Role of Cholinoceptors Recycling in Short-Term Potentiation of Cholinosensitivity of Command Neurons in Edible Snail

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Exocytosis inhibitor Exo 1, dynamin inhibitory peptide (inhibitor of endocytosis), and colchicine disturb short-term potentiation of cholinosensitivity of defensive behavior command neurons in edible snail induced by rhythmic electrical orthodromic stimulation. We hypothesize that the short-term potentiation of cholinosensitivity in the extrasynaptic membrane develops due to incorporation of extra cholinoceptors into neuron plasmalemma as a result of enhanced recycling of the internalized cholinoceptors with participation of microtubules.

Key Words: *Exo 1, inhibiting peptide dynamin; colchicine; potentiation of cholinosensitivity; edible snail command neurons*

Rhythmic electrical stimulation of the foot of edible snail induces short-term sensitization of the defense reaction provoked by tactile input [1,2]. Potentiation of this reaction and enhancement of cholinosensitivity of the defensive behavior command neurons induced by rhythmic orthodromic stimulation of *n. intestinalis* with the same parameters as in the behavioral experiments are similar in dynamics [1,2,6]. Therefore, the increase in cholinosensitivity of the plasmalemma of defensive behavior command neurons can be involved in into the neuronal mechanism of the systemic defense reaction.

Since inhibitors of protein synthesis anisomycin and saporin do not prevent potentiation of neuronal cholinosensitivity, it is hardly possible that synthesis of cholinoceptors and their incorporation into plasma membrane underlie potentiation of cholinosensitivity of command neurons in edible snail [1]. An important mechanism of regulation of G-protein-associated receptors, including muscarinic receptors included, is their internalization into the cytoplasm [10,15]. In HEK-293 cells, the muscarinic cholinoceptors are internalized into clathrin-coated vesicles and recycle into the plasma membrane. This internalization pathway depends on the concerted action of β-arrestin, c-Src, and the GTPase dynamin, which catalyses budding of vesicles from the plasma membrane [15]. Internalization and recycling of transmitter receptors are functionally coupled to cytoskeleton microtubules [8].

Our aim was to test the hypothesis that the mechanism of short-term potentiation of cholinosensitivity can involve up-regulation of recycling of cholinoceptors internalized in the cytoplasmic pool into the neuronal plasma membrane with participation of cytoskeletal microtubules.

MATERIALS AND METHODS

The experiments were carried out on identified defensive command neurons LPa2, LPa3, RPa3, and RPa2 [3] of Crimean edible snail *Helix lucorum* under stop-flow conditions in the chamber, where semi-intact preparation of CNS—visceral sac was bathed in physiological saline containing (in mM):

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100 NaCl, 4 KCl, 10 $CaCl_2$, 4 $MgCl_2$, and 10 Tris HCl (pH 7.5-7.7). The neural peripharyngeal ring was placed into a 1-ml perfusion chamber and the visceral sac into a 41-ml bath. After enzyme treatment of the ganglia (0.5% digestase, Seatec, for 20-60 min at room temperature) the connective tissue sheathes were removed.

Acetylcholine (Ach) was applied locally by ionophoresis to the dorsal surface of neuron soma at the rate of 10^{-1} min. Transmembrane ionic current induced by local application of ACh (Ach-currents) were recorded [1,2,5,6].

This current was recorded using the method of two-electrode voltage clamp connecting the outside solution to the virtual ground. The traces were recorded in a PC using a CONAN 3.0 software. The data were obtained on 93 neurons (4 LPa2, 42 LPa3, 46 RPa3, and 1 RPa2) in 93 preparations. Resting potential, neuron input resistance, and holding potential were -52.96±1.12 mV, 4.18±0.27 MΩ, and -75 mV, respectively.

Changes in the amplitude of Ach-currents were analyzed after electrical stimulation of *n. intestinalis* (0.5 μA current amplitude, 30 msec pulse duration, 2 Hz repetition rate, 2 min duration of stimulation). Rectangular pulses were applied via bipolar metal electrodes made of nichrome wire 0.1 mm in diameter (80 MΩ electrode resistance).

The study used the following chemicals: Exo 1 (2-(4-Fluorobenzoylamino)methylbenzoate), a reversible inhibitor of exocytosis (Sigma); *2*) dynamin inhibitory peptide DIP (consisting of 10 amino acids QVPSRPNRAP) also referred to as peptide P4, a competitive inhibitor of clathrin-depending endocytosis, which blocks binding of dynamin GTPase with amphiphysin (Tocris); and *3*) colchicine, a plant membrane-penetrating alkaloid, which destroys cytoskeletal microtubules by binding to tubulin and preventing its polymerization (Sigma). Exo 1 (2 mM) was dissolved in 6% DMSO (Sigma) and 1 M potassium acetate. DIP (0.5 mM) and colchicine were dissolved in 2 M potassium acetate and physiological saline, respectively. The neurons were loaded with Exo 1, DMSO (in control experiments) or DIP for 60-80 min before testing by passive diffusion from intracellular microelectrodes for membrane potential recording (tip resistance 44.17±2.76 MΩ). Colchicine was added to perfusion solution (0.1 mM, 60-min exposure before resting).

The following experimental protocols were used: *1*) spontaneous change of Ach-current amplitude under the action of DMSO without rhythmic stimulation (control series, n=13); *2*) change of Ach-current amplitude after rhythmic stimulation of *n. intestinalis* under the action of DMSO (experimental series, n=11); 3) spontaneous change of Ach-current amplitude under the action of Exo 1 without rhythmic stimulation (control series, n=11); *4*) change in Ach-current amplitude after rhythmic stimulation of *n. intestinalis* under the action of Exo 1 (experimental series, n=10); *5*) spontaneous change of Ach-current amplitude under the action of DIP without rhythmic stimulation (control series, n=14); *6*) change of Ach-current amplitude after rhythmic stimulation of *n. intestinalis* under the action of DIP (experimental series, n=14); *7*) spon-

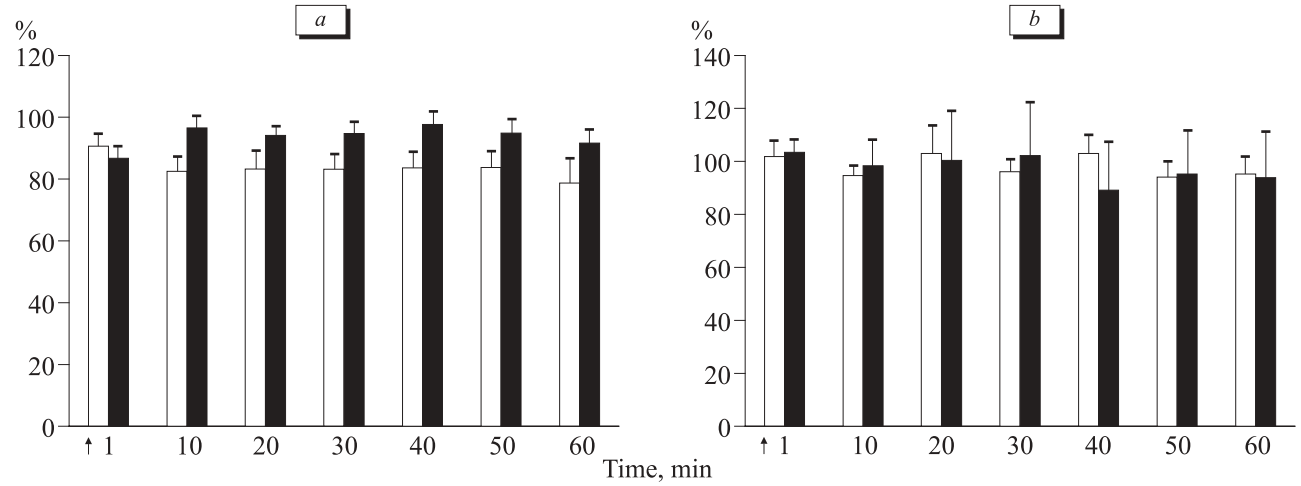

Fig. 1. Effect of intracellular application of DMSO (*a*) and Exo 1 (*b*) on potentiation of Ach-current amplitude in command neurons of edible snail after rhythmic orthodromic stimulation of *n. intestinalis*. Here and in Fig. 2: ordinate: amplitude of Ach-current, % of last (control) Ach-current recorded before rhythmic stimulation in the experimental series. In series without conditioning stimulation, 100% amplitude was taken from the record corresponding by the time to the control response in the experimental series. Light bars: control series without rhythmic electrical orthodromic stimulation; dark bars: after rhythmic stimulation. Arrows mark application of conditioning electrical stimulation.

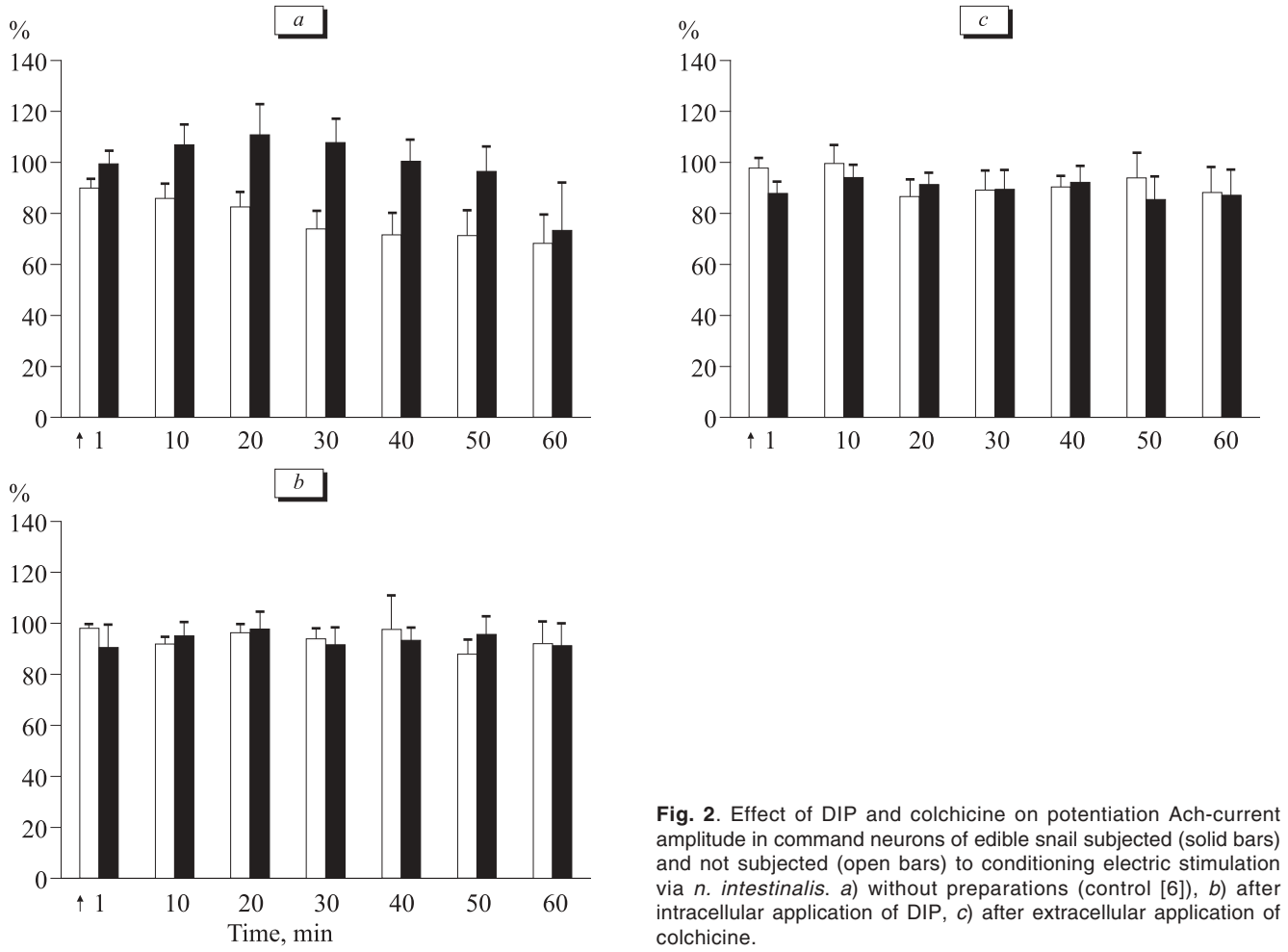

Fig. 2. Effect of DIP and colchicine on potentiation Ach-current amplitude in command neurons of edible snail subjected (solid bars) and not subjected (open bars) to conditioning electric stimulation via *n. intestinalis*. *a*) without preparations (control [6]), *b*) after intracellular application of DIP, *c*) after extracellular application of colchicine.

taneous change of Ach-current amplitude under the action of colchicine without rhythmic stimulation (control series, n=11); *8*) change of Ach-current amplitude after rhythmic stimulation of *n. intestinalis* under the action of colchicine (experimental series, n=9).

The arithmetic means and standart deviations were calculated. The results were analyzed statistically using nonparametric Wilcoxon tests and sign test for paired comparison (STADIA 6.2 software).

RESULTS

Intracellular injection of 6% DMSO did not prevent potentiation of cholinosensitivity: a significant difference was revealed between the control (n=13) and experimental (n=11) series (p<0.05, Fig. 1, *a,*)

Exo 1 disturbed potentiation of neuronal cholinosensitivity (Fig. 1, *b*). No differences between the control (n=11) and experimental (n=10) series were revealed.

Similar to Exo 1, DIP also prevented potentiation of neuronal cholinosensitivity (Fig. 2, *a*, *b*). No differences between the control (n=14) and experimental (n=14) series were revealed.

Colchicine also prevented potentiation of neuronal cholinosensitivity (Fig. 2, *a*, *c*). No differences between the control (n=11) and experimental (n=9) series were revealed.

The antagonizing effect of Exo 1 on potentiation of cholinosensitivity probably attests to the involvement of cholinoceptor recycling via their exocytosis into potentiation of Ach-current. The effect of DIP on potentiation of neuron cholinosensitivity can be explained as follows: after DIP-induced inhibition of spontaneous endocytosis-mediated internalization of cholinoceptors, their recycling from the depleted pool of internalized receptors decreased, which could be the cause of prevention of Ach-current potentiation. Inhibition of Ach-current potentiation with colchicine indicates involvement of microtubules into this process.

These findings suggest participation of recycling of internalized cholinoceptors and involvement of cytoskeletal microtubules into cellular mechanism of potentiation of cholinosensitivity in the

soma of edible snail command neurons. We can hypothesize that orthodromic rhythmic stimulation increasing serotonin content in the hemolymph [11] stimulates incorporation of additional extrasynaptic cholinoceptors into somatic membrane by their exocytosis from the reserve cytoplasmic pool of internalized cholinoceptors.

Incorporation of extra glutamate receptors into the postsynaptic membrane in guinea pig CA1 hippocampal field neurons via vesicular exocytosis is considered as a possible mechanism of long-term potentiation of evoked postsynaptic currents in pyramidal neurons [13]. Exocytosis-mediated incorporation of extra AMPA-receptors into postsynaptic membrane is considered as the most probable mechanism of serotonin-induced short-term (40 min) potentiation of glutamate-induced depolarization of the motoneurons in Aplysia siphon [9] and monosynaptic glutamatergic sensorimotor excitatory postsynaptic potentials (EPSP) of these neurons [12]. However, it cannot be excluded that serotonin can induce the exocytosis-mediated Ca^{2+}-dependent increase in receptor sensitivity to glutamate. The role of microtubules in long-term potentiation of excitatory postsynaptic currents in rat hippocampal neurons was demonstrated [14].

Since defensive behavior command neurons of edible snail have both extrasynaptic [5] and postsynaptic [4,7] cholinoceptors, up-regulation of cholinoceptors recycling can increase the efficiency of interneuronal traffic for a short period during sensitization of the defensive reflex by elevation of sensitivity of the extrasynaptic and probably subsynaptic membrane regions to the neurotransmitter without synthesis of new transmitter receptors.

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REFERENCES

1. M. S. Abramova, A. A. Moskvitin, and A. S. Pivovarov, *Zh. Vyssh. Nervn. Deyat*., **56**, No. 3, 355-362 (2006).
2. M. S. Abramova, V. L. Nistratova, A. A. Moskvitin, and A. S. Pivovarov, *Ibid.*, **55**, No. 3, 408-415 (2005).
3. V. N. Ierusalimskii, I. S. Zakharov, T. A. Palikhova, and P. M. Balaban, *Ibid.*, **42**, No. 6, 1075-1089, (1992).
4. T. A. Palikhova, M. S. Abramova, and A. S. Pivovarov, *Byull. Eksp. Biol. Med*., **142**, No. 9, 244-247, (2006).
5. A. S. Pivovarov and E. I. Drozdova, *Neirofiziologiya*, **24**, No. 1, 77-86 (1992).
6. A. S. Pivovarov, E. I. Drozdova and A. A. Moskvitin, *Zh. Vyssh. Nervn. Deyat*., **49**, No. 6, 990-998 (1999).
7. A. G. Ter-Markaryan, T. A. Palikhova, and E. N. Sokolov, *Ibid.*, **40**, No. 1, 183-184 (1990).
8. G. Cheng, F. Qiao, T. N. Gallien, *et al., Am. J. Physiol. Heart Circ. Physiol.*, **288**, No. 3, H1193-H1202 (2005).
9. R. A. Chitwood, Q. Li, and D. L. Glanzman, *J. Physiol*., **534**, Pt. 2, 501-510 (2001).
10. J. M. Edwardson and P. G. Szekeres, *Life Sci*., **64**, No. 6-7, 487-494 (1999).
11. J. Levenson, J. Byrne, and A. Eskin, *J. Neurosci.,* **19**, No. 18, 8094-8103 (1999).
12. Q. Li, A. C. Roberts, and D. L. Glanzman, *Ibid.*, **25**, No. 23, 5623-5637 (2005).
13. P. M. Lledo, X. Zhang, T. C. Sudhof, *et al., Science,* **279**, No. 5349, 399-403 (1998).
14. H. D. Lux and N. S. Veselovsky, *Neurosci. Lett.,* **178**, No. 2, 231-234 (1994).
15. C. J. van Koppen, *Biochem. Soc. Trans.,* **29**, Pt. 4, 505-508 (2001).