Inhibition of Contractions of Nerve Processes in Calcium-Free Medium

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 149, No. 2, pp. 232-235, February, 2010
Original article submitted April 22, 2009

The possibility of blocking contractile activity of damaged neurites was studied on viable isolated neurons from *Lymnaea stagnalis* mollusks. Retraction was blocked in more than 80% cells plunged in Ringer's solution free from Ca^{2+} or containing 20 mM $CoCl_2$. Nimodipine and nitrendipine significantly inhibited neurite contractions. Inhibition of neurite contractile activity can be useful for reducing diastasis after nerve crossing.

Key Words: *isolated neuron; neurite contraction; calcium channel blocker; nimodipine; nitrendipine*

Retraction of neurites is an important process during normal development of the nervous system and in neurodegenerative processes [13]. Retraction and facilitation of arborization of apical dendrites of pyramidal neurons in hippocampal field CA3 and disorders in synaptic organization of mossy fibers develop in insulin-dependent diabetes [7,8].

It was hypothesized that retraction mechanism is involved in the formation of diastasis after nerve crossing and injury to cerebral conduction tracts [2]. Nerve diastasis is formed due to not only elastic characteristics of the glia and connective tissue of brain membranes, but also due to retraction of nerve fibers. Hence, not only stimulation of regeneratory capacity of the nerve, but also prevention or minimization of the gap between the peripheral and central stumps of the damaged nerve are essential for reduction of diastasis, formation of minimum cicatrix, and facilitation of nerve regeneration.

Contraction requires the presence of Ca^{2+} and kinase phosphorylating one of the myosin light chains [1]. Changes in the cytoskeleton associated with nonmuscular mobility of cells are initiated by Ca ions [11]. Contraction in the growth cone can be triggered by an increase in intracellular Ca^{2+} concentration [5,15]. The entry of Ca^{2+} in electrostimulated cells is assumed to be responsible for massive retraction of Retzius neuron nerve processes (NP) growing on laminin substrate [10]. Hence, the entry of extracellular Ca^{2+} through the neurolemma should be blocked in order to inhibit calcium mechanism triggering NP contraction.

We carried out experiments aimed at blocking of Ca^{2+} entry into the cell using calcium-free Ringer's solution, Ca channel blockers $CoCl_2$, nimodipine, and nitrendipine.

MATERIALS AND METHODS

The study was carried out on living neurons isolated from the peripharyngeal ring ganglia of 86 fresh water mollusks (*Lymnaea stagnalis*). Before isolation of the ganglia, the shell was destroyed, the mollusk body was pinned, and the peripharyngeal ganglionic ring was removed through a scissors cut and plunged in 0.4% pronase for 40 min. This treatment destroyed the connective tissue of the sheath and glial cells, this promoting the release of solitary neurons. After pronase treatment, the ganglia were pipetted in Ringer's solution, the isolated neurons were transferred into a mini-box with the slide for the bottom.

The isolated neurons were examined in phase contrast using a computer microvideodevice. Neuronal

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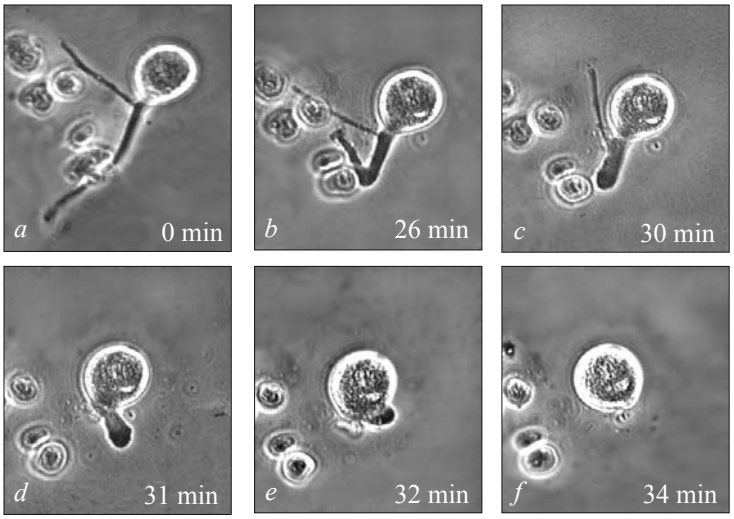

Fig. 1. Dynamics of NP contractions in isolated neuron from *Lymnaea stagnalis* mollusks. Here and in Figs. 2, 3: time from the start of videorecording is shown. Phase contrast, ×200.

behavior was recorded with a Moticam 1000 videocamera attached to the MBI-13 inversion microscope tube (LOMO Plant). The videocamera and the microscope lamp were switched on and off through a timer.

Retraction of NP was inhibited by calcium-free Ringer's solution, calcium-free Ringer's solution with nimodipine (10^{-6} g/ml), calcium-free Ringer's solutions with $CoCl_2$ (20, 5, and 1.2 mM), Ringer's solution with nimodipine (10^{-6} g/ml), and Ringer's solution with nitrendipine (10^{-6} g/ml).

RESULTS

Contractile activity of NP was observed in all cases in the control (Ringer's solution; Fig. 1). It manifested in gradual rounding of terminal portion of the neurite, then a club-shaped formation developed, which we called the retraction bulb. The velocity of NP contraction greatly varied (from 0.034 to 5.9 μ/min) in different neurons irrespective of their body and fiber diameters and in the course of one experiment. The contraction could develop with inhibition and delays, but commonly the NP were completely retracted into the cell body.

The retraction bulbs appeared at the ends of damaged fibers in Ringer's medium free from Ca^{2+} (Fig. 2, *a*). Later, some of these neurons did not contract (Table 1). NP contractions were inhibited in 87.5% cells in Ca^{2+}-free medium (Fig. 2, *a*). In 12.5% of these, retraction stopped not immediately, but after 45 min and later. In 12.5% cases, the nerve fibers retained their contractile activity. In some experiments, these contractions of NP were due to the presence of fragments of destroyed cells (containing calcium) in the box in poorly purified medium.

In some cases, retraction of short pricky NP was observed, but no contractions of the initial neurite. In small neurons, fine neurites covered by varicosities

TABLE 1. Inhibition of NP Contractions under Conditions of Blockade of Ca^{2+} Entry into Neuron *In Vitro*

Experiment conditions		Retraction, % of cases		
		present	absent	slight, partial
Ringer's solution		100	0	0
Ca^{2+}-free Ringer's solution		12.5	75	12.5
Ca^{2+}-free Ringer's solution with nimodipine, 10^{-6} g/ml		0	100	0
Ringer's solution with $CoCl_2$	20 mM	8.7	60.9	30.4
	5 mM	16.6	33.3	50
	1.2 mM	50	0	50
Ringer's solution with nitrendipine, 10^{-6} g/ml		50	25	25
Ringer's solution with nimodipine, 10^{-6} g/ml		41.7	58.3	0

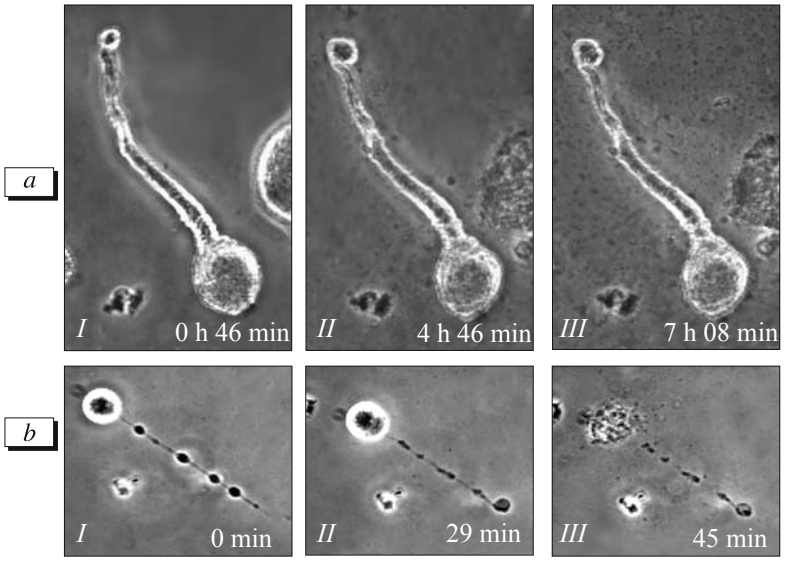

Fig. 2. No contractions of the process in Ca^{2+}-free Ringer's solution over 7 h (*a*) and at the stage of degeneration of fine nerve fiber in the absence of its retraction (*b*).

underwent Waller's degeneration without modifying the length of their NP (Fig. 2, *b*). The period of observation varied from 45 min to 13 h. Degenerative changes in the soma of neurons developed after 3-8 h.

Hence, Ca^{2+} ions are involved in initiation of neurite contractile activity, and contraction is virtually impossible without them. No contractile reaction of processes was observed after addition of nimodipine (10^{-6} g/ml) to Ca-free Ringer's solution (Table 1).

Contractions were completely or partially blocked in almost all fibers after addition of $CoCl_2$ to Ringer's solution at a concentration of 5 mM. The retraction was blocked in 80.3% fibers at $CoCl_2$ concentration of 5 mM and only in only 50% fibers at a concentration of 1.2 mM (Table 1). No retraction bulbs were seen on nerve fibers of isolated neuron (Fig. 3); no fiber contractions were seen over 14 h 32 min of observation, when the body of the cell exhibited pathological changes in its contour. Neurites looked like optically dense compact structures. The diameter of the neuron soma slightly increased and optically dense submembrane aggregations appeared. In some cases, the contractions were blocked incompletely. However, retraction bulb did not appear at the end of the process.

Nimodipine and nitrendipine characterized by the inhibitory effect on Ca^{2+} channels also inhibited (to a certain measure) retraction of NP. The inhibitory effect of nimodipine and nitrendipine was detected in 50 and 58.3% cases, respectively (Table 1).

Hence, study of the behavior of damaged neurons after dissociation of the ganglion revealed active contractile reaction of NP, eventuating in their complete retraction into the body of the neuron. Presumably, NP retraction processes play an important role in vital activity and disease of the normal nervous system.

This is in line with the findings of authors observing retraction activity of NP. It was shown that cytokine IFN-γ, which is stimulated in nerve injury, chronic inflammation, disseminated sclerosis, and infection by human immunodeficiency virus, inhibits dendrite growth in cultured sympathic and hippocampal neurons of rat embryo and decelerates synapse formation [4]. Blockers of Ca^{2+} channels γ-conotoxin, nimodipine, and flunarizine inhibit neurite retraction [3,14]. NP retraction can be prevented by nicardip-

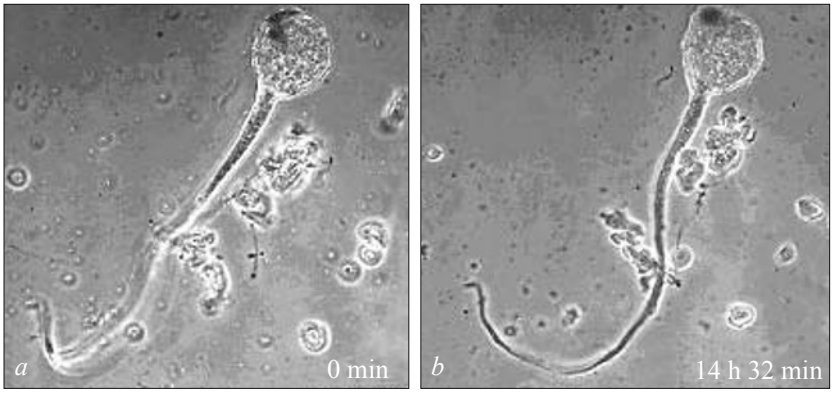

Fig. 3. No retraction bulbs and neuron contractions in Ringer's medium with 20 mM $CoCl_2$ over 14 h. The first (*a*) and last (*b*) images of the preparation.

ine, a blocker of L type Ca^{2+} channels involved in the growth of processes and plasticity [9].

The contraction process can be regulated by Ca^{2+} channel blockers. Hence, our data and findings of other authors provide a theoretical background suggesting the possibility of inhibiting NP contraction in injuries of the peripheral nerves and CNS nerve tracts and reducing diastases in their ruptures.

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