did not cause the desensitization of their receptors,
enabled us to visualize the kinetics of JSTX-V interaction with the membrane channel-receptor complexes of isolated hippocampal neurons and analyze the dependence of this interaction on the concentration of JSTX-V and JSTX-3.

The velocities of the irreversible blocking of gCRC by the studied antagonists were close to exponential (Fig. 6). It allowed determining the time constants of τ decline for various concentrations of the antagonists. The dependency of 1/τ on concentration of JSTX-V venom and JSTX-3 toxin were studied and subsequent calculations were done. Within the limits of experimental accuracy, the dependency for venom JSTX-V was demonstrated as a linear one approximated by a direct line passing through “0” of axes, corresponding to a simple mechanism of irreversible blocking

\[ P + T \rightarrow PT \]  

where \( P \) is the receptor locus of gCRC, \( T \) is the molecule-antagonist of the receptor (here: JSTX-V), \( PT \) is gCRC blocked by the antagonist (here: JSTX-V), \( k \) is the constant of the velocity of blocking.

In this case, the constant of blocking velocity is linked with the concentration of the antagonist:

\[ 1/τ = k[T] \]  

This effect was registered for JSTX-V, and value \( k \) for JSTX-V, calculated in these experiments, was \( k = 4.4 \times 10^3 \text{ µL/(units} \cdot \text{s)} \).

The same dependency of KK-activated currents blocking kinetics on JSTX-3 concentration is presented in Fig. 6B. This dependency is close to linear one, its approximating direct line is higher then “0” of coordinate axes. Such dependency corresponds to irreversible blocking:

\[ P + T \xleftrightarrow[{k_1}][{k_{-1}}] PT \]  

where \( k_1 \) and \( k_{-1} \), respectively, corresponded to velocities of direct and reversed reaction of toxin binding to gCRC. The dependency of \( t \) on the concentration corresponds to the mechanism:

\[ 1/τ = k_1[T] + k_{-1} \]  

The experimental data shown in Fig. 4C, D allowed estimating two velocity constant rates for the interaction of JSTX-3 with channel-receptor complexes according to the slope of the direct line and its intersection with the axis of ordinate:

\[ k_1 = 2.1 \times 10^3 \text{ L/(mol}\cdot\text{s)} \]  

\[ k_{-1} = 2.7 \times 10^{-2} \text{ 1/s} \]  

Measurement of the kinetics of JSTX-3 “washing” during the recovery of KK-activated ionic currents allow estimating the value independently in another way. Such measurements gave value \( k_{-1} = 1.3 \times 10^{-2} \text{ s}^{-1} \) (number of neurons \( n = 4 \)) that is close in order to the value obtained from the plot in Fig. 4C, D. Thus, the value of the dissociation constant of interaction between the reversibly acting JSTX-3 component and receptors corresponds to the interval from \( K_d = 6.2 \times 10^6 \text{ mol/L} \) to \( 1.3 \times 10^5 \text{ mol/L} \).

Assuming that the isolated active fraction is the main active component of JSTX-V, by comparison of direct constants of venom and its fractions interaction with channel-receptor complexes, it is possible to estimate the content of the toxin in previously proposed conditional unit:

1 unit corresponds to 2.1 µmol of the toxin.
**Fig. 6.** Numerical dependence of the action of JSTX-V and JSTX-3: **A** – dose–effect relationship for Glu-activated currents in control (1) and under the influence of $5.0 \cdot 10^{-5}$ units/$\mu$L of JSTX-V on the cell. Current values at the peak points were normalized to the maximum. The curves are single-bond isothersms with the values of $K_0 = 1.1 \cdot 10^{-3}$ mol/L (1) and $K_0 = 2.35 \cdot 10^{-4}$ mol/L (2); **B** – concentration dependence of the kinetics of KK-activated currents blocking with JSTX-3 toxin. Absciss axis – JSTX-3 concentration in mol/L; ordinate axis – $1/\tau$, s$^{-1}$. Each point corresponds to the value $1/\tau$ obtained from the mean values of 2–4 neurons, $V_{\text{hold}} = -100$ mV. Vertical segments show the standard errors of measurement. Direct lines were drawn using the least squares method; **C, D** – volt-ampere characteristics of Glu- and KK-activated ionic currents in control (C) and under the influence of $5.0 \cdot 10^{-5}$ units/$\mu$L of JSTX-V on the neuronal membrane (D). The concentration of each agonist was 1 mmol/L. The extracellular solutions contained NaCl = 156 mmol/L, the intracellular solutions – KF = 100 mmol/L and TrisCl = 30 mmol/L. Records C and D were made on different neurons. Direct lines were plotted using the least squares method. Reversal potentials of currents were close equal to C $29$ mV and 31 mV for Glu- and KK-activated currents, respectively, and to D $21$ mV and $32$ mV for JSTX-V.

**Comments:** Glu – glutamate, KK – kainate

**Рис. 6.** Числова залежність дії JSTX-V і JSTX-3: **A** – залежність доза–ефект для Glu-активованих струмів у контролі (1) і за дії на клітину $5.0 \cdot 10^{-5}$ од./мкл JSTX-V. Значення струмів у точках піків нормалізовані щодо максимальної. Криві являють собою ізотерми одномісного зв'язування зі значенням $K_0 = 1.1 \cdot 10^{-3}$ моль/л (1) і $K_0 = 2.35 \cdot 10^{-4}$ моль/л (2); **B** – Концентраційна залежність кінетики блокування KK-активованих струмів токсином JSTX-3. По осі абсцис – концентрація JSTX-3 моль/л, по осі ординат – $1/\tau$, с$^{-1}$. Кожна точка відповідає значенням $1/\tau$, що отримані зі середніх значень на 2–4 нейронах, $V_{\text{hold}} = -100$ мВ. Вертикальними відрізками показані середньоквадратичні помилки виміру. Прямі проведені за методом найменших квадратів; **C, D** – вольт-амперні характеристики Glu- і KK-активованих іонних струмів у контролі (C) і за дії на мембрану нейрона JSTX-V $5.0 \cdot 10^{-5}$ од./мкл (D). Концентрація кожного з агоністів була 1 ммол/л. Позаклітинні розчини містили NaCl = 156 ммол/л, внутрішньоклітинні KF = 100 ммол/л, Tris-Cl = 30 ммол/л. Записи C і D отримані на різних нейронах. Прямі проведені методом найменших квадратів. Потенціали реверсії струмів близькі і становили для Glu- і KK-активованих струмів відповідно 29 мВ і 31 мВ у контролі та D 21 мВ і 32 мВ за дії JSTX-V.

**Примітки:** Glu – глутамат, KK – кайнат
This estimation of concentration is in good agreement with those obtained previously based on the assumption that the molecular weight of the toxin corresponds to 500 Da. Thus, the used JSTX-V concentrations were in the interval from $2.0 \times 10^{-8}$ to $2.0 \times 10^{-4}$ mol/L.

The dependency of $1/\tau$ on the concentration of toxin JSTX-3 – antagonist of KK-activated currents – is demonstrated in Fig. 6B. Similarly to the previous case of JSTX-V, this dependence was linear and can be approximated by a direct line, but passed above the coordinate “0” reflecting a simple irreversible blocking mechanism.

**Components of chemo-activated currents that were not blocked by JSTX-V venom.** We demonstrated that JSTX-V and JSTX-3 did not block Glu- and KK-activated currents completely; after their action was unblocked, “residual” components of currents remained. After the action of JSTX-V, the values of the unblocked component amplitudes reached 2/3 of the initial amplitudes (Figs. 2–4).

A series of experiments was devoted to the investigation of the properties of these unblocked currents’ components. The current-voltage characteristics of the “residual” components of Glu- and KK-activated currents after JSTX-V blocking were obtained. These voltage-current characteristics are shown in Figs. 6C, D. They were linear for both agonists before and after the action of antagonists, and the reversing potentials of these currents varied slightly (the records of currents are shown in Figs. 6C, D). Therefore, the nature of the unblocked current components did not change as a result of JSTX-V blocking action.

Also, the dose–effect relationship was investigated for binding of Glu molecules to gCRC both before JSTX-V blocking and after it. Similarly to control dependencies, they could be approximated as single bond isotherms and demonstrated the absence of cooperativity. The value of dissociation constant $K_d$ for the glutamate with the receptor obtained from the dose-effect was $K_d = 2.36 \times 10^{-4}$ mol/L. It was slightly lower than the $K_d$ value of glutamate in the control experiments before the venom influence, but didn’t differ significantly according to value order: $K_d = 1.1 \times 10^{-3}$ mol/L for Glu and $K_d = 5.0 \times 10^{-4}$ mol/L for KK.

**Biological requirements of Nephila clavata and physiological effects of JSTX-V, JSTX-3.** We studied chemo-activated transmembrane electric currents in the membranes of rat hippocampal neurons and influenced them by antagonists from *N. clavata* – integral venom JSTX-3 and its main active component toxin JSTX-3. The purpose of such experiments was to investigate electrophysiological characteristics of gCRC, the mechanisms of gCRC functioning, and study the details of Glu-R chemical structure. The main results of our study are summarized in Tables 1 and 2 that present the mathematical descriptions of the blocking effects. These antagonists in the used concentrations under the voltage-clamp conditions decreased the amplitudes of Glu-, KK- and QL-activated currents. The kinetics of activation and desensitization (in case of Glu and QL) of currents were not affected by these antagonists.

It should be emphasized that the action of both antagonists depended on the holding transmembrane potential. Such dependency may evidence that it was the ion channel of gCRC channel-receptor complex that was blocked. The kinetics of gCRC antagonists’ actions demonstrated the following regularity: when the concentration of the antagonist in the solution increased, the velocity of blocking increased too (the rate of blocking increases with the increasing of toxins’ concentrations). Because of partial reversibility of JSTX-3 action, the $K_d$ value could be calculated – $6.2 \times 10^6$ mol/L (Table 2).
The comparison of general characteristics of KK-activated ionic currents blocking by integral venoms from Nephila clavata and Argiope lobata and toxins (their main active components) isolated from them

| Antagonist | Effect | Blocking: irreversible | Currents’ amplitudes suppression (%) | Currents’ amplitudes recovery (%) | Potential-dependence of ant influence on gCRC | Antagonist influences on activated state | Antagonist influences on inactivated state | Antagonist influences on kinetics | Kinetics of antagonist influence described by 1 or 2 exponents |
|------------|--------|------------------------|--------------------------------------|----------------------------------|-----------------------------------------------|------------------------------------------|-----------------------------------------|----------------------------------|---------------------------------------------------|
| JSTX-V     | +      | 34.0                   | +                                    | +                                | +                                            | +                                        | -                                       | -                                | 1 1                                               |
| JSTX-3     | +      | 6.0                    | +                                    | +                                | -                                            | -                                        | -                                       | -                                | 1 1                                               |
| AR-V       | +      | 14.4                   | +                                    | +                                | -                                            | -                                        | -                                       | -                                | 2 1                                               |
| AR         | +      | 19.0                   | 77.0                                 | +                                | +                                            | -                                        | -                                       | -                                | 2 1                                               |

Comment: KK – kainate; gCRC – glutamate channel-receptor complex

The problem of an adequate selection of enzymes for the process of brain neurons pretreatment before gCRC studies with the use of antagonists from N. clavata and A. lobata require additional study. Drawing from our experience, the use of proteases from...
Aspergillus oryzae demonstrated the best effects: amplitudes of Glu- and KK-activated ionic currents were 11.8 times higher after the influence of A. oryzae proteases than after pretreatment of the object with traditionally used trypsin, pronase or collagenase (separately or in mixtures) [20, 24]. The standard methods of the enzymatic treatment demonstrated noticeably worse results: currents’ amplitudes were lower, neurons were in a worse condition (dead or they died quickly during electrophysiological experiment), “noises” in the experimental electrical circuits were higher and sometimes masked useful signal. Thus, proteases of A. oryzae caused more pronounced effects.

Proteases are known to be present in spiders’ venoms in large quantities [6, 22, 35]. During the bite, along with the toxins a spider introduces into the victim’s body a digestive secret with proteases and a significant number of free amino acids, mainly glutamate [22, 35]. These substances were present in our experimental chambers during the experiments with the registration of Glu-, KK-activated ionic currents as well as molecules of proteases that could remain in the experimental conditions in the gCRC vicinity in spite of “washing”. Such a combination of substances may lead to the formation of a specific molecular system that is similar to the one in the living nature. In the process of evolution, such a molecular system could have appeared to satisfy biological effects for the “spider-victim” pair. There is a possibility that in the course of our experiments, we recreated the close-to-natural conditions that are the best for gCRC blocking with Araneidae antagonists (from N. clavata as well as A. lobata). In such a case, the formation of a molecular system “gCRC – glutamate – antagonist – proteases” might be needed for studying the effects of such substances. Future investigations can confirm or disprove the verity of the suggested hypothesis.

CONCLUSIONS

1. The effects of Nephila clavata integral venom were studied in the concentrations of \(10^{-8}–10^{-4}\) units/\(\mu L\), the effects of JSTX-3 in the concentrations of \(10^{-6}–10^{-5}\) mol/L. These antagonists in the used concentrations under the voltage-clamp conditions decreased the amplitudes of Glu, KK, QL-activated currents. The kinetics of activation and desensitization (in case of Glu, QL) of currents were not affected by these antagonists.

2. The blocking effect of JSTX-V was irreversible; the JSTX-3 effect was slightly reversible. For both antagonists, the constant rates for the velocity of blocking (direct reaction) and the velocity of currents’ amplitudes recovery for JSTX-3 were calculated. Because of partial reversibility of JSTX-3 action, the \(K_d\) value can be calculated: \(6.2\cdot10^{-6}\) mol/L.

3. JSTX-V and JSTX-3 action depended on the holding transmembrane potential; this might be the evidence that the ion channel of gCRC channel-receptor complex was blocked.

4. Some of the registered effects were similar for both JSTX-V and JSTX-3: one-exponential type of gCRC blocking and blocking of gCRC in an activated state; but JSTX-3 can block gCRC in the inactivated state too. Some physiological effects of JSTX-V and JSTX-3 were explained; they reflect biological necessities of N. clavata.

5. JSTX-3 toxin demonstrated unique blocking effects; that is why it was proposed for the role of a “universal gCRC marker”.

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6. The use of proteases from *Aspergillus oruzae* for hippocampal neurons pretreatment demonstrated the best effects: the amplitudes of Glu- and KK-activated ionic currents were 11.8 times higher than after this object pretreatment with the traditionally used trypsin, pronase or collagenase. In such a way, close-to-natural conditions, which are the best for gCRC blocking with Araneidae antagonists from *N. clavata* and *A. lobata*, were modeled. The formation of the molecular system: “gCRC – glutamate – antagonist – proteases” might be important for the investigation of gCRC antagonists’ effects.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: This article does not contain any studies with human subjects performed by the any of the authors.

Animal studies: All institutional, national and institutional guidelines for the care and use of laboratory animals were followed.

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ЕЛЕКТРОФІЗІОЛОГІЧНІ ДОСЛІДЖЕННЯ ВПЛИВУ ОТРУТИ І ТОКСИНУ NEPHILA CLAVATA НА МЕМБРАНИ НЕЙРОНІВ ГІПОКАМПА ПІСЛЯ ОБРОБКИ ЇХ ПРОТЕАЗАМИ ASPERGILLUS ORYZAE

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Вступ. Застосування токсинів артропод в електрофізіологічних експериментах є важливим для медичної практики, проте експериментальних даних стосовно цієї теми недостатньо. Метою цієї статті було представити експериментальні дані про
вплив отрути павуків *Nephila clavata* та її головного активного компоненту – токсіну JSTX-3 на глутаматний канало-рецепторний комплекс.

**Методи.** Кашіат було застосовано як агоніст глутаматних канало-рецепторних комплексів, оскільки він ініціював у мембранах гіпокампа шурів трансмембранні електричні струми, що не десенситизувалися; на тлі таких струмів можна було добре досліджувати дію антагоністів глутаматних канало-рецепторних комплексів. Усі досліджені хімічні речовини аплікували на мембрани перфузованих пірамідних нейронів гіпокампа, застосовуючи методику “фіксації концентрації” та фіксації потенціалу.

**Результати та обговорення.** Встановлено, що цілісна отрута і токсин JSTX-3 блокують глутаматні канало-рецепторні комплекси. Ампілітуди електричних трансмембранних струмів, активованих глутаматом, каїнатом, квісквалатом, зменшувались (іноді до нуля) після аплікації антагоністів глутаматних канало-рецепторних комплексів на мембрану гіпокампа шурів в умовах фіксації потенціалу. Ці антагоністи не впливають на кінетику активації та десенситизації (у випадку глутамату, квісквалату) трансмембранних електричних струмів. Вплив цілісної отрути *Nephila clavata* досліджували за концентрацій 10^{-8}–10^{-4} од./мкл, а ефекти JSTX-3 – за концентрацій 10^{-6}–10^{-5} моль/л. Цілісна отрута ніколи повністю не блокувала досліджувані струми, а тільки зменшувала їх ампілітуду до певних рівнів. Цілісна отрута блокувала глутаматактивовані струми до 36±15 % початкових величин, каїнатактивовані – до 34±16 %. Навпаки, JSTX-3 блокував іонні струми, активовані цими агоністами практично повністю (до 6±3 %) за потенціалу -100 mV. Блокуючі ефекти цілісної отрути були незворотними, на відміну від частково зворотної дії JSTX-3. Відмінності між антагоністами також полягали в кількісних характеристиках їхньої блокуючої дії. Досліджено вплив антагоністів на ступінь блокування струмів та відновлення їх способом “відмивання”, на залежність “доза–ефект”, на активовані та інактивовані рецептори, на кінетику дії антагоністів, на константи дисоціації блокаторів зі зворотною дією того.

**Висновки.** Зроблено висновки щодо механізмів дії цих речовин на глутаматний канало-рецепторний комплекс, а також проведено порівняння спричинених ефектів. Обговорено вплив попередньої обробки нейронів гіпокампа протеазами Aspergillus oryzae під час підготовки їх до дослідів. Створено фізичну модель молекулярної системи “глутаматний канало-рецепторний комплекс – глутамат – антагоніст – протеази”.

**Ключові слова:** Araneidae, отрута, токсин, антагоніст глутаматних рецепторів, трансмембранний електричний струм

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