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ORIGINAL PAPER

Express Method for Determination of Low Value of Trans-membrane Potential of Living Cells with Fluorescence Probe: Application on Haemocytes at Immune Responses

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Abstract The method for measurement of trans-membrane potential of cell membrane was evaluated for the case of low potential value using fluorescence probe 4-(4-dimethylaminostyryl)-1-methylpyridinium, DSM. The method is based on comparative titration of cells with probe in buffers containing Na^+ or K^+. The apparent trans-membrane potential obtained with this way is a result of K^+-Na^+ pump activity. The presented approach allowed measuring the low value of potential with 1–2 mV of accuracy without additional calibration procedures. The method was applied for investigation of potential of cell membrane of haemocytes of *Galleria mellonella* larvae. The value of potential of intact insect's haemocytes was found in the range from −10 to −20 mV. The change of potential value of haemocytes was investigated under model immune response and natural envenomation and parasitizing. The obtained deviations of cell membrane potential were in good correlation with changes of activity of main immune reactions, described in literature and obtained by us earlier.

Keywords Potential sensitive probe · K+−Na+ pump · Immune response · Parasitizing

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Introduction

The cell membrane plays a role of barrier from outer environment and possesses the functions, which are determined by membrane-bound proteins. One of them is K-Na ATPase that provides the necessary ion concentration inside of cell [1, 2]. The ions regulate the activities of membrane and intracellular enzymes and, therefore, play an important role in vital functions of living cells. The value of potential, which reflects the intracellular ion concentrations, often is considered as living status of cells. In high throughput screening, the most popular approaches are based on the application of potential sensitive fluorescence probes [3]. These methods are good described but mainly are able to determine the high potential values. The minimum value of potential that can be determined by described fluorescence methods can be estimated as −20 mV. It arises from contribution of false signals of different natures (autofluorescence, scattering etc.) to observed fluorescence.

We were interesting in measurement of the cell membrane potential of insect's blood cells, haemocytes, and its changes at immune responses. These cells are immunocompetent and react on any pollutants, infection etc., which attack the insect penetrating in the haemolymph. The immune response is the series of highly specific biochemical processes including pattern-recognition receptors located on the membrane surface [4]. These mechanisms are investigated widely in contrast with role of physicochemical properties of membrane such as its electrostatic properties. Some researchers investigated the surface charge of membrane that is to be responsible for haemocytes adhesion [5]. The very important property of cell membrane is its trans-membrane potential that is widely investigated for

mammalian. For invertebrates, the value of potential and its role is neither measured nor investigated so much for now. We could only propose that value of this potential is quite low, and for its investigation we should have an adequate method for its determination.

For these purposes, we developed and applied an express method, which was suitable for determination of potential of absolute value below of 20 mV without additional calibration procedures. As a fluorescent probe, we applied 4-(4-dimethylaminostyryl)-1-methylpyridinium, DSM, which is potential sensitive and used for determination of potential of cell and mitochondrial membranes [6].

Materials and Methods

Chemicals

Lypopolysaccharide (LPS), zymosan, HEPES, NaCl, KCl were purchased from Sigma (USA). DSM was kindly provided by Dr. N. Kolosova (Institute of Cytology & Genetics of RAS SB). Stock solution of DSM in water was about 1 mM, is its ultimate solubility. All solutions were prepared with bidistillate deionized water.

Insects, Parasitoid and Activation

A laboratory population of *Galleria mellonella* was maintained at 28 °C on an artificial medium containing corn meal (22,5 %), bee honey (12,5 %), glycerol (12,5 %), bees' wax (12,5 %), wheat flour (10 %), milk solid (12,5 %), yeast (5 %) and glass distillate water (12,5 %). Larvae of 5th or 6th stages were used in the study.

Ectoparasitoid *Habrobracon hebetor* were reared on 6th stage larvae of the *G. mellonella*. Adult stages of parasitic wasps were maintained at 28 °C with a photoperiod of 14 h on a diet of 12 % liquid honey.

For natural envenomation, the adult parasitoids were placed into glass jars containing *G. mellonella* larvae for 3 h. Then, the larvae of *G. mellonella* were quickly removed from jars, wiped with 70 % ethanol and kept in the Petri dishes with paper discs until measurements.

For parasitic invasion, the larvae of *G. mellonella* were kept in the Petri dishes with the adult parasitoids for 1 day until measurements.

For in vivo activation of immune response, an aliquot of LPS or zymosan suspension (4 μl, 1 mg/ml) was injected in hemocoel of every larva (*G. mellonella*) in the experimental group of 30 insects. As a control group, the same number of insects was injected with the same aliquots of saline. Injected insects were incubated for an hour until measurements.

Venom Sample Preparation

The wasps *Habrobracon hebetor* were mechanically stimulated to inject some drop of venom on glass plate covered with sterile parafilme. The drops of venom from five females were combined and dissolved in 5 μl of saline. Samples were immediately collected and stored at −20 °C. The venom samples were 6.4 and 32.3 times diluted with HEPES buffer (140 mM NaCl, 5 mM KCl, 6 mM glucose, 10 mM HEPES, pH 7.2) for in vitro activation of haemocytes of larvae of *Galleria mellonella*.

Haemocyte Collection and Sample Preparation for Measurements

In experimental groups of 30 *G. mellonella* larvae, the 15 μl of haemolymph from every larvae were collected in tube with cooled (on ice bath) anticoagulant (62 mM NaCl, 100 mM glucose, 10 mM EDTA, 30 mM trisodium citrate, 26 mM citric acid, pH 4.6) (AC) by cutting the third proleg with microscissors and drawing haemolymph into the tip of a pipette [7]. The sample was centrifuged at 500 g for 5 min at 4 °C to pellet haemocytes. Precipitate was resuspended and washed three times in cool AC and once in HEPES buffer, and finally resuspended in 520 μl of HEPES buffer to content about 10^7 cells/ml.

Heamocytes suspension was divided into two equal parts, centrifuged at 500 g for 5 min at 4 °C to pellet haemocytes. One part was resuspended in normal HEPES buffer, and another part was resuspended in K^+-HEPES buffer (140 mM KCl, 6 mM glucose, 10 mM HEPES, pH 7.2). The aliquots (48 μl) of each suspension were placed in fluorimetric cells of microplate reader. Four fluoremetric cells were occupied by samples in normal buffer and other fours cells by samples in K^+-HEPES buffer. The fluorescence measurements were made upon titration of samples by DSM solutions in the same buffer as suspension.

For in vitro activation experiments, an aliquot of LPS or zymosan suspensions (60 μl, 1 mg/ml) or diluted venom samples were added to heamocytes suspension obtained as described above. Suspensions were incubated for 30 min until measurements.

Instrumental

Fluorescence spectra of DSM were recorded on spectrofluorimeter VARIAN Cary Eclipse (Australia). Measurements of concentration of DSM in aqueous solution ($\varepsilon_{450}=3\cdot10^4\ M^{-1}cm^{-1}$) were performed on spectrophotometer Shimadzu UV-2401 (Japan).

The photometric measurements were performed on home-made 8-well microplate reader. Measurement mode is bottom irradiation, top reading. The irradiation source is

ultra bright LED (LUXEON K2-PR14) which has maximum emission at 455 nm with band width of 20 nm. The detecting system is equipped with band-pass filter wheel. The intensity of irradiation was modulated with frequency of 5–10 Hz followed by signal detection on the same frequency. That allows detecting only irradiation-related signal and, therefore, to exclude the contribution of false signals such as dark signal, offset or side illumination of photomultiplier. Acquisition time of single measurement was 10 s. Total time of measurements of eight samples including titration was about 30–60 min.

Results and Discussion

Properties of Fluorescence Probe DSM

DSM is a multipurpose fluorescent probe, which is applied for investigation of biological membrane [6, 8]. In aqueous solution, it is weekly fluorescent (Q=0.0017) but in viscous media and upon adsorption on membrane, the quantum yield of DSM increases significantly. DSM is a cation, therefore, its binding with cell membrane depends on surface charge and trans-membrane potential.

The emission spectrum of DSM depends on its localization in the membrane. Usually, two main regions of probe localization in the membrane are considered: surface and intra-membrane. These regions of localizations have different polarity that results in different fluorescent spectra [9, 10]. On the Fig. 1 are shown the spectra of excitation and emission of DSM in the water-glycerol mixture as model of surface localization and in the octanol as a model of intra-membrane localization.

Measurement of the Trans-membrane Potential of Cell Membrane of Haemocytes with DSM

Upon addition to cells, DSM accumulates with time course subsequently in the cytosol and mitochondria that was used for determination of trans-membrane potentials. This is valid for other potential sensitive probes and allowed to measure the sum of cell membrane and mitochondrial potentials. To extract cell membrane potential, the measurements are to be performed in different buffers, sodium and potassium.

As a rule, the probes are incubated with cells for 20–30 min for penetration of probe into cell. In our experiments, we performed measurements shortly after addition of DSM to haemocytes suspension. DSM absorbed rapidly on membrane, in sub-second time scale, and resulted in decreasing of fluorescence yield. In this case, the probe should be localized on the membrane surface and inside of cell membrane, without penetrating into cell. We tested the further penetration of DSM that takes about 30 min resulting in final increasing of fluorescence of about 10 %–30 % depending on probe concentration (Fig. 2).

The fluorescence signal of haemocyte suspensions versus added DSM is demonstrated on Fig. 3. We applied band-pass filter at 560 nm (band width of 15 nm) that corresponds to emission of intra-membrane probe (see Fig. 1). Measurements were performed in different buffers, sodium and potassium (Fig. 3a). The fluorescence signal in potassium buffer exhibited the lower signal. That demonstrated the presence of trans-membrane potential of haemocytes as a result of K-Na ATPase activity in sodium buffer.

The shown titrations were performed until concentration of DSM of 100–120 μM. At the final concentrations, the absorption of DSM in solution is significant that results in

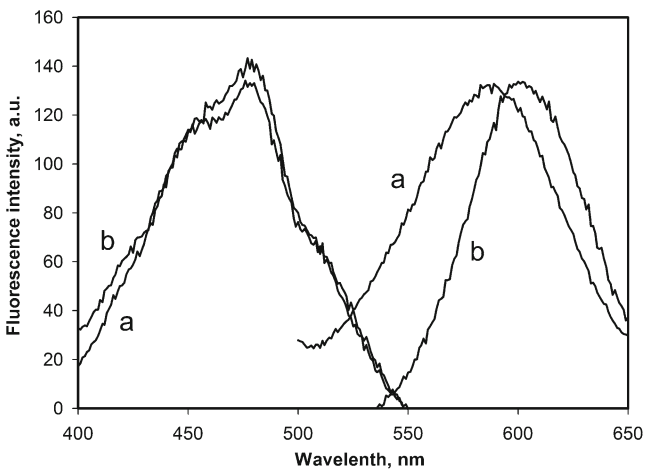

Fig. 1 Spectra of excitation/emission of DSM in octanol (**a**) and glycerol-water mixture (80 % w/w) (**b**). For trans-membrane potential determination, the band-pass filter at 560 nm was applied

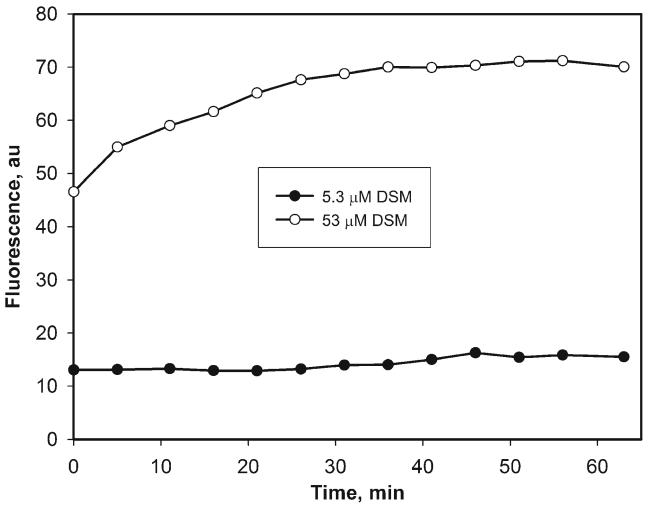

Fig. 2 The kinetics of fluorescence of DSM measured right after its addition to haemocytes suspension. The green band of fluorescence at 560 nm was recorded

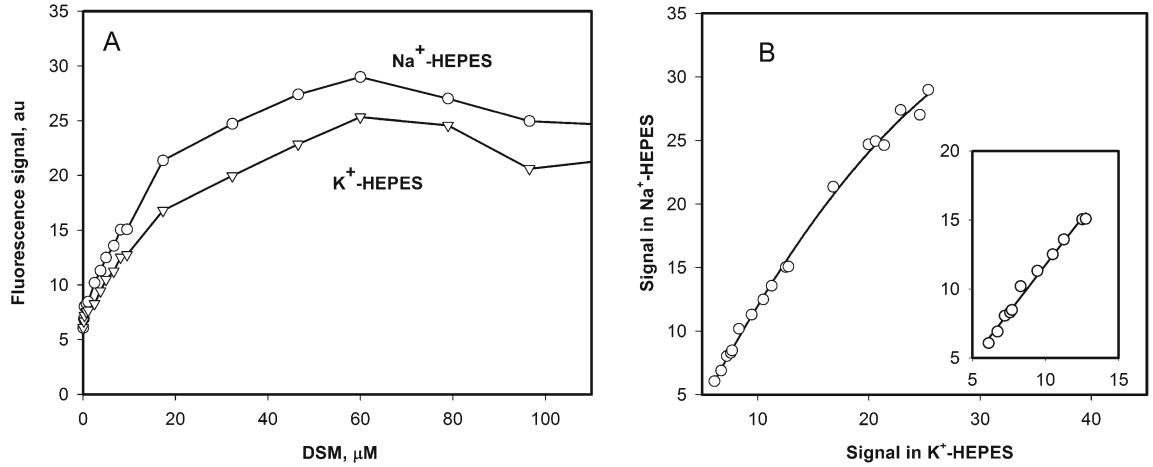

Fig. 3 **a** Dependence of fluorescence intensity of DSM in suspension of haemocytes versus added concentration of DSM in different buffers: Na^+-HEPES and K^+-HEPES. **b** Plotting of fluorescence signal in Na^+-HEPES versus signal in K^+-HEPES buffer. In the insert, the linear part of this curve is shown, which corresponds to the concentration of DSM below 10 μM. The slope of the linear part of curve was used for calculation of the value of trans-membrane potential using Eq. 1

decreasing the intensity of irradiation in 2–3 times as was tested experimentally (data not shown). This phenomenon is called as effect of internal filter and results in decreasing of observed fluorescence intensity at high concentration (see Fig. 3a). It made impossible to provide a reasonable analysis of experimental titration curves. We were able only to estimate the equilibrium concentration of DSM of process of adsorption on haemocytes membrane of 50 μM. It corresponds to binding constant of $2\times10^4 M^{-1}$ For comparison, this value for liposome from lecithin is 10^3 M^{-1} [9] and for erythrocytes is $2\times10^6 M^{-1}$ [11].

To quantify the difference of data in the different buffers, the experimental data were plotted from each other, namely, signals in sodium buffer versus signals in potassium buffer (Fig. 3b). This curve was linear in the beginning that corresponded to the range up to 10 μM of DSM. The value of slope is equal to the ratio of concentrations of DSM in the membrane in different buffers. Taking into account that the extracellular concentration of DSM is the same in all samples at a single data point, the value of the slope can be used for determination of trans-membrane potential using Nernst equation:

$$\Delta\Psi = \frac{kT}{e}\ln\left(\frac{DSM_{Na}^{membrane}}{DSM_{K}^{membrane}}\right) = \frac{kT}{e}\ln(K) \qquad (1)$$

where $\Delta\Psi$ is trans-membrane potential; e is elementary charge; $DSM_{Na,K}{}^{membrane}$ is concentration of DSM bound

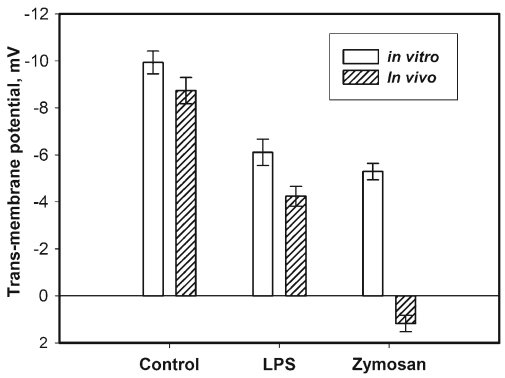

Fig. 4 The apparent trans-membrane potentials of haemocytes of *G.mellonella* after addition of immune activators LPS and Zymosan to cells suspension in vitro; and after 1 h of injection of immune activators LPS and zymosan to insects, in vivo. The data are presented as the mean ± standard deviation

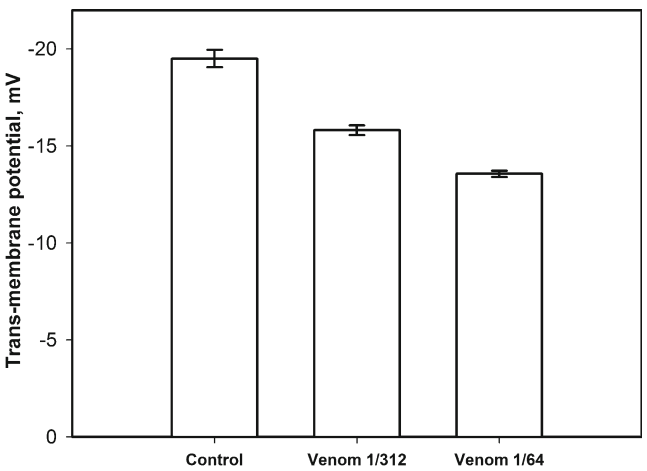

Fig. 5 The apparent trans-membrane potentials of haemocytes of *G. mellonella* after addition of *Habrobracon hebetor* venom to cells in vitro. Venom was extracted from wasps as described in Materials and Methods and was diluted in 312 and 64 times. The data are presented as the mean ± standard deviation

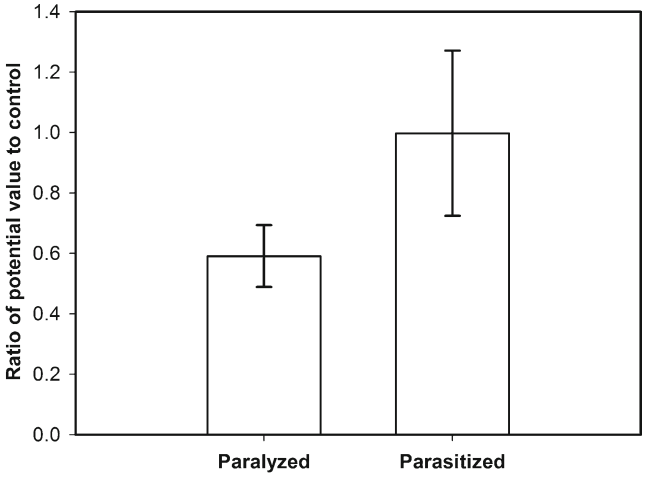

Fig. 6 The ratios of trans-membrane potentials of cell membrane of haemocytes of parasitized and paralyzed *G. mellonella* to control. The data are presented as the mean ± standard deviation. Paralyzed insect data are statistically different from the control ($p<0.05$, $N=3$)

with haemocyte in Na- or K-HEPES buffers; K is slope of curve shown on Fig. 3b (insert).

The value of slope of the curve shown in Fig. 3b (insert) is equal to 1.45 that corresponds to −9.5 mV of potential value. The accuracy of determination of potential from the slope is 1–2 mV. The decreasing of slope at concentration of DSM higher then 10 μM demonstrated the decreasing of potential value due to accumulation of probe in the membrane. In the present investigation of trans-membrane potential of haemocytes, we applied the titration in the range of linear dependence.

Such data presentation and analysis have a few benefits. The slope depends only on the ratio of the probe concentration whereas other sources of fluorescence are compensated. These are systematic errors of measurements, irradiation related false signals (scattering, auto fluorescence and unbound probe signals) and influence of effect of internal filter. The dark signal and side illumination were eliminated instrumentally as described in Materials and Methods. As a result, we managed to exclude the influence of false signals that is considered as a main factor determining the low limit of potential determination with fluorescence probe methods.

Classical Nernst equation implies the ratio of intracellular concentration of ions to extracellular. In our case, DSM is located inside of cell membrane. So, obtained value of potential could be considered as effective intra-membrane potential. For the calculation of the true trans-membrane potential, the consideration the profile of potential and DSM localization distribution is required. For now, we can propose that the true value of potential is proportional and quite close. On the other hand, we observed the value of effective potential of suspension of haemocytes, which consists of different types of cells. The proposed approach was intended to apply for investigation of deviation of this apparent value of potential under different affections on insect haemocytes.

Influence of the Model Immune Response on Trans-membrane Potential of Haemocytes of *Galleria mellonella* Larvae

To activate the immune response in haemocytes, we applied lipopolysaccharide and zymosan, which are the standard immune activators. LPS is major component of outer membrane of gram-negative bacteria, its addition to suspension of blood cell simulates the bacterial infection. Zymosan is a polysaccharide contained in cell wall of some kind of yeast. In general, it is considered as activator of phagocytosis. In the process of activation, these species are recognized by receptors located on the outer membrane of blood cells [4]. We investigated the effect of these immune activators on the value of trans-membrane potential of haemocytes.

We applied two types of activation in vitro and in vivo. In case of in vitro activation, the species of activator affect directly on cell demonstrating the initial stage of immune response. In vivo activation is performed by injection of activator to insects (see Materials and Methods). It is considered as the prolonged effect of immune response.

The results of experiments are shown on Fig. 4. The activation of immune response causes the decreasing of potential in all cases but in different degrees. For in vitro experiments, the decreasing was 40–50 % for both LPS and zymosan activations. In vivo activation with LPS resulted in 50 % of decreasing. Injection with zymosan resulted in phagocytosis that was appeared in visible melanization of larvae. In this case, the complete degradation of membrane potential of haemocytes was detected (Fig. 4).

Table 1 The results of typing of haemocytes of *G. mellonella* affected by *Habrobracon hebetor* in compare with controls

	Plazmatocyte (Pl)	Granulotocyte (Gr)	Oenocytoid (Oe)	Prohaemocyte (Pr)
Control	45 %	46 %	5 %;	4 %.
Paralyzed	35 %	58 %	1.5 %	4.5 %
Control	40 %	53 %	4 %	3 %
Parasitized	34 %	59 %	4 %	6 %

Study of Trans-membrane Potential of Haemocytes of *Galleria mellonella* Larvae Paralyzed and Parasitized by Ectoparasitoid *Habrobracon hebetor*

Habrobracon hebetor is the ectoparasitoid of many species of insects from different orders but the main hosts are from the order Lepidoptera [12]. The venom of the female of *Habrobracon hebetor* has a neurotoxic effect on insects. Paralytic activity of *Habrobracon hebetor* venom is mediated by three partially characterized proteins and other unknown factors, which affect on host endocrine and metabolic activities [13, 14]. Female wasps inject paralyzing venom into *Galleria mellonella* larvae, and then deposit between 3 and 20 eggs on the outside of the host.

We investigated the change of trans-membrane potential of haemocytes of *G. mellonella* under the influence of *Habrobracon hebetor* effective agents both in vitro and in vivo experiments. In vitro experiments were performed by adding two different doses of purified venom to isolated haemocytes of *G. mellonella*. The obtained results demonstrated that the venom causes the decreasing of potential. The degree of decreasing was in direct dependence of the venom concentration added (Fig. 5).

For in vivo experiments, the haemocytes were collected from the *Galleria mellonella* larvae, which were preliminary paralyzed (by natural envenomation) and parasitized as described in Materials and Methods. The paralyzed insects exhibited the decreased potential values (Fig. 6). This is in good correlation with result obtained in vitro (Fig. 5). The data for parasitized insects didn't allow to make definite conclusion due to significant standard deviation of data. In this case, we can propose that parasitizing could affect on potential in different degree but does not cause the complete degradation, in compare, for example, with affection of zymosan (Fig. 4).

It is to note that the paralyzing of larvae of insects by venom inhibits the mechanisms of immune response that was proved for some kind of insects [15–17]. As a result, the melanization is suppressed and, therefore, the encapsulation of parasitoid's eggs doesn't take place. In our recent experiments, we demonstrated that the envenomation of *Galleria mellonella* larvae by female of *H. hebetor* also suppresses the main immune reactions including the rate of melanization [18]. We can suggest that, on the one side, the venom affects directly to the haemocytes causing decreasing of activity K-Na ATPase as was observed for phenoloxidase [18]. On the other side, the envenomation suppresses phagocytosis and, as a result, prevents the complete degradation of trans-membrane potential in contrast for the effect of zymosan in vivo (Fig. 4). The mechanism of observed degradation of trans-membrane potential in phagocytosis is under discussion. We could only propose that it does not relate with total inhibition of activity of K-Na ATPase but is a result of destroying of membrane of haemocytes. For the case of parasitizing, it's required the haemocytes stay alive and preserve certain level of activity during parasitoid eggs incubation period.

In vivo experiments were attended by procedure of typing of haemocytes shown in Table 1. These results didn't reveal any significant changes in cell type distribution. The only difference was observed for enocytoids but theirs content varied from 1.5 % to 5 %. Such small concentration of the only type of cells cannot affect on changes of membrane potential of total haemocytes suspension. Thus, we can propose that the decreasing of potential is not cell type selective but is a result of the common affection of envenomation on K-Na ATPase activity in the processes of immune response.

Conclusion

In the present work, we applied a simple express method for determination of trans-membrane potential of cell membrane that allows measuring the value of potential from few millivoltes with reasonable accuracy and without additional calibration. In the presented interpretation, this method doesn't pretend for serious investigations of physical-chemical properties of trans-membrane potential of itself. However, it could be easy extended by applying different probes and experimental conditions, if desired. We are interesting in comprehensive studies of livings cells that requires involving different approaches. The measurements of trans-membrane potential of cell membrane could be considered as one of them.

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