Study of the Vesicular Cycle in Nerve Structures in Somatic Muscle of Earthworm (Lumbricus terrestris)

Kazan Medical State University, Kazan, Tatarstan, Russia
*e-mail: euroworm@mail.ru
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Abstract—In the muscle wall of the earthworm Lumbricus terrestris, with the aid of fluorescent endocytotic dyes FM1-43, FM2-10, and FM4-64, there are revealed fluorescent spots 1–2 µm in diameter that represent clusters of “synaptic boutons.” Application takes place onto ganglia of the abdominal nerve chain of the Dil membrane probe capable of translocation by axoplasmic transport; the subsequent (next day) staining of nerve structures with the endocytotic marker FM4-64 showed the complete superposition of fluorescence of these dyes fluorescing in different specter areas. The fluorescent marker DiBAC₄(3) revealed an enhancement of fluorescence of nerve elements with increase of K⁺ concentration in the extracellular medium. Use of FM2-10 showed that, the higher the K⁺ content in solution and, accordingly, the nerve cell depolarization, the faster the release of the marker and, on the contrary, the slower the process in the absence of K⁺ in the medium. In the Ca²⁺-free solution and in the presence of the Ca²⁺ chelator BAPTA or BAPTA-AM, there uptake and release of FM2-10 are blocked, but only after preliminary 40-min incubation in such solution. In clusters of synaptic boutons, exo- and endocytosis processes take place that are also preserved under conditions of rest. This vesicular cycle depends on the membrane potential of nerve structures and on the content of K⁺ and Ca²⁺ in the medium, the calcium sensor working most likely by the “all or nothing” principle.

Keywords: earthworm, fluorescent markers, “synaptic boutons,” vesicular cycle
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INTRODUCTION

Somatic cells of the earthworm muscle wall are innervated segmentwise by neuronal processes of the abdominal nerve chain. In synaptic structures there are revealed vesicular structures resembling those in motor terminals of vertebrate animals (Rosenbluth, 1972; Farnesi, Vagnetti, 1975), whereas, in muscle cells, miniature spontaneous postsynaptic currents are recorded (Volkov et al., 2007). With the aid of the FM1-43 fluorescent dye, synaptic zones were found optically in the earthworm muscle wall, which were identified by the presence of fluorescent spots several µm in diameter (Shimizu et al., 1999). Meanwhile, information about the mechanisms of the exoendocytotic cycle in nerve elements of the annelid primary cross musculature is practically absent.

The goal of the present work was a study of the peculiarities of the vesicular cycle in synaptic structures in the earthworm muscle walls with the use of fluorescent markers.

MATERIALS AND METHODS

Experiments were carried out on somatic muscle of the internal side of the cutaneous-muscular sac of the earthworm Lumbricus terrestris. Freshly prepared, 10- to 15-segment-long fragments purified from celomic organs were placed into a modified Dreves–Pax solution (Volkov et al., 2001) at room temperature of the following ion composition (mol/l): 16 Na⁺, 3 K⁺, 6 Ca²⁺, 93 Cl⁻, 43 SO₄²⁻, 2 Tris, pH 7.4. The ion composition of the solutions was modified taking into account the constancy of ion strength and osmolality. An increase or a decrease of K⁺ or Ca²⁺ was compensated for by the corresponding change of Na⁺ concentration. To bind extracellular Ca²⁺, we used BAPTA or BAPTA-AM, at concentrations of 0.05 and 0.5 mmol/l, respectively. In several experiments, to dye nerve ending, we used a Dil fluorescent membrane probe emitting in the green spectrum area (exciting photofilter 470–500 nm, emissional filter 530–570 nm). Crystal of the dye was placed onto ganglia of the abdominal nerve chain. Subsequently, with aid of axoplasmic transport and lateral diffusion, the dye was spread from neuronal soma by nerve conductor and stained nerve structures (Nguyen et al., 2007).

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fluorescing in the green spectrum (Mennerick et al., 2010). In the process of cell membrane depolarization, the dye input into the cytoplasm is enhanced, which is accompanied by an increase of fluorescence. Hyperpolarization of the membrane leads to the opposite effect.

After isolation of the muscle preparation, it was placed into a bath for experiments with 2 ml of potassium-free solution. Then 0.5 µl (2 µmol/l) DiBAC₄(3) were added and kept for 40 min. The intensity of fluorescence was measured each 2 min for 10 min. After this, the solution was replaced consecutively by 3, 10, and 40 mmol/l K⁺, respectively, and the procedure of measurement of intensity of fluorescence was repeated.

FM1-43 and FM4-64 membrane fluorescent dyes were used at a concentration of 2–3 µmol/l, as well as FM2-10 at a concentration of 22–24 µmol/l, which, during endocytosis, turn out to inside the newly formed vesicles (“loading”) (Betz, Bewick, 1993; Zefirov et al., 2003). Loading of the dye was performed in Dreves–Pax solution for 3 min. To reduce the background fluorescence after loading with the dye, the preparation was kept for 10 min in solution without perfusion. To determine the dynamics of the loss of the dye, the preliminarily loaded nerve endings were submitted to action of solutions with different K⁺ concentrations (0, 3, 10, and 40 mmol/l) by recording a decrease of the fluorescence intensity for 40–50 min. To provide dissociation of the dye from membranes, ADVASEP-7 reagent was used at a concentration of 10 µmol/l. Each series of experiments was performed on 10–12 earthworms. Statistical processing of data was performed with use of Student’s criterion and ANOVA.

Fluorescence was observed with the aid of a DP71 CCD videocamera (Olympus, Germany) and an Olympus BX51 microscope (Japan) supplied with an LUMPLFL 60 XW objective and a DSU confocal system. In work with fluorescent markers, we used an exciting photofilter of 470 ± 10 nm and an emission filter transmitting light with a wavelength of more than 515 nm. The fluorescence intensity was estimated in relative units of the pixel fluorescence (with the use of Cell P and ImagePro software). In work with markers, the following way of the fluorescence estimation was used. The value of the background fluorescence was determined as the mean fluorescence intensity in a square of 50 × 50 pixels in the site of the image without a nerve ending. The fluorescence of the nerve ending was determined after subtraction of the background fluorescence (Petrov et al., 2008). The fluorescence intensity was estimated as the mean fluorescence of all pixels in the contour of the central site (40 µm) of individual nerve terminal or as the mean fluorescence of all pixels in the contour of individual spots (fluorescence of spots).

The following reagents were used: BAPTA (1,2-bis(2-aminophenoxy)-N,N,N,N-tetraacetic acid), BAPTA-AM (1,2-bis(2-aminophenoxy) ethane-N,N,N,N-tetraacetic acid acetoxyethyl ester), Dil (dialkylcarbocyanine, lipophilic tracer sampler Kit), and DiBAC₄(3) (bis-(1,3-dibutylbarbituric acid)trimethine oxonol) from Sigma (United States); FM1-43 (N-(3-triethylammoniumpropyl)-4-(4-diethylamino)styryl)pyridinium dibromide), FM4-64 (N-(3-triethylammoniumpropyl)-4-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide), FM2-10 (N-(3-triethylammoniumpropyl)-4-(4-diethylamino)styryl) pyridinium dibromide), and ADVASEP-7 (sulfonated b-cyclodextrin C42H70-n035(C4H6SO3Na)n from Biotium (United States).

RESULTS

Identification of synaptic structures in earthworm muscle. Earlier it was shown that use of FM1-43 fluorescent dye revealed fluorescent spots in the earthworm muscle wall, which, in the authors’ opinion, suggest a possibility of application of the dye for visualization of the processes occurring in synaptic structures in annelids in the same way as in vertebrate animals (Shimizu et al., 1999).

Incubation of the muscle preparation for 3 min in solution with normal K⁺ content in the presence of FM1-43 dye led to the appearance of stained structures reminiscent of a bunch of grapes. Each bunch was composed of several tens of spots of oval shape and diameter of 1–2 µm (Fig. 1a). Clusters of spots are located in a strict order in immediately by thickenings of the abdominal nerve chain: in each metamere of the animal body, there were found two such clusters located symmetrically at both sides from the medial line. This agrees with ultrastructural studies of myoneural synapses of the annelid somatic musculature (Farnesi, Vagnetti, 1975), as well as with data on optical detection of synaptic structures in earthworm muscle with the aid of fluorescent dyes (Shimizu et al., 1999; Mizutani et al., 2003). Since the diameter of the synaptic boutons of nerve terminals is known to amount approximately to 1 µm (Fernandes-de-Miguel, Drapeau, 1995), it can be suggested that the here-revealed grape bunches represent agglomerations or clusters of synaptic boutons, where an individual spot represents one or possible several synaptic terminals (Shimizu et al., 1999).

To confirm the connections of the revealed structures with the abdominal nerve chain, we performed the following experiments. The Dil membranous probe fluorescing in the green spectrum area (530–570 nm) was applied onto ganglia of the abdominal nerve chain. By diffusing in the membrane, it was translocated for 1 day from nerve cell bodies to nerve endings by filling the entire volume. The preparation labeled with Dil was loaded with an FM4-64 endocytotic marker with red fluorescence (the excitational photofilter 530–570 nm, the emissional photofilter 710–750 nm). Meanwhile, both Dil and FM4-64 dyed the same structures (Fig. 2). In other words, the