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BIOCHEMISTRY, BIOPHYSICS AND MOLECULAR BIOLOGY

Synaptosome-Associated Protein 25 (SNAP25) Synthesis in Terminal Buttons of Mouse Motor Neuron

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Abstract—Previously, we formulated the hypothesis of compartmentalized protein synthesis in axons of motor neurons. In the axon hillock, along the entire length of the axon and in its ending, specific proteins are locally synthesized, which ensure the function of each compartment. In support of this hypothesis, in this work we studied the local protein synthesis in mouse motor nerve ending.

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It is currently believed that proteins in neurons are synthesized not only in the perikaryon and dendrites but also in the axon [1]. Taking into account the fact that the functions of the axon in different sections are specific (the nerve impulse is generated in the axon hillock and conducted along the entire length of the axon, whereas neurotransmitters are secreted only in its endings), the hypothesis of the compartmentalized protein synthesis in the axon was formulated [2]. In other words, there is reason to believe that the proteins that ensure the functioning of the given axonal compartment are preferentially synthesized in different axon regions. On the basis of above, it can be assumed that the proteins that ensure the neurotransmitter exocytosis are synthesized in the terminal branches of the axon. Following this hypothesis, in the experiments on the neuromuscular preparation of the rat, we first immunohistochemically detected the ribosomal protein L26 in the motor nerve endings of the axon by electron microscopy [3]. It is known that proteins of the SNARE family (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) are involved in the neurotransmitter secretion in the nerve terminal. One of the proteins of the SNARE complex is the SNAP25 protein (synaptosome-associated protein of 25 kDa), which is predominantly localized in the plasma membrane and is involved in the Ca^{2+}-dependent fusion of synaptic vesicles with the presynaptic membrane. Since the half-life of SNAP25 is only 16 h [4], constant renewal of this protein for normal functioning of the synapse is required. Therefore, the demonstration of the fact of SNAP25 synthesis in axon endings can be a good confirmation of the validity of the hypothesis of the compartmentalized protein synthesis in the axon.

One of the modern methods used to investigate protein functions is RNA interference, which allows local inhibition of protein production by degrading its mRNA. Previously, using this method, we have demonstrated the existence of an RNA interference mechanism in the axon and the possibility of using a short interfering RNA (siRNA) to study protein synthesis in nerve endings [5, 6].

At the first stage of this study, to obtain direct evidence of the possibility of protein synthesis in the presynaptic area, the recombinant mRNA of the green fluorescent protein (GFP) was injected into the motor nerve endings, after which specific fluorescence was detected. At the second stage of the study, to confirm the local synthesis of the membrane fusion protein SNAP25 in synaptic vesicles, we detected the disturbance of the quantum secretion of neurotransmitter acetylcholine in the neuromuscular preparation after the injection of a siRNA complementary to SNAP25 mRNA into the nerve endings.

The experiments were performed on isolated neuromuscular preparations of m. levator auris longus of C57BL/6N mice (Pushchino animal breeding facility). The preparation was placed in an experimental cuvette and transfused with Ringer's solution containing 137.0 NaCl mM, 5.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11.0 mM $NaHCO_3$, and 11.0 mM glucose (pH 7.3–7.4). To prevent muscle contractions when recording the single-quantum endplate currents (EPCs), the experiments on studying the electrophysiological activity of the neuromuscular preparation were performed using saline with a reduced calcium content (0.5 mM) and

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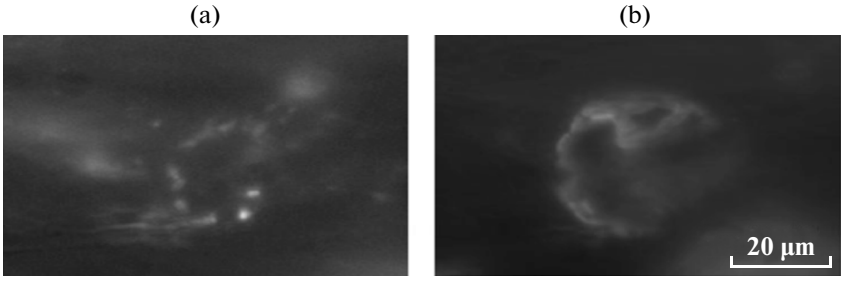

Fig. 1. Injection of the green fluorescent protein mRNA into the mouse motor nerve ending. (a) Microphotograph of the mouse motor nerve ending 24 h after the GFP mRNA microinjection in the fluorescence mode. (b) Microphotograph of the mouse nerve ending in the fluorescence mode. The postsynaptic nicotinic receptors were stained with the labeled α-bungarotoxin (the same preparation).

an increased magnesium content (5.0 mM). Experiments were performed at a temperature of 20.0 ± 0.3°C, which was maintained using Peltier elements.

GFP mRNA was synthesized using the mScript mRNA Production System kit (MSC11610, Cambio, Great Britain) according to the instructions of the manufacturer. The Monster Green Fluorescent Protein phMGFP Vector plasmid (E6421, Promega, United States) was used as a template DNA. GFP mRNA was injected into the nerve ending at a concentration of 0.38 μg/μL. In the experiments with the inhibition of protein synthesis by the RNA interference mechanism, the motor nerve ending in the test series ($n = 4$) was injected with 100 nM siRNA complementary to the mRNA for the SNAP25 synaptosome-associated protein (sc-36516, Santa Cruz Biotechnology, Inc., United States), and the motor nerve ending in the control series ($n = 4$) was injected with a solution of corresponding inactive siRNA (100 nM, sc-37007, Santa Cruz Biotechnology, Inc.). GFP mRNA and siRNA were dissolved in 200 mM of KCl preliminarily subjected to sterilizing filtration.

Microinjections of RNA into the nerve ending were performed under a visual control with an Olympus BX 51 microscope (Olympus Corp., Japan) equipped with a water-immersion lens (magnification, ×60) as described in [7]. An injection micropipette with a resistance of 150–200 mOhms containing an aliquot of the injection material was brought close to the ending using a Piezo-Patch PPM5000 micromanipulator (WPI, United States). Using the "penetration" mode of the micromanipulator, the membrane of the nerve ending was penetrated and injected with approximately 100 pL of siRNA or GFP mRNA using a PV820 pneumoinjector (WPI). To ensure visual verification of the quality of microinjections and the absence of lesions of the presynaptic membrane, siRNA solutions were supplemented with the indifferent fluorescent dye Alexa fluor 448 (Invitrogen, United States).

After the microinjection with GFP mRNA, the neuromuscular preparation was placed in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin solution (PanEco, Russia) and cultured for 24 h. The fluorescence study revealed specific fluorescence in the nerve ending area, unambiguously indicating the synthesis of GFP in axon endings. Additional staining of the neuromuscular preparation with α-bungarotoxin conjugated with the fluorescent label showed that the specific fluorescence is observed at the sites where the postsynaptic acetylcholine receptors are located (Fig. 1).

The synaptic activity before and after the siRNA injection was recorded by the standard microelectrode technique. The spontaneous and induced EPCs were recorded after nerve stimulation at a frequency of 15 pps. The mean quantum content of the EPC was calculated "directly" as the ratio of the number of recorded EPCs to the number of stimulation pulses. It was found that, 4 h after the injection with siRNA complementary to the SNAP25 mRNA, the mean quantum content of EPC decreased ten times (0.30 ± 0.04 quanta in the experiment and 0.03 ± 0.02 quanta in the control, $n = 4$, $p < 0.05$). Injections with the control siRNA had no significant effect on the mean quantum content of the EPC (0.46 ± 0.13 quanta before injection and 0.40 ± 0.15 quanta after injection, $n = 4$, $p > 0.05$, Fig. 2).

The results of this study showed that SNAP25 is synthesized in the motor nerve endings and that the level of SNAP25 mRNA affects the activity of exocytosis of the neurotransmitter. In addition, these results suggest that, in the terminal branches of the axon of the motor neuron of mammals, "work" the molecular mechanisms that are responsible not only for protein synthesis, but also for the degradation of mRNA.

Thus, in this study, we have confirmed the validity of the hypothesis of the compartmentalized protein synthesis in mammalian motor neuron axons. The results of the study of the GFP synthesis led us to conclude that the motor nerve ending contains all the components required for protein synthesis, and the experiments with the injection of siRNA complementary to the SNAP25 mRNA confirmed the existence of local protein synthesis of proteins of the

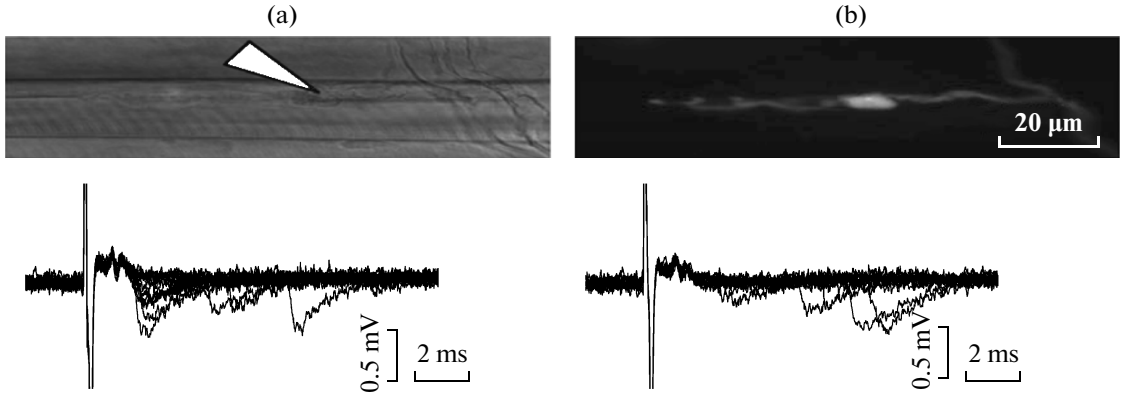

Fig. 2. Injection of siRNA complementary to the mRNA of the SNAP25 synaptosome-associated protein into the mouse motor nerve ending. (a) Microphotograph of the mouse nerve endings in the "bright field" illumination mode and the superposition of the induced nerve ending currents and EPCs after the stimulation of the motor nerve at a frequency of 15 pps in the control. The arrow indicates the site of siRNA microinjection. (b) Microphotograph of the nerve ending in the fluorescence mode and the superposition of induced currents of the nerve ending and EPCs after the stimulation of the motor nerve with a frequency of 15 pps 4 h after the microinjection with the complementary siRNA. Data were obtained for the same preparation.

SNARE family, which ensure the exocytosis of the neurotransmitter.

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