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Concentration of cytosolic calcium in hemocytes of the greater wax moth larvae *Galleria mellonella* during the cellular immune response

Динамика кальция в цитозоле гемоцитов личинок *Galleria mellonella* при клеточном иммунном ответе

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Key words: *Galleria mellonella*, hemocytes, cytosolic calcium, phagocytosis, encapsulation.

Ключевые слова: иммунитет насекомых, гемоциты, цитозольный кальций, фагоцитоз, капсулообразование.

Abstract. Changes in the concentration of cytosolic calcium ($[Ca^{2+}]_i$) have been observed in *Galleria mellonella* (Lepidoptera: Pyralidae) hemocytes on early stages of the development of encapsulation and phagocytosis. The comparative analysis of the dynamics of cytosolic calcium concentration on the different stages of capsule formation on the nylon implants and the phagocytosis of bacterial cells has been carried out. During encapsulation of the implant in the hemocoel of *Galleria mellonella* larvae an increase of free calcium ions (by 1.8, $p < 0.001$) was recorded within the first hour. At 4 h after implantation a decrease of free calcium ions (by 2.5, $p < 0.001$) was detected in cytoplasm of blood cells. The development of phagocytosis of *Escherichia coli* was also accompanied with an increase in cytosolic calcium (by 2.5–3, $p < 0.001$) within 1.5 h followed by a 50 % decrease ($p < 0.001$) in 2 h compared to the control level. These results suggest that $[Ca^{2+}]_i$ is released in hemocytes within 1–1.5 h during the capsule formation and phagocytosis.

Резюме. Проведён сравнительный анализ динамики уровня цитозольного кальция ($[Ca^{2+}]_i$) во время таких процессов, как образование капсулы вокруг нейлонового волокна (имплант) и фагоцитоза бактерий *Escherichia coli*, меченых FITC. Инкапсуляция импланта в гемоцеле личинок вощиной огнёвки *Galleria mellonella* сопровождалась увеличением в 1,8 раз ($p < 0{,}001$) уровня свободных ионов кальция в течение первого часа. Через 4 часа после введения импланта отмечали снижение в 2,5 раза ($p < 0{,}001$) уровня кальция в цитоплазме клеток крови. Развитие процесса фагоцитоза также сопровождалось выбросом в 2,5–3 раза ($p < 0{,}001$) кальция в цитозоль на протяжении 1 часа, с последующим снижением ($p < 0{,}001$) в 2 раза к двум часам по сравнению с уровнем у контрольных насекомых. Полученные результаты свидетельствуют, что при активном формировании капсулы и интенсивном фагоцитозе происходит повышение уровня эндогенного кальция в гемоцитах.

Introduction

Normal function of eukaryotic cells is impossible without calcium ions. Much of the vital activity of eukaryotic cell is regulated by calcium signaling, based on the low concentration of cytoplasm $[Ca^{2+}]_i$ in a resting cell [Tkachuk, 2000; Carafoli, 2002; Ganitkevich, 2003]. Different effects on cells, including the immunocompetent cells, in the first place result in a sharp increase of Ca^{2+} ions in cell cytoplasm [Tojo et al., 2000; Melendez, 2005]. This happens due to the work of Ca^{2+} channels, which provide entry for the ions down their concentration gradient, and the system of active transport of Ca^{2+} (Ca-pumps, Na/Ca-transporters, etc.) [Tkachuk, 2000; Ganitkevich, 2003; Dushay, 2009]. Long-term influence of high concentrations of calcium, however, is lethal for a cell and the influx of Ca^{2+} into the cytoplasm activates feedback systems that provide inactivation of the channels and the removal of Ca^{2+} ion excess [Tkachuk, 2000; Ganitkevich, 2003; Dushay, 2009]. Active research has been done on the role of calcium ions in the transmission of nerve impulses, regulation of hormonal level, and also in osmoregulation and hemolymph circulation in invertebrates [Iwanaga et al., 1998; Ganitkevich, 2003; Zakarian et al., 2003]. Hematopoietic tissue cells,

pericardial cells, as well as fixed and circulating freely hemolymph cells — hemocytes, which play the key role in immune response of insects, participate in the formation of cellular immune response of insects [Kurata, 2006; Marmaras, Lampropoulou, 2009; Tsakas, Marmaras, 2010]. Hemocytes take part in phagocytosis, encapsulation and nodule formation. Phagocytosis and encapsulation are directly related to activation of the cytoskeleton of hemocytes, of which conformational changes are impossible without the participation of calcium ions [Smaili et al., 2000; Tojo et al., 2000; Melendez, 2005; Dushay, 2009].

The formation of capsule is followed by active destruction of immunocompetent cells, during which the content of granules is released and first and foremost prophenoloxidase and calcium ions [Marmaras et al., 1996; Melendez, 2005; Dushay, 2009]. Therefore, in invertebrates the participation of calcium ions is required for cell immune reactions to take place [Stanley et al., 1999; Tojo et al., 2000; Willott et al., 2002; Tsakas, Marmaras, 2010]. Although the dynamics of changes in the calcium level in the cytoplasm of immunocompetent cells of insects during the protective reactions, is little studied.

In the present work we have observed changes of the free calcium concentration in blood cells of the great wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) during the process of encapsulation and phagocytosis of foreign bodies.

Materials and Methods

Insects. A laboratory population of *Galleria mellonella* (Lepidoptera: Pyralidae) was maintained at 28 °C on 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.

Preparation of hemocytes suspension. To prepare a hemocyte suspension, hemolymph was collected in plastic tubes containing cooled (+4 °C) anticoagulant [AC; 62 mM NaCl, 100 mM glucose, 10 mM EDTA, 30 mM sodium citrate, 3.26 mM hydroxyl tricarballylic acid (citric acid), pH 4.6. Then the hemolymph was centrifuged for 5 min. at 500 g the pellet obtained was re-suspended and the hemocytes were washed three times in cold AC for 5 min. at 500 g and once with HEPES-buffer, pH 7.2 (140 mM NaCl, 5 mM KCl, 6 mM glucose, 10 mM HEPES; pH adjusted to 7.2 NaOH).

Measurement of cytosolic calcium concentration. The measurement of cytosolic calcium concentration was performed with Fura 2 AM fluorescent indicator. The lymph was collected into cooled anticoagulant free from EDTA (AC +4 °C). About 250 µl of AC^- samples of 15 larvae were pooled. 20 µl of hemolymph from each larva was extracted. The hemocytes were washed out three times in AC^- for 5 min. at 500 g and once in HEPES-buffer, 7.2 pH. After the last wash the volume of hemocyte suspension was adjusted to 500 µl in HEPES–buffer with Fura 2 AM at the final concentration of 3 µM.

The prepared suspension was incubated in the dark for 45 min at 28 °C with continuous intensive shaking. After the incubation the hemocytes were washed out two times with loading buffer (LB; 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ x $6H_2O$, 10 mM HEPES, 2.6 mM $NaHCO_3$, 10 mM glucose] and for 15–20 min were balanced subjected to continuous shaking. Before the measurement was performed, cells had been washed out with 3B again and the hemocyte suspension had been pipetted into plate wells for spectrophotometric testing. The measurement was carried out with the help of spectrofluorometer VARIAN Cary Eclipse (Australia) at excitation 340 nm and emission 505 nm.

Phagocytosis assay. Phagocytic activity was assessed using FITC-labeling *Escherichia coli*. Bacterial cells were injected in hemocoel (10^5 bacterial cells of each larva). The phagocytic cell activity was investigated at 15 min, 30 min, 1 h and 2 h after the injection. The hemolymph was collected into ice-cold anticoagulant saline and centrifuged. The supernatant was discarded, and hemocytes were washed out three times in anticoagulant saline, and then the cells were diluted in HEPES buffer (0.15 M, pH 7.2). Hemocyte monolayers were placed in fresh wells for 15 min. in the dark at 28 °C. Afterwards the monolayers were fixed with 0.25 % glutare aldehyde (10 min.) and washed out in distillate water. Then the monolayers with drops of Trypan blue were analyzed under fluorescence phase-contrast microscope (Axioscop 40). All the hemocytes present in a photo frame area were counted, and the cells with fluorescent bacteria were distinguished. This process was repeated 10 times.

Changes of cytosolic calcium during the phagocytic activity were assessed using inactivated *Escherichia coli*. Bacterial cells were injected in the hemocoel (10^5 bacterial cells of each larva). Before bleeding, larvae were washed out with distilled water and then with 70 % (v/v) ethanol to sterilize their surface. The larvae were bled by cutting the posterior segment and applying a gentle pressure in order to extrude a drop of the hemolymph. To assess the dynamics of cytosolic calcium, the hemolymph was collected at 15 min., 30 min., 60 min. and 120 min. after the injection. The hemolymph of 15 larvae was pooled in one sample. The analysis of cytosolic calcium concentration in hemocytes suspension was measured using the aforementioned method.

Encapsulation assay. To assess the thickness of a newly formed capsule, the plastic implants (2 mm long and 0.25 mm in diameter with a nodule at the end) were removed at the intervals of 15 min., 30 min., 1 h, 4 h and 24 h, dehydrated in a graded acetone series and then embedded in Epon-Araldite resin mixture for 24 h at 60 °C. Semi-thin sections were cut from the prepared blocks with a LKB Ultratome III. A thickness of the melatonic capsule was measured with light mic-

rioscope (Axioscop 40, Carl Zeiss, Germany).The dynamics of cytosolic calcium was measured at the intervals of 15 min., 30 min., 1 h, 4 h and 24 h. The hemolymph of 15 larvae was pooled in one sample. The analysis of cytosolic calcium concentration in hemocytes suspension was measured using the aforementioned method.

Statistics. Obtained data have been presented as average ± its standard error. To test the normality of sampling the Wilk, Shapiro W criterion was used. The encapsulation rate, phagocytic activity and calcium ions concentration were tested with one-way ANOVA, followed by Tukey's post-hoc tests to identify specific differences between means (Statistica 6.0).

Results

While investigating the dynamics of changes in the cytosolic calcium level by inserting the implant into the hemocoel of *G. mellonella* larvae, we registered a slight rise (by 1.2) of free calcium ions concentration during the encapsulation at the first 15 min ($F = 11.70$, d.f. = 11, $p = 0.005$) and 30 min ($F = 8.95$, d.f. = 14, $p = 0.0098$) followed by a significant increase (by 1.8, $F = 99.97$, d.f. = 11, $p < 0.001$) of the registered values after 1 h (Fig. 1). In 4 h after implant insertion we noticed a decrease of the number of calcium ions in the cytoplasm of blood cells ($F = 58.85$, d.f. = 10, $p < 0.001$) with an increase of the number of calcium ions in 24 h of encapsulation of implants ($F = 149.08$, d.f. = 20, $p < 0.001$) (Fig. 1).

Analysis of the capsule thickness showed that already in 15 min after implant insertion a formation of the capsule with 5.4 ± 1.4 μm thick walls (Fig.1) takes place. In 30 min the wall thickness increases to the values of 6.6 ± 2.01 μm ($F = 12.89$, d.f. = 48, $p < 0.001$) and it is evident that during the next 4 h it does not change further (Fig. 1). In 24 h the thickness of capsule wall was 9.2 ± 2.2 μm, which has definitely exceeded the previous value ($F = 28.75$, d.f. = 48, $p < 0.001$).

While analyzing the dynamics of cytosolic calcium concentration during the process of phagocytosis within the first 1 h after the injection of bacterial cell suspension, we have detected an increase (by 3; $F = 28.10$, d.f. = 10, $p < 0.001$) in 15 min (by 1.5, $F = 29.33$, d.f. = 14, $p < 0.001$) in 30 min and (by 2.5, $F = 226.20$, d.f. = 14, $p < 0.001$) in 1 h of the cytosolic calcium level followed by a control level at the 2 hours (Fig. 2). At the same time we have noticed that the intensity of phagocytosis reaches its maximum values by the end of the first hour compare with 15 min ($F = 386.25$, d.f. = 12, $p < 0.001$) and 30 min ($F = 71.59$, d.f. = 12, $p < 0.001$) after the injection of target cells. By the end of the second hour, the recognized percentage of phagocytosis decreased by 1.5 against to 1 h after injection ($F = 171.41$, d.f. = 11, $p < 0.001$) (Fig. 2).

Discussion

Calcium is known to be an integral factor that increases the activity of phenoloxidase and proteolytic enzymes, which trigger prophenoloxidase cascade, a coagulogen, building a clot around an object [Tacahashi, Enomoto, 1987; Pech, Strand, 2000; Lavine, Strand, 2002]. In addition, capsule formation around a foreign body is followed by the flattening and destruction of hemocytes, i.e. apoptosis, which is directly connected to the emission of calcium into cytosol. It has been known that these reactions are followed by the release of calcium into the surrounding area, inclu-

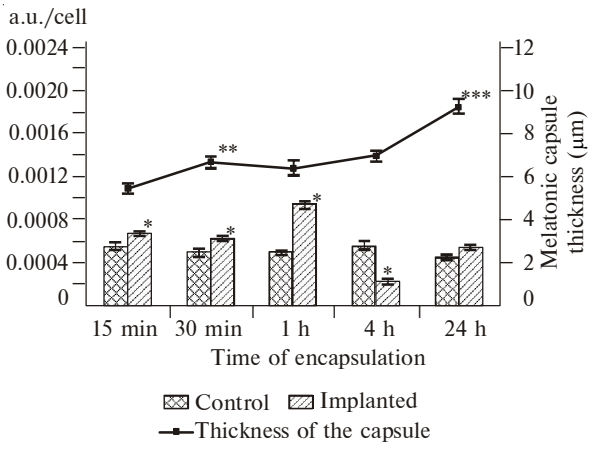

Fig. 1. Cytosolic calcium concentration in hemocytes of control *G. mellonella* larvae and larvae implanted by a nylon implant [*$p < 0.001$ in comparison with control larvae; **$p < 0.001$ in comparison with larvae at 15 min of encapsulation; ***$p < 0.001$ in comparison with larvae at 4 h of encapsulation].

Рис. 1. Уровень цитозольного кальция в гемоцитах *G. mellonella* при развитии процесса инкапсуляции нейлонового импланта [*$p < 0{,}001$ в сравнении с контрольными насекомыми; **$p < 0{,}001$ в сравнении с инкапсуляцией через 15 мин.; ***$p < 0{,}001$ в сравнении с инкапсуляцией через 4 часа].

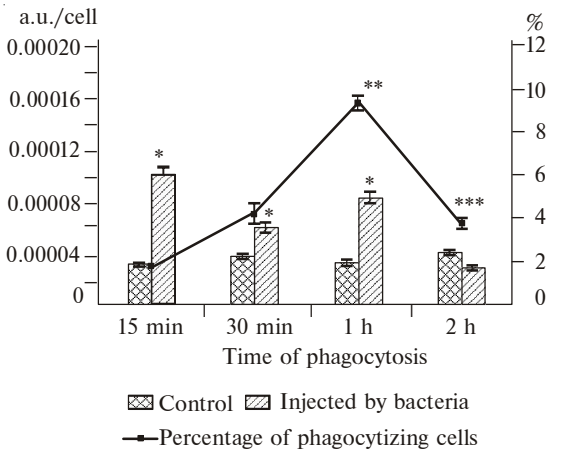

Fig. 2. Cytosolic calcium concentration in hemocytes of control *G. mellonella* larvae and larvae injected by target cells [*$p < 0.001$ in comparison with control larvae; **$p < 0.001$ in comparison with larvae at 15 and 30 min of phagocytosis; ***$p < 0.001$ in comparison with larvae at 1 h of phagocytosis].

Рис. 2. Уровень цитозольного кальция в гемоцитах *G. mellonella* при фагоцитозе бактериальных клеток [*$p < 0{,}001$ в сравнении с контролем; **$p < 0{,}001$ в сравнении с 15 и 30 мин. фагоцитоза; ***$p < 0{,}001$ в сравнении с 1 ч фагоцитоза].

ding that of the destroyed cells [Smaili et al., 2000; Tojo et al., 2000; Willott et al., 2002; Dushay, 2009]. The manifested active capsule layer formation during the first 0.5 h seems to explain the registered emission of cytosolic calcium during the first hour after implant insertion.

It is known that for a particle to internalize into a phagosome receptor engagement and a complex sequence of events that require the kinase activation, alterations in the phospholipid metabolism, remodeling of the active cytoskeleton and acceleration of membrane traffic. These processes are always followed by an increase of intracellular calcium [Jaconi et al., 1990; Yang et al., 2000; Tejle et al., 2002; Brooks, Dunphy, 2005; Melendez, 2005; Nordenfelt, Tapper, 2010]. The absence of release of Ca^{2+} are likely to be determined by the fact that initiation of phagocytosis is not always required at the early stages of recognition of a foreign target, but it surely does play an important role in the formation and destruction of phagosome and apparently in the identification of an antigen [Jaconi et al., 1990; Carafoli, 2002; Tejle et al., 2002; Melendez, 2005; Tsakas et al., 2010]. It is known that before a particle will dip into phagosome the interaction of receptors of the immune cells with target is necessary. This interaction invokes the kinase activation, changes of phospholipid metabolites, remodeling of the active cytoskeleton and acceleration of membrane traffic. These processes always accompanied by the increase in concentration of cytoplasmic Ca^{2+} [Jaconi, 1990; Yang et al., 2000; Tejle et al., 2002; Melendez, 2005; Nordenfelt, Tapper, 2010].

Thus, the present study of *G. mellonella* first describes the changes in cytosolic calcium concentration during the capsule formation and phagocytosis. Based on the obtained results we suggest that the basic reactions related to the hemocyte activation, their conformational changes and the synthesis of secondary messengers of cell immune response of *G. mellonella* larvae occurs within the first 1–1.5 h after the activation of cell immune response.

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