Cholinergic Receptor Exocytosis under Conditions of Depression of Acetylcholine-Induced Current in Edible Snail Neurons in Cellular Analogue of Habituation

D. A. Makhnovskiy, G. B. Murzina*, and A. S. Pivovarov

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 4, pp. 410-413, April, 2012
Original article submitted February 11, 2011

Exocytosis inhibitor Exo 1 potentiates depression of acetylcholine-induced inward current in defensive behavior command neurons of edible snail, when acetylcholine is applied rhythmically to the soma in cellular correlate of habituation. A mathematical model presupposing different receptor localization in the cell and regularities of their translocations made it possible to analyze the dependence of acetylcholine-induced current depression on a number of intracellular processes. It was concluded that depression of choline-sensitive extrasynaptic zones on the membrane of defensive behavior command neurons in edible snail in cellular correlate of habituation is partially determined by attenuation of exocytosis of internalized cholinergic receptors.

Key Words: *Exo 1; exocytosis; depression of acetylcholine-induced current; edible snail command neurons; habituation*

Rhythmic local application of acetylcholine (ACh) to the soma of defensive behavior command neurons of edible snail results in reversible depression of choline-sensitive extrasynaptic zones in cellular analogue (correlate) of behavioral habituation [5,13,14]. Calculations of receptor states, including membrane-coupled and intracellular receptors, using a mathematical model demonstrated that depression of choline-sensitive neurons can be associated with reduced number of membrane-coupled cholinergic receptors (CR) due to their enhanced internalization and attenuation of CR recycling from the cytoplasm [3]. Intensification of CR endocytosis in depression of choline sensitivity in command neurons was discovered experimentally [3]. The objective of our study included experimental verification of the role of extrasynaptic CR exocytosis in depression of neuronal soma choline sensitivity in cellular analogue of habituation.

MATERIALS AND METHODS

Experiments were carried out on identified neurons LPa3, LPa2, RPa3, and RPa2 [1] of edible snail *Helix lucorum* (Crimea population) on the isolated ganglion preparation. These neurons control defensive behavior [2]. Their membrane contains extrasynaptic [6] and postsynaptic [4,7] CR.

Methods of making isolated ganglion preparation, transmembrane current registration and local ACh application are described in details previously [3].

The study employed compound Exo 1, reversible exocytosis inhibitor (IC_{50} ~ 20 μM), a blocker of vesicular transport between endoplasmic reticulum and Golgi apparatus [10]. Exo 1 (2 mM) was dissolved in 6.7% DMSO and 0.2 M potassium acetate (all Sigma). Intracellular electrode for potential recording was filled with this solution (electrode impedance 7-118 MΩ, 48.8±5.4 MΩ). Neurons were loaded with Exo

Department of Higher Nervous Activity, M. V. Lomonosov Moscow State University, Moscow; *Laboratory of Mathematical Neurobioligy of Learning, Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Moscow, Russia. ***Address for correspondence:*** as_pivovarov@mail.ru. A. S. Pivovarov

0007-4888/12/1534-0424 © 2012 Springer Science+Business Media, Inc.

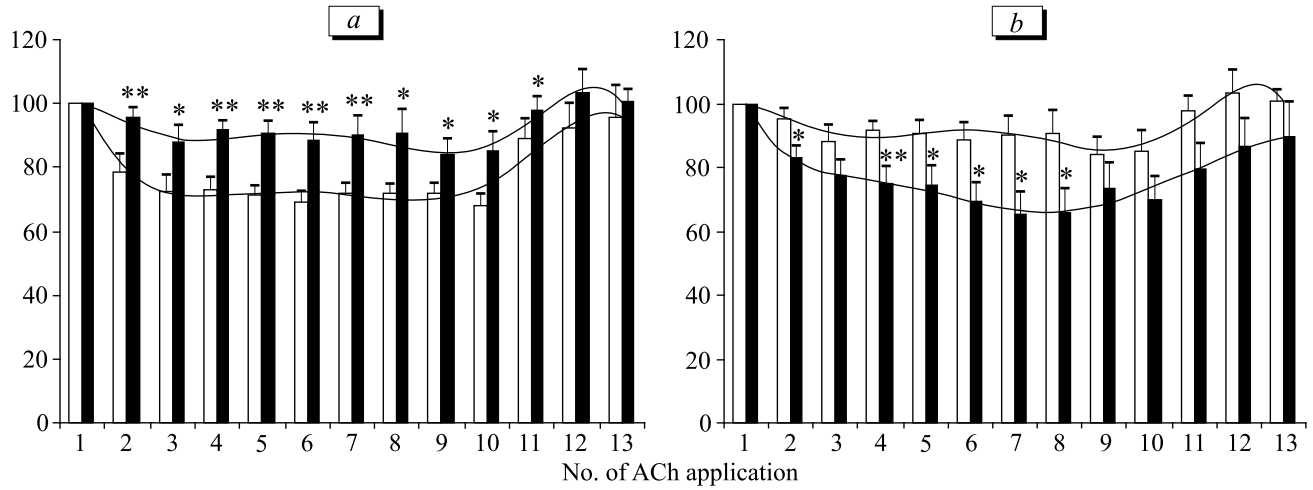

Fig. 1. Effects of DMSO and Exo 1 on the dynamics of neuronal ACh-current depression in cellular correlate of habituation. Cumulative results of all experiments are presented. Intervals between stimuli 1-10 and 10-13 were 3 and 5 min, respectively. Ordinate: ACh-current amplitude (mean±SEM) in % of ACh-current in response to the first ACh application in the series. *a*) intracellular DMSO injection (6.7%, dark bars) without pharmacological exposure (control, light bars). *b*) intracellular Exo 1 injection (2 mM in microelectrode, dark bars), intracellular DMSO injection (6.7% in microelectrode; control, light bars). **P*<0.05, ***P*<0.01 in comparison with the control (unpaired Wilcoxon test). Continuous curve: six-degree polynomial approximation.

1+DMSO (6.7% DMSO for control) by passive diffusion for 60-80 min before the testing.

Experiment included: 1) baseline rhythmic ACh applications with 5 min interval; 2) series of 10 more frequent (3 min interval) ACh injections with the same ionophoresis parameters; 3) return to rhythmic ACh applications with 5 min interval. Experiments were carried out under the conditions of stopped flow of physiological solution. Results were obtained on 33 neurons (19 LPa3, 7 RPa3, 4 LPa2, 3 RPa2) in 33 preparations. Cell membrane potential: (-38)-(-68) mV (-53.8±1.5 mV); fixation potential -75 mV. Neuron input impedance: 0.9-15.8 MΩ (7.1±0.8 MΩ).

Statistical STADIA 6.3/basic (NPO Informatics and Computers) and SigmaStat 3.5 (Systat Software Inc.) software were used. Arithmetic mean and standard error were calculated. Significance of the effects was assessed using unpaired Wilcoxon and Student's tests.

We used a mathematical model presuming receptor localization on the cell membrane, in formed vesicles, and in recycling vesicles [3]. Modified model included additional consideration for receptor transport from the endoplasmic reticulum to the membrane and receptor degradation. The choice of rate constants for receptor transition to different states was determined by the rates of membrane receptor transport to the forming vesicles (k_1), receptor endocytosis (k_3) and exocytosis (k_4), receptor transport from endoplasmic reticulum (k_6), and receptor degradation (k_5) and was carried out on the basis of experimental findings, according to which time constant for receptor endocytosis is few minutes, and the constant for exocytosis is 2-3 times longer.

RESULTS

Since Exo 1 was dissolved in DMSO, a preliminary control series with 6.7% DMSO was performed (on 10 neurons, n=10) after its intracellular exposure. DMSO altered depression curve of acetylcholine-induced current (ACh-current) in comparison with the series without pharmacological exposure (n=11; Fig. 1, *a*; Table). DMSO attenuated ACh-current depression (decreased initial rate of depression and increased depression level in the series) without affecting the time of attaining

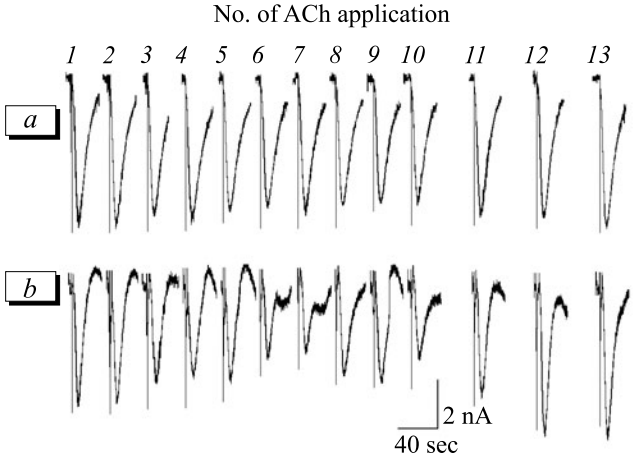

Fig. 2. An example of Exo 1 effects on ACh-current depression in command neurons in cellular model of habituation. ACh-currents in response to rhythmic ACh applications with 3-min (stimuli 1-10) and 5-min (10-13 stimuli) intervals are presented. *a*) control series after intracellular DMSO (6.7%) injection into the RPa3 neuron, *b*) series after intracellular Exo 1 (2 mM) injection into the LPa3 neuron. Holding potential -75 mV.

TABLE 1. Effects of Inhibitor Exo 1 on ACh-Current Depression Parameters in Neurons in Cellular Correlate of Habituation

Series of repeated ACh applications	ACh-current depression			Level of ACh-current recovery after completion of series of rhythmic ACh applications, %		
	initial speed, %/min	time to plateau, min	mean level, %	5 min	10 min	15 min
Without treatment; *n*=11 (control for series 2)	7.23	6	71.21±0.62	89.34±6.41**	92.25±7.77**	95.58±10.28**
DMSO, 6.7%; In; *n*=10 (control for series 3)	1.55	6	88.65±1.98***	97.73±4.60*	103.50±7.41*	100.80±3.83*
Exo 1, 2 mM+DMSO, 6.7%; In; *n*=12	5.96	15	71.31±3.30***	79.36±8.26	86.36±8.82	89.62±10.99

Note. In: intracellular exposure, n: number of neurons in the series. Means (for initial rate of ACh-current depression) and means±SEM (for ACh-current depression at the plateau and during recovery of ACh-current amplitude) are presented. Significance of the differences between the mean values of ACh-current depression was evaluated using Student's test; significance of ACh-current restoration in comparison to the response to the 10th stimulus in the series of rhythmic ACh-applications was evaluated using unpaired Wilcoxon test (*P<0.05; **P<0.01, ***P<0.001).

the plateau and accelerated recovery of reduced ACh-current amplitude.

Addition of Exo 1 into the intracellular microelectrode (n=12) potentiated ACh-current depression (increased the initial rate of depression and reduced depression level in the series), prolonged the time of attaining the plateau and insignificantly reduced the extent of spontaneous ACh-current recovery (Fig. 1, *b;* Fig. 2; Table).

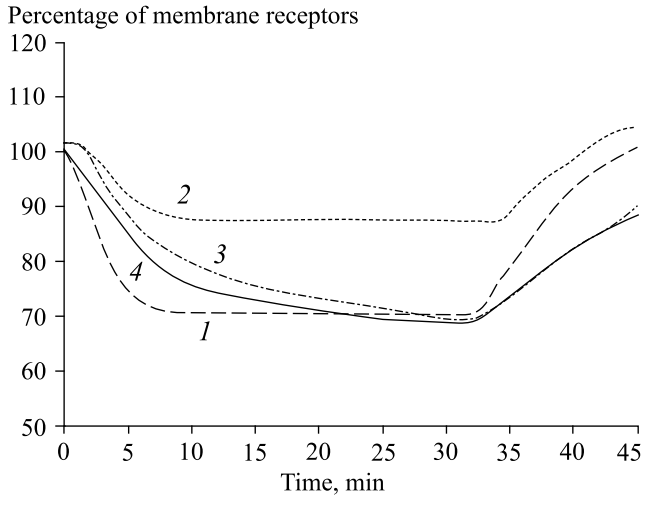

Fig. 3. Calculated curves for membrane receptors determining depression of ACh-current (obtained using the mathematical model). *1*) without pharmacological exposure; *2*) under the effect of DMSO (new control level for depression of ACh-current); *3* and *4*) under the effect of Exo 1 only on receptor trafficking from endoplasmic reticulum to the Golgi apparatus (*3*) and under presumable effect of Exo 1 also on the receptor transport to the membrane (*4*).

Modeling demonstrated that the estimated curve, similar to experimental curve of CR desensitization following intracellular DMSO injection, can be obtained after reduction of receptor transition rate constants (k_1 by 1.8 times and k_4 by 1.45 times) used to calculate the control CR desensitization rate without pharmacological exposure (Fig. 3, curves *1*, *2*). This suggests that changes in receptor desensitization curve under the effect of DMSO (considered in our study as the control in relation to Exo 1 effects) is apparently associated with DMSO effects on primembrane endo- and exocytosis of receptor.

Experimental data suggests that intracellular Exo 1 injection results in increased CR desensitization. According to published data, Exo 1 inhibits receptor trafficking from the endoplasmic reticulum to the Golgi apparatus [10]. Estimations showed that the decrease in receptor trafficking from the endoplasmic reticulum to the membrane (modeled by 2.5-fold reduction of the k_6 constant) alone will increase desensitization (Fig. 3, curve *3*). However, complete similarity with experimental curve was not observed: the initial rate of decline of estimated curve is lower than of experimental one. It can be explained by the fact that initial decline rate for the curve is determined by the rate of membrane receptor trafficking to the forming vesicles and by the rate of endocytosis and exocytosis receptor, since the time constant for the receptor transport from endoplasmic reticulum to the membrane is higher that time constants for this processes. Calculations suggest that the initial decline rate increases with decreasing k_4 constant reflecting receptor exocytosis from endosomes to the membrane, *i.e.* deceleration of

receptor recycling. In this case, the calculated curve corresponds to the experimental curve if k_6 decreases 1.5-fold and k_4 decreases 1.2-fold in comparison with the corresponding constants used for the control curve (Fig. 3, curves *2, 4*). Despite the absence of direct experimental evidences of Exo 1 effects on the rate of receptor recycling from endosomes (its inhibiting effects is demonstrated only for vesicle trafficking from the endoplasmic reticulum to the Golgi apparatus via modulation of Arf1, ADP-ribosylating factor 1) [10], we can hypothesize how Exo 1 affects the receptor recycling process. Apart from Arf1, ADP-ribosylating factor family includes also Arf6, the factor that is now most intensively studied [9]. Arf1 regulates vesicle transport between endoplasmic reticulum and Golgi apparatus and inside Golgi apparatus, then Arf6 controls the transport between membrane receptors and endosomes and has an effect on cytoskeleton reorganization in some cells [9]. It has been found that Arf6-GTP facilitates recycling of clathrin/AP-2 complex back to the neuronal synaptic membrane [11]. Arf6 is present on endosomal membrane, and in some cases it regulates exocytosis process and formation of clathrin vesicles on the plasma membrane [8]. The presence of similar domains in Arf6 and Arf1 structures [12] allows their interaction with identical proteins [9], and therefore, it cannot be excluded that Exo 1 inhibits not only Arf1-associated processes, but also similar Arf6-associated processes. After addition of Exo 1, the match between the experimental and estimated curves occurs with decrease in receptor vesicle transport from their intracellular depot (endoplasmic reticulum and endosome area) to the neuronal membrane, from whence the number of membrane receptors decreases and desensitization (its initial speed and maximal value) increases. Therefore, decrease in neuronal soma sensitivity to ACh in cellular analogue of habituation develops as a result of decrease of membrane CR number due to attenuation of internalized CR recycling.

This study was supported by Russian Foundation for Basic Research (grant No. 09-04-00304-a).

REFERENCES

1. V. N. Ierusalimskiy, I. S. Zakharov, T. A. Palikhova, and P. M. Balaban, *Zh. Vyssh. Nerv. Deyat*., **42**, No. 6, 1075-1089 (1992).
2. O. A. Maksimova and P. M. Balaban, *Neuronal Mechanisms of Behavior Plasticity* [in Russian], Moscow (1983).
3. D. A. Makhnovskiy, M. S. Tret'yakova, G. B. Murzina, and A. S. Pivovarov, *Zh. Vyssh. Nerv. Deyat*., **60**, No. 2, 244-254 (2010).
4. T. A. Palikhova, M. S. Abramova, and A. S. Pivovarova, *Byull. Exp. Biol. Med.*, **142**, No. 9, 244-247 (2006).
5. A. S. Pivovarov, *Zh. Vyssh. Nerv. Deyat*., **42**, No. 6, 1271-1286 (1992).
6. A. S. Pivovarov and E. I. Drozdova, *Neirofiziologiya*, **24**, No. 1, 77-86 (1992).
7. A. G. Ter-Markaryan, T. A. Palikhova, and E. N. Sokolov, *Zh. Vyssh. Nerv. Deyat*., **40**, No. 1, 183-184 (1990).
8. J. G. Donaldson, *J. Biol. Chem*., **278**, No. 43, 41,573-41,576 (2003).
9. J. E. El-Annan, D. Brown, S. Breton, *et al*., *Am. J. Physiol. Cell Physiol*., **286**, No. 4, C768-C778 (2004).
10. Y. Feng, S. Yu, and T. K. Lasell, *et al*., *Proc. Natl. Acad. Sci. USA*, **100**, No. 11, 6469-6474 (2003).
11. M. Krauss and V. Haucke, *Methods Enzymol*., **404**, 388-398 (2005).
12. S. Pausqualato, J. Menetrey, M. Franco, and J. Cherfils, *EMBO Rep*., **2**, No. 3, 234-238 (2001).
13. A. S. Pivovarov, *Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol*., **110**, No. 3, 229-240 (1995).
14. A. S. Pivovarov, *Modern Problems of Cellular and Molecular Biophysics*, Eds. Ayrapetyan S.N., North A.C.T. Yerevan, 69-81 (2001).