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COMPARATIVE AND ONTOGENIC PHYSIOLOGY

Effects of Nimodipine, Calcium-Free Medium and Colchicine on Electrogenesis of Neurosecretory Retzius Cells in the Leech *Hirudo medicinalis*

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Abstract—Retzius neuron (RN) in the medicinal leech is known to function as a typical neurosecretory cell. This study addresses the relationship between its two functions, neural and paracrine. It was shown that synaptic activation of RN at 710-Hz frequencies causes the neurophysiological process of habituation. Experiments conducted in calcium-free solution and in that containing nimodipine and colchicine (which block somatic exocytosis of serotonin under these experimental conditions) demonstrate alterations in the electrophysiological characteristics of RN: the rate of spontaneous impulse activity (IA), action potential (AP) amplitude, and synaptic stimulation threshold. Under these conditions, RN generates AP to every stimulus even at frequencies of 7 to 10 Hz. Thus, while somatic exocytosis of serotonin is blocked, habituation does not develop. It is suggested that habituation of RN to high-frequency synaptic stimulation is mediated by the concurrent effects of two factors — stimulatory (via synaptic activation) and inhibitory (via autoinhibition).

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Key words: habituation, leech neurosecretory Retzius neuron, impulse activity, nimodipine, colchicine.

Abbreviations: RN—Retzius neuron, AP—action potential, IA—impulse activity.

INTRODUCTION

Neurons of invertebrates regularly serve experimental models to study physiological processes in the brain of vertebrates. One of these models is the leech Retzius neuron (RN), which is a typical neurosecretory cell. As any neuron, it receives an electrical signal, transduces it and transmits synaptically to other neurons [1, 2]. As a secretory cell, RN synthesizes serotonin and releases it to the ganglionic extracellular space [3, 4]. The interplay of these two functions, neural and paracrine, has long been in the focus of attention [5, 6]. Specifically, it was shown that stimulation of RNs by an intracellular electrode at a frequency of 10 Hz evokes translocation of neurosecretory granules toward the inner surface of the plasma membrane and somatic exocytosis of serotonin. This process is mediated by Ca^{2+} entering neuron upon AP generation [6, 7]. We demonstrated that synaptic stimulation of RNs at the same frequency triggers a neurophysiological reaction of habituation [8]. However, the mechanism behind this electrophysiological response and its probable relationship with the neurosecretory function of RNs are still unstudied.

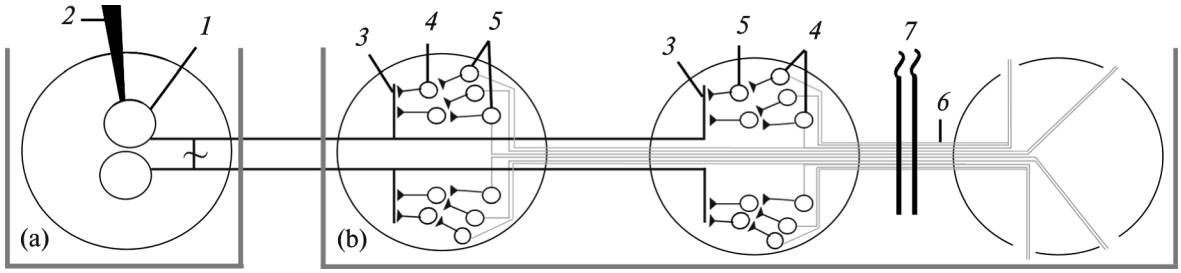

Fig. 1. Diagram of the medicinal leech abdominal nerve chain preparation. (a) 1st experimental chamber with the ganglion and Retzius neurons (RN); (b) 2nd experimental chamber with the third, fourth and fifth ganglia of the abdominal nerve chain; (*1*) RN in the second ganglion; (*2*) recording electrode; (*3*) RN processes in the third and fourth ganglia with postsynapses; (*4*) interneurons with presynapses activating RN; (*5*) sensory neurons; (*6*) processes of sensory neurons in the interganglionic connective; (*7*) stimulation electrodes.

The aim of this study was to find out if the RN habituation to high-frequency synaptic stimulation can be explained by autoinhibition.

MATERIALS AND METHODS

Experiments were carried out on the medicinal leech *Hirudo medicinalis* raised at the KNM Biofactory (St. Petersburg, Russia). Animals were narcotized in cold water and dissected from the abdominal side; part of the abdominal nerve chain composed of seven ganglia was excised and placed into a plastic chamber. In the second ganglion, the connective-tissue capsule was dissected and the ganglion itself was fixed on a rubber support. The preparation was vitally stained with 0.01% neutral red which clearly visualizes the bodies of two large RNs on the ganglionic surface. The extracellular gold glass-isolated recording microelectrode was led up to one of them under control of the MBS-10 microscope. The remaining part of the nerve chain was placed into a nearby chamber with bipolar stimulating electrodes and covered with a vaseline-soaked cotton ball to prevent drying.

The nerve connective between the fourth and fifth ganglia was stimulated by rectangular current pulses with a strength twice as high as the threshold one, duration of 0.2 ms, and frequencies from 1 to 10 Hz. As shown previously [9], under these conditions there occurs stimulation of dendritic synapses of RNs whose somata are located in the second ganglion while dendrites in the fourth. The design of experiments (Fig. 1) shows that chemical agents were applied directly onto the RN somata while synapses were unaffected. The frequency of spontaneous and evoked IA of RNs, the AP amplitude, and the synaptic activation threshold were determined. Since the threshold (the strength of the stimulatory current that evokes the generation of IA) was normally variable in different preparations, its magnitude was taken for 100% while its alterations under the effect of agents were expressed as a percentage to the normal level. Ringer solution was then preplaced by the solutions containing nimodipine and colchicine. In the first series of experiments, the standard Ringer solution for leeches (130 mM NaCl, 4 mM KCl, 1.8 mM CaCl, 48 mM glucose; pH 7.4) was replaced by a Ca^{2+}-free solution to block Ca^{2+} influx to RN. In the second series of experiments, the leech Ringer solution was added with 10 μM nimodipine (Sigma), a L-type Ca^{2+}-channel antagonist. In the third series, the leech Ringer solution was added with 0.5 mM colchicine (Sigma), a microtubule depolymerizer, to arrest the translocation of neurosecretory granules from the deeper somatic compartments to the RN plasma membrane. In each series of experiments, the electrical characteristics of 50 RN were analyzed, by 10 cells per each frequency of synaptic stimulation.

Electric stimulation of the nerve fiber was implemented using the ESU-1 stimulator, and the signal was recorded with the aid of the UBP1-02 amplifier. Experimental data were visually analyzed on the S1-93 oscillograph (USSR) and GDS-806S digital oscillograph (GW Instek, Taiwan), then recorded and saved in the computer. For frequency analysis, the Microsoft Office Excel 2003 application was used. The data were presented as arithmetical means and their errors; the sig-

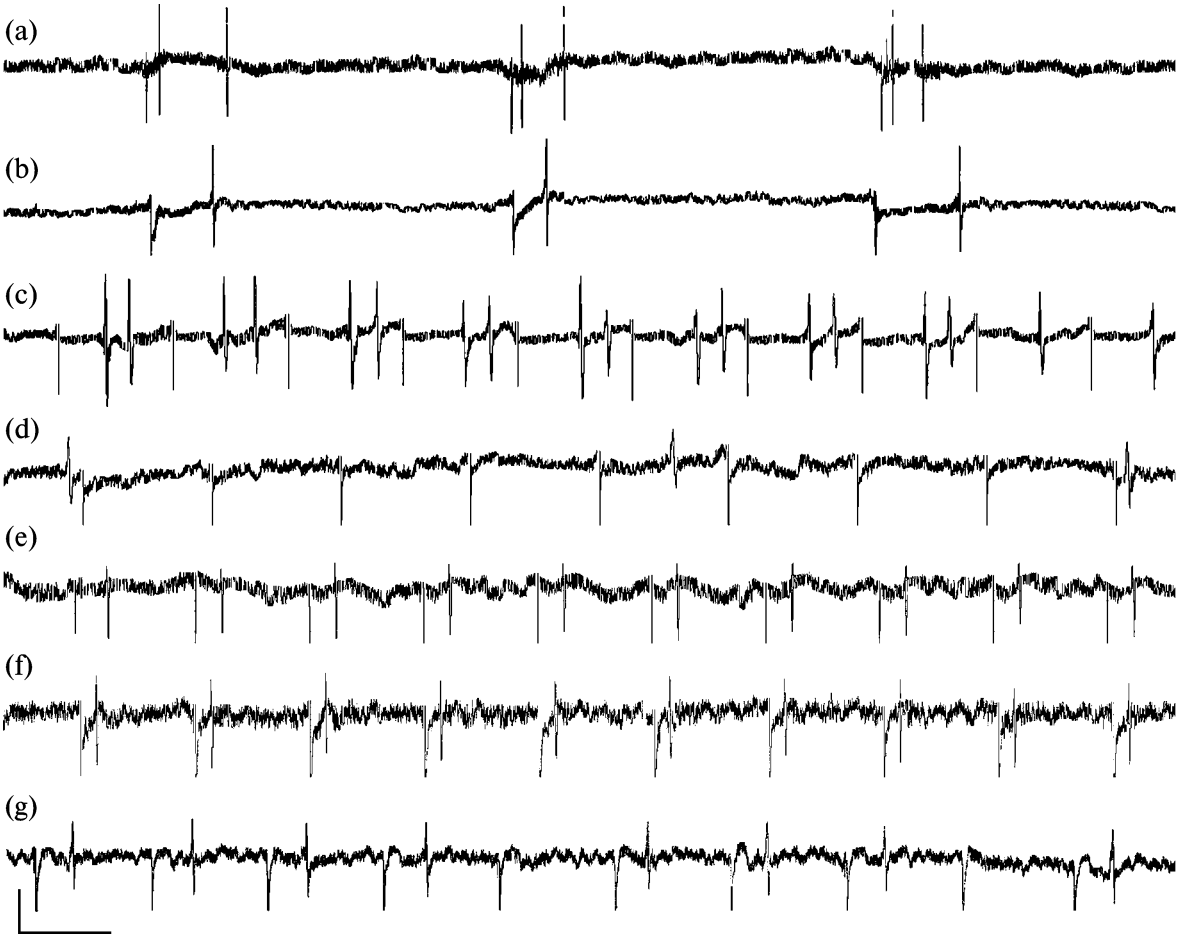

Fig. 2. Response of leech Retzius neuron to synaptic activation. (a) Response of Retzius neuron (RN) to first three pulses of stimulatory current in activation by a frequency of 3 Hz; (b) response of RN to stimulation by a frequency of 3 Hz (2nd minute of stimulation); (c) response of RN to a train of first pulses of stimulatory current at a frequency of 10 Hz; (d) stimulation of RN by a frequency of 10 Hz (2^{nd} minute of stimulation); (e) changes in IA in response to stimulation by a frequency of 10 Hz in Ca^{2+}-free medium; (f) changes in IA in response to stimulation by a frequency of 10 Hz in nimodipine solution; (g) changes in IA in response to stimulation by a frequency of 10 Hz in colchicine solution. Calibration: 25 μV, 0.1 s. Dots mark artefacts.

nificance of differences (p) was determined using one-way ANOVA.

RESULTS

Control. In the quiescent state, RN showed a spontaneous IA with a frequency from 0.1 to 0.3 imp/s. In control, during synaptic activation, RN always responded to the first stimulatory cue with several APs. For example, the 3-Hz activation evoked 6 while the 10-Hz one already 17 APs (Figs. 2a, 2c). Then, over the first minute of stimulation, the frequency of evoked APs gradually decreased and in the second minute entered the response plateau. The data recorded during the last 10 s of the plateau were analyzed. It was found that the frequency of evoked IA of RNs recorded in the second minute of stimulation depended on that of the cues. At a frequency of synaptic stimulation from 1 to 5 Hz, RN responded with an AP to each stimulatory cue (Fig. 2b). At a pulse frequency from 7 to 10 Hz, there was a transformation of the rhythmicity of evoked IA: RN responded not to each cue (Fig. 2d). Table 1 (line 1) shows a dependence of the frequency of evoked IA on that of synaptic stimulation. Our experiments indicate that in control, upon synaptic stimulation of RN by the frequencies from 7 to 10 Hz, there develops a neurophysiological process that can be characterized as a habituation [10]. These data are

Changes in impulse activity of leech Retzius neuron in response to synaptic stimulation by frequencies from 1 to 10 Hz

Series \ Activation frequency	1 Hz	3 Hz	5 Hz	7 Hz	10 Hz
Control $n = 30$	1.05 ± 0.13	3.0 ± 0.0	5.0 ± 0.0	3.64 ± 0.18	3.07 ± 0.2
Ca^{2+}-free solution $n = 14$	1.0 ± 0.0	3.0 ± 0.0	5.0 ± 0.0	7.0 ± 0.0	10.0 ± 0.0
Nimodipine $n = 14$	1.0 ± 0.0	3.0 ± 0.0	5.0 ± 0.0	7.0 ± 0.0	10.0 ± 0.0
Colchicine $n = 14$	1.7 ± 0.1	3.2 ± 0.1	5.1 ± 0.1	6.3 ± 0.3	7.9 ± 0.5

consistent with the previously published [8].

Experiments in Ca^{2+}-free medium. Incubation of nerve ganglia and IA recording in the Ca^{2+}-free medium led to a significant decrease in the spontaneous spike amplitude from 49.2 ± 1.01 (norm) to 38.3 ± 1.44 μV ($p < 0.001$). The frequency of spontaneous IA significantly increased from 0.17 ± 0.03 (norm) to 0.33 ± 0.06 imp/s ($p < 0.05$). The synaptic activation threshold in Ca^{2+}-free medium decreased on average by 21.6% ($p < 0.05$). Under these conditions, synaptic activation of RN by the frequencies from 7 to 10 Hz caused no transformation of evoked IA (see Table). RN responded with an AP to each stimulatory cue (Fig. 2e). Descriptive statistics of the obtained data is presented in Table.

Experiments in nimodipine solution. Incubation of nerve ganglia in the nimodipine solution led to a significant decrease in the spontaneous spike amplitude from 47.5 ± 1.3 to 34.0 ± 1.6 μV ($p < 0.001$). The frequency of spontaneous IA significantly decreased from 0.20 ± 0.02 to 0.13 ± 0.02 imp/s ($p < 0.05$). The synaptic activation threshold of RN significantly increased on average by 30.1% ($p < 0.001$). Synaptic activation of RN by the frequencies from 7 to 10 Hz caused no transformation of IA (see Table), and RN responded with an AP to each stimulatory cue (Fig. 2f).

Experiments in colchicine solution. Incubation of nerve ganglia in colchicine solution led to a significant decrease in the spontaneous spike amplitude from 46.9 ± 1.1 (norm) to 37.4 ± 1.4 μV ($p < 0.001$). The frequency of spontaneous IA significantly increased from 0.22 ± 0.04 (norm) to 0.75 ± 0.1 imp/s ($p < 0.001$). The synaptic activation threshold significantly decreased in the experiment by 17.6% ($p < 0.001$).

Synaptic activation of RN by the frequencies from 7 to 10 Hz caused transformation of evoked IA, and RN responded with an AP not to each stimulatory cue (Fig. 2g; see Table). However, we cannot qualify this response as a habituation because it does not meet the basic criterion of this process, namely a gradual decrease in the impulse response at the increasing frequency of the stimulatory cue [10]. It was revealed that evoked IA of RN in the colchicine solution is significantly lower when activated by a frequency of 10 Hz than the RN response in the Ca^{2+}-free medium or nimodipine solution at the same activation frequency (Fig. 3).

DISCUSSION

Our experiments demonstrate that incubation of RN in the Ca^{2+}-free solution causes alterations in the functional state of the cell (increased frequency of spontaneous IA and decreased threshold), indicative of an increased excitability of RN under these conditions. These data support the known properties of extracellular Ca^{2+} to alter neuronal excitability [11, 12].

In the nimodipine solution, the frequency of spontaneous IA decreases while the synaptic activation threshold increases, indicative of a decreased excitability of RN under the effect of the antagonist of L-type Ca^{2+} channels. Apparently, this is due to a decreased Ca^{2+} concentration in the intracellular submembrane zone. In the Ca^{2+}-free and nimodipine solutions, the spontaneous spike amplitude decreases, reflecting the known involvement of Ca^{2+} (along with Na^{+}) in the generation of APs in RN [13–15]. The lack of Ca^{2+} influx to RN does not prevent spike generation in

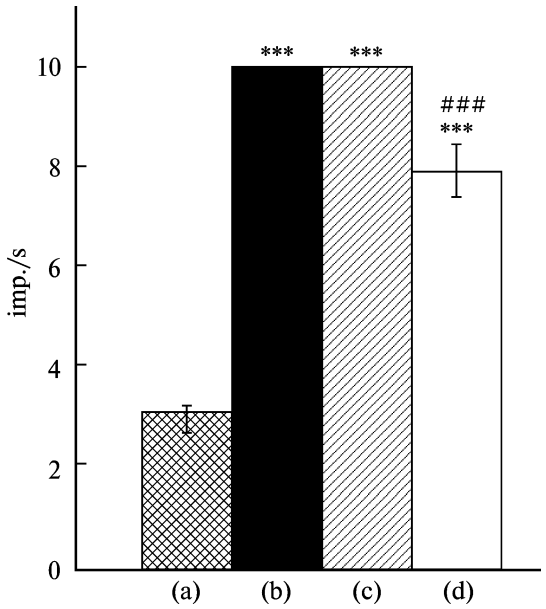

Fig. 3. Frequency of IA of leech Retzius neuron in activation by a frequency of 10 Hz. (a) Control; (b) Ca^{2+}-free medium; (c) nimodipine; (d) colchicine; ***—significant differences from control, $p < 0.001$; ###—significant differences between the effect of colchicine on evoked responses of RN and those of Ca^{2+}-free and nimodipine solutions, $p < 0.001$.

a wide range of stimulatory frequencies, however, habituation does not develop in this situation. Thus, our experiments both with Ca^{2+}-free and nimodipine solutions demonstrate that Ca^{2+} plays a crucial role in the development of habituation of RN to high-frequency synaptic stimulation.

Intracellular Ca^{2+} is commonly accepted to be a universal second messenger involved in the regulation of multiple intracellular processes [16]. The RN soma has a lot of serotonin-containing vesicles that RN releases to the ganglionic extracellular space [3]. Using a laser confocal microscopy with a fluorescent dye for Ca^{2+}, it was shown that intracellular stimulation of RN by a pulse train at 10 to 20 Hz results in a Ca^{2+} buildup to the central regions of the cell [7]. At the same time, during the generation of single spikes, the Ca^{2+} concentration rises significantly only in dendrites and axons [7], i.e. in the regions relevant to synaptic activity. Based on the outcome of synaptic studies which clearly demonstrated that the interaction between Ca^{2+} and the cytoskeleton stimulates neurosecretory vesicular transport to the presynaptic membrane, it was suggested that somatic exocytosis in neurosecretory RNs may proceed in the same way [17]. In other words, serotonin-containing vesicles may be actively transported to the plasma membrane as supported in our experiments with colchicine [18]. Really, during intracellular stimulation of RNs by the frequencies from 10 to 20 Hz, serotonin-containing vesicles diffuse toward the RN plasma membrane [4, 5, 19]. Somatic exocytosis was observed to occur under the same conditions of RN stimulation [5].

Serotonin in RN alters the state of the ganglionic neurons whose response (excitation or inhibition) depends on the type of serotonin receptors on their plasma membranes [20, 21]. Serotonin receptors were found to be present on the outer membrane of RNs [22]. Since serotonin is an inhibitory neurotransmitter in RNs [23, 24], it may cause an inhibition of these cells in an autoinhibitory manner [23].

In our experiments, we used colchicine to block serotonin exocytosis and to reveal the differential involvement of serotonin and synaptic stimulation in the development of habituation. The effect of colchicine does not prevent Ca^{2+} influx to the cell. Under these conditions, there occurs a transformation of the neural impulse response to high-frequency synaptic activation but habituation as a neurophysiological process does not develop. Thus, we are free to suggest that upon synaptic activation by the frequencies from 7 to 10 Hz habituation forms by the two factors: excitatory via synapses and inhibitory via autoinhibition. In both cases, Ca^{2+} plays an important role.

Previously, we demonstrated that the formation of the neurophysiological reaction of habituation relies mostly on the molecular properties of the channels for inward Na^+ current. There occurs an inactivation of TTX-resistant Na^+ channels and an alteration of the state of the inactivation gating system of TTX-sensitive inward Na^+ current channels [1]. Which intracellular cascades are involved in the concurrent action of these two factors, excitatory and inhibitory? The answer to this question is of great importance as it would allow a deeper understanding of how the neurosecretory cell participates both in the synaptic and paracrine regulation of the organism through differential responding to synaptic stimulation by different frequencies.

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