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CHARACTERIZATION OF PYRETHROID – INSECT ION CHANNEL INTERACTIONS VIA CYCLIC VOLTAMMETRIC TECHNIQUE

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In this study we present novel methodology for studying the interaction of biologically active substances with ion channels reconstructed in bilayer lipid membranes (BLM). We utilized reconstruction of grasshopper Na^+ channels in planar BLM and cyclic voltammetric technology for this purpose. BLM constructed from general lipids of bovine brain shows in NaCl as well in KCl solution a typical curve of cyclic voltammetry. In BLM reconstructed with grasshopper cephalothorax membrane proteins the capacitance and the resistance shifted to 2.2 μF/cm^2 and 160 MΩ (against to 0.2 μF/cm^2 and 1.3 GΩ for BLM free from membrane proteins), respectively, for BLM prepared in 1 mm diameter hole in NaCl solution, while remaining constant in KCl. The results indicate about insect sodium channels reconstruction in BLM.

We used the developed methodology for determination of apparent dissociation constant (K'd) of ligand-macromolecule interaction for the model of pyrethroid insecticide and ion-channel. The resistance of BLM with reconstructed ion channels was used for quantitative characterization of mentioned interaction. Determination of K'd values for ligand – ion channel complexes, using the asymptotic method of none-linear regression analysis, reviled 40.3±8.7 μM and 104.9±16.9 μM for known pyrethroids deltamethrin and cypermethrin, respectively.

Cyclic voltammetry – BLM reconstructed Na^+ channels – pyrethroid – apparent dissociation constant

Աշխատանքում ներկայացված է նոր մեթոդաբանություն երկշերտ լիպիդային թաղանթներում (ԵԼԹ) վերակառուցված իոնական անցուղիների հետ կենսաբանական ակտիվ միացությունների փոխազդեցության ուսումնասիրման համար: Այդ նպատակի համար օգտագործվել է ԵԼԹ-ում վերակառուցված մորեխի Na^+-ական անցուղիները և վոլտամպերմետրական տեխնիկան: Ցլի ուղեղի ընդհանուր լիպիդային ֆրակցիայի կիրառմամբ ստացված ԵԼԹ-ն NaCl-ի, ինչպես նաև KCl-ի լուծույթներում տիպիկ ցիկլիկ վոլտամպերմետրիայի կորեր է ցույց տալիս: 1 մմ տրամագծով անցքի վրա NaCl-ի լուծույթում ստացված և մորեխի կրծքագլխի թաղանթային սպիտակուցներով վերակառուցված ԵԼԹ-ների ունակությունները և դիմադրությունները փոխվում են համապատասխանաբար մինչև 2,2 մկՖ/սմ^2 և 160 ՄՕհմ (համեմատած թաղանթային սպիտակուցներից ազատ ԵԼԹ-ների 0,2 մկՖ/սմ^2 և 1,3 ԳՕհմ բնութագրերի հետ), մնալով անփոփոխ KCl-ի լուծույթում: Արդյունքները վկայում են ԵԼԹ-ներում միջատների իոնական անցուղիների վերակառուցման մասին:

Մշակված մեթոդաբանությունը օգտագործվել է պիրետրոիդային ինսեկտիցիդ — մակրոմոլեկուլ մոդելի դեպքում լիգանդ-մակրոմոլեկուլ փոխազդեցության դիսոցման թվացյալ հաստատունի (K'd) հաշվարկման համար: Նշված փոխազդեցության քանակական բնութագրման համար օգտագործվել է վերակառուցված իոնական անցուղիներով ԵԼԹ-ների դիմադրությունը: Ոչ գծային ռեգրեսիոն անալիզի ասիմպտոտիկ մեթոդի կիրառմամբ լիգանդ-իոնական անցուղի կոմպլեքսի K'd-ի որոշումը հայտնի պիրետրոիդների՝ դելտամետրինի և ցիպերմետրինի համար ցուցաբերել է համապատասխանաբար 40,3(8,7μM և 104,9(16,9 M արժեքներ:

Հիմնաբառեր՝ Ցիկլիկ վոլտամպերմետրիա – ԵԼԹ-ում վերակառուցված Na+-ական անցուղիներ – պիրետրոիդ – թվացյալ դիսոցման հաստատուն

В работе представлена новая методология для изучения взаимодействия биологически активных соединений с ионными каналами, реконструированными в бислойных липидных мембранах (БЛМ). Для этой цели мы использовали реконструирование Na^+ каналов кузнечиков в БЛМ и вольтамперметрическую технологию. БЛМ, полученный с применением общей липидной фракции мозга быка в растворе NaCl, а также KCl, показывает типичные кривые циклической вольтамперметрии. В БЛМ, реконструированной мембранными белками цефалоторакса кузнечиков емкость и сопротивление меняются до 2,2 мкФ/см^2 и 160 МОм (против 0,2 мкФ/см^2 и 1,3 ГОм для БЛМ, свободной от мембранных белков) соответственно для БЛМ приготовленной в поре диаметром 1 мм в растворе NaCl, оставаясь неизменным в KCl. Результаты указывают на реконструирование ионных каналов насекомых в БЛМ.

Мы использовали разработанную методологию для определения кажущейся константы диссоциации (K'd) взаимодействия лиганд – макромолекула для модели пиретроидный инсектицид и ионный канал. Сопротивление БЛМ с реконструированными ионными каналами использовали для количественной характеристики указанного взаимодействия. Определение значений K'd для комплексов лиганд – ионные каналы, с применением асимптотического метода нелинейного регрессионного анализа, выявило 40,3(8,7 μM и 104,9(16,9 M для известных пиретроидов делтаметрина и циперметрина соответственно.

Циклическая вольтамперметрия – Na+каналы, реконструированные в БЛМ – пиретроид – кажущаяся константа диссоциации

This article is dedicated to the memory of Dr. Rafik Brutyan, the pioneer of BLM electrochemical investigations in Armenia.

The selective conductance of sodium ions across the plasma membrane is known to be involved in the propagation of the action potential in vertebrate and invertebrate neuronal cells [9]. The critical role of the sodium channel in the functioning of the nervous system has made it the target of a diverse array of toxins during evolution. Experimental methods for revealing the potency of neuronal drugs via electrophysiological techniques are essential in biology and pharmacology. Particularly, experiments revealing the influence of drugs on potential-dependent channel opening and closing (gating) are of special interest.

A remarkable technological development occurred in the ion channel field in the late 1970s, when Neher and Sakmann successfully recorded acetylcholine receptor single-channel currents from the denervated frog skeletal muscle [10]. This technique was further improved by the development of gigaohm patch-clamps [7]. The two main advantages of patch clamps are: 1) whole-cell currents of any receptors and channels can be recorded by voltage clamp using practically any type of cells, including neurons, myocytes, and lymphocytes, and 2) single-channel currents can be recorded with a high degree of precision. Consequently, the patch-clamp technique has become the basic tool for studying native ionic channels, and it is extremely popular in cellular neuropharmacology or neurotoxicology since the effects of any chemicals on any receptors/channels can be studied.

The benefits of patch clamps in studying the ion channels is appealing, however, it is sometimes difficult to adapt the object of interest to path clamp; the problem being to extend this type of analysis to smaller, noncylindrical, cellular structures that would not allow insertion of metal wires. Moreover, unlike the plasma membrane, intracellular membranes are usually not stable enough to withstand mechanical manipulation by glass electrodes during seal formation and rupturing of the membrane [2]. The alternative choice is based on planar BLM reconstructed ionic channels.

Therefore, accurate reconstruction of a functional ion translocation system from the minimal number of components required to mimic an active membrane would certainly help general understanding of membrane excitability. One of the most useful approaches, in this respect, is the reconstruction of the simple membrane system using self assembled lipid bilayers or liposomes [3].

Recently, we presented an alternative approach for ion channels reconstitution in conventional planar BLM [16]. Combining this approach with Tien's cyclic voltammetric technology of metal supported BLM characterization [1], we were able to record integral electrical characteristics of in BLM reconstructed insect sodium channels. Direct addition of mixture of bovine general brain lipids and isolated insect ion channel proteins, leads to self assembly and reconstruction of native ionic channels in BLM. Herein, we use this model to characterize the mode of action and affinity characteristics of pyrethroid insecticides on reconstructed insect sodium channels.

Pyrethroids are synthetic derivatives of pyrethrins, toxins contained in the flowers of some *Chrysanthemum* species. Pyrethroids that are widely used as an insecticide present low mammalian toxicity and high biodegradability. They primarily affect the sodium channels of excitable tissues, prolonging the Na^+ current [11,12,13]. This action underlies the neurotoxic basis for sensorimotor changes in both vertebrates and invertebrates. In addition, pyrethroids may produce cardiotoxic effects mediated by I_{Na} prolongation in cardiac myocytes [11]. Nevertheless, since global use of pyrethroids is increasing, especially in regions burdened by arthropod-borne diseases, a rise in human acute pyrethroid exposure has occurred [6]. Hence, the understanding of pharmacological and toxicological spectrum of this class of insecticides is of considerable interest.

The aim of this work is to develop new experimental methodology for studying the interaction of biologically active substances such as newly synthesized pyrethroids and other drug candidates with in BLM reconstructed ion channels/receptors. The method is based on determination of the membrane resistance – the integral characteristics of BLM involving reconstructed ion channels.

Materials and methods. *Bovine brain lipids* were isolated by method developed by Folch et al. [5]. Obtained lipids were kept frozen at 253K under argon atmosphere before use.

Planar BLMs were obtained by pouring 10-20 μL hexane-dissolved bovine brain lipids (20-25 mg/mL) under the 1 mm diameter vertical hole, separating electrode chambers. The chambers were filled with 0.1 M NaCl or KCl solution containing 20 mM tris-HCl buffer, pH 8.2.

Grasshopper's cephalothorax membrane proteins were solubilized as described in the modified method of Moskowitz et.al. [8]. Grasshoppers were collected in summer and kept frozen at 253K before use. 10 g of grasshoppers cephalothorax was homogenized in 50 ml buffer A (0.2 mM PMSF, 2 mM EDTA, 5 mM mercaptoethanol, 50 mM tris-HCl, pH 8.2) in MPW-302 homogenizer (Poland) at 2000 rpm during 15 min at 277K. The homogenate was centrifuged at 20000 g, 20 min and supernatant was discarded. The pellet was added to another 20 mL buffer A supplemented with 0.2 % triton X-100 (solubilization buffer B). The obtained suspension was processed as described above. After centrifugation at 20,000 g for 20 min, supernatant was collected and used in all experiments as membrane protein fraction.

Concentration of the membrane protein adjusted to 10 mg/mL with solubilization buffer B. Membrane protein reconstructed BLM was obtained as described above by bovine brain lipids added grasshoppers membrane proteins in 10/1 ratio (V/V).

To study the pyrethroid action on BLM reconstructed sodium channels the corresponding compounds were dissolved in acetone in 10 mg/mL concentration. Each series with different pyrethroid concentrations was made in one experiment with the same insect protein reconstructed membrane using increasing concentration of the chemical. The acetone dissolved chemical was added in front chamber near the opposed to the BLM wall of the chamber. The acetone action on conductivity and capacitance was studied in separate series of experiment, with a new insect protein reconstructed BLM. In these experiments the same increasing acetone concentrations, as in mother experiment, were used.

The results obtained for acetone action were subtracted from corresponding results obtained in main experiment after normalization for the point of zero acetone concentration.

All measurements were performed by Tiens cyclic voltammetric method on CH Instruments Model 600 voltammetric analyzer. Electrochemical experiments were carried out using a conventional two-compartment three-electrode electrochemical cell equipped with the working electrode, an auxiliary electrode and an Ag/AgCl/KCl(sat) as reference electrode (fig. 1(A)). A personal computer was used for data storage and processing. To compensate the influence of outside electromagnetic fields the chamber was placed in metallic box. All the measurements were carried out at 298±1K.

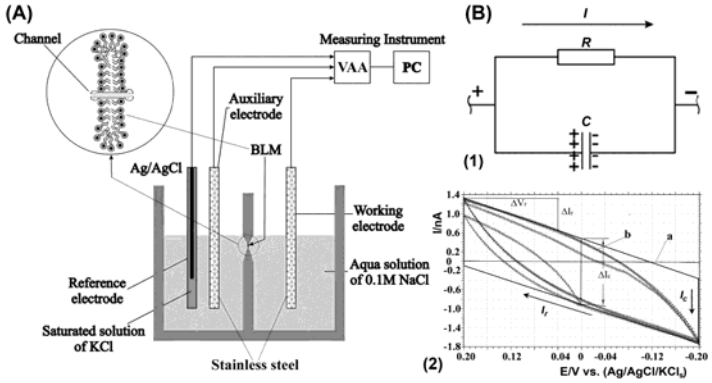

Fig.1. (A) Schematic diagram of the electrochemical chamber adapted for recording the electrochemical characteristics of BLM by VAA. (B) Principles of cyclic voltammetry. (1) – BLM representation as an electrical circuit consisting of R and C. (2) – Schematic representation of cyclic voltammetry, where I is total current, I_r is current through the R, I_c is current through the C, E is potential.

The membrane resistance (Rm) and capacitance (Cm) were calculated according to [1]. The obtained results are the mean of at least three independent experiments. The apparent dissociation constant (K'd) of ligand – macromolecule interaction was determined by asymptotic method of none-linear regression analysis using SPSS 16.0.

Results and Discussion. Typical cyclic voltammetric diagrams of BLM without and with reconstructed insect Na channels are presented in fig. 2.

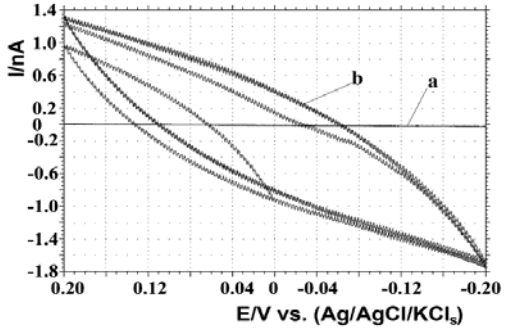

Fig. 2. Representative cyclic voltammograms of BLM prepared from general bovine brain lipids (a) and the BLM with reconstructed grasshopper's membrane protein (b); scan rate = 30 mV/s; cycle repeats (segment) = 7; sample interval = 0.001 V.

The obtained data show that BLM formed in 1 mm round hole displays a typical curve of cyclic voltammetry, with C_m= 0.2 μF/cm^2 and Rm= 1.3 GΩ. When BLM was formed by addition of grasshopper cephalothorax membrane proteins a 11-fold increase in capacity and a nearly 8.1-fold decrease in resistance were observed.

The used channel proteins of trans-membrane nature produce polar side pieces on BLM surface. They cause the sorption and accumulation of existing ions and increase their concentration immediately on BLM surface resulting in an increasing of the membrane capacity. As a result the mentioned above parameters shifted to C_m= 2.2 μF/cm^2 and R_m= 160 MΩ for BLM prepared in NaCl solution, respectively, while remaining constant in KCl. It should be mentioned, that used protein concentration is optimal (is corresponding to saturation), since its subsequent 10-fold dilution prior to 10/1 lipid-protein mix preparation doesn't substantially affect on obtained BLM voltammetric characteristics (data not shown).

Deltamethrin and cypermethrin caused the decrease of resistance of grasshopper cephalothorax membrane protein reconstructed BLM, hence increasing the Na^+ current through in BLM reconstructed channels. The action of deltamethrin and cypermethrin on the resistance of grasshopper cephalothorax membrane protein reconstructed BLM is presented in fig. 3(a) and fig. 4(a), respectively.

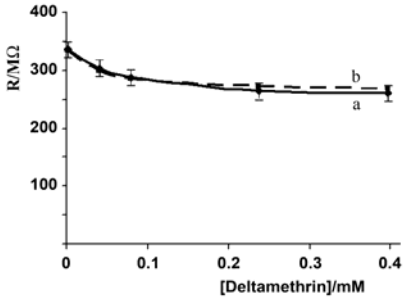

Fig. 3. Changes in resistance of BLM (R/Ω) with reconstructed grasshopper cephalothorax membrane proteins as a function of deltamethrin concentration ([Deltamethrin]/mM). (a) – experimental curve with data standard deviations, (b) – theoretical curve with K'd calculated by none-linear regression analysis using SPSS 16.0.

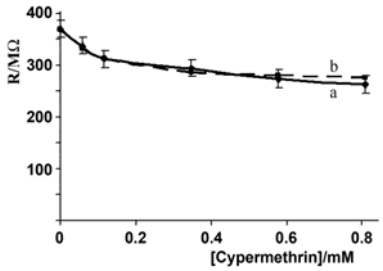

Fig. 3. Changes in resistance of BLM (R/Ω) with reconstructed grasshopper cephalothorax membrane proteins as a function of cypermethrin concentration ([Cypermethrin]/mM).
(a) – experimental curve with data standard deviations,
(b) – theoretical curve with K'd calculated by none-linear regression analysis using SPSS 16.0.

The obtained data look like reflecting Mass action low (1) for ligand-macromolecule complex dissociation process (M.L=M+L) [4].

$$K'_d = [M][L] / [M,L] \qquad (1)$$

where K'd is apparent dissociation constant, [M], [L] and [M.L] are concentrations of in BLM reconstructed macromolecule, ligand and ligand-in BLM reconstructed macro-molecule complex, correspondingly.

As in case of ligand excess the saturating ligand concentration [M.L]max is equal to total concentration of macromolecule ([M]0), or to sum of ligand bound and unbound macromolecule concentrations ([M.L]+[M]), the normalized decrease of resistance is equal to molar part of ligand-macromolecule complex ([M.L]/ [M.L]max), we can easily obtain the equation (2).

$$(R - R_{SS}) \Big/ (R_0 - R_{SS}) = [M,L] \Big/ ([M] + [M,L]) = [L] \Big/ (K'_d + [L]) \qquad (2)$$

where RSS is resistance of BLM with reconstructed ion channel before ligand addition, R0 is the same BLM resistance in ligand excess (when molar part of LMC is equal to 1).

Calculated changes in resistance of grasshopper cephalothorax membrane protein reconstructed BLM as a function of deltamethrin or cypermethrin concentration are presented in fig. 3(b) and fig. 4(b), respectively. It is obvious, that used model correctly reproduced experimental data. It will be mentioned, that obtained near 20 % increase of conductance in

Result of pyrethroid addition coincided with values of signals of integral nature (inactivation timecourse of parameters from guinea pig ventricular myocytes in presence of 10 μM fenpropathrin) known in other objects [11].

The K'd of ligand-macromolecule interaction determined by asymptotic method of none-linear regression analysis were 40.3±8.7 μM and 104.9±16.9 μM for deltamethrin and cypermethrin, respectively. The analogous parameters for deltamethrin, known as inducer of large and prolonged tail currents on voltage gated sodium channels, are arranged in interval of 5 nM – 1 mM for vertebrate DRG neurons [12], Drosophila melanogaster [15], cockroach [14] etc. In our model the relative high K'd for studied pyrethroids may be associated with the fact of working with preparations of crude grasshopper membrane proteins and with the possible changes in ion channels conformation during their reconstruction in BLM. We are going to study this phenomenon in our future researches.

The obtained results are valuable from the point of view that developed method allows to study the LMI using the integral voltammetric characteristics of BLM with reconstructed ion channels.

We have adapted the technique initially developed by Tien and coauthors for the study of different reduction-oxidation reactions in metal supported BLM, for in planar BLM reconstructed membrane proteins. Presented model was stable during the time of experiment (up to 30 min). Proposed methodology is unique, since it allows characterizing ligand/ion channel interaction via simple cyclic voltammetric technique. So, the ion channel reconstructed membrane integral characteristic – R_m was used for calculating the value of apparent dissociation constant of LMC. It was adapted to study the action of deltamethrin and cypermethrin on in BLM reconstructed ion channels.

The proposed methodology can be used to develop strategies for new drug design, to evaluate methods for drug candidates screening on the model of isolated, purified and in BLM reconstructed target ion channels. Moreover, the method can be used for creation of quantitative parameters databases for SAR evolution.

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