Electrical Activity of NO-Producing Neuron Depends on NO Level

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NO-producing neuron exhibited an excitatory response to the decrease in NO concentration, which was induced by NO synthase inhibitor N-nitro-L-arginine or specific NO acceptor 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. Addition of NO donors to the medium inhibits neuronal activity. The excitatory effects of N-nitro-L-arginine and 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide were preserved, while the inhibitory action of NO donors significantly decreased after isolation of the neuron. These findings indicate that NO regulates activity of these neurons by the negative feedback mechanism. This regulation includes the following complementary mechanisms: (1) endogenous mechanism of cell self-activation in response to the decrease in NO concentration; and (2) exogenous mechanism of cell-mediated inhibition in response to NO excess.

Key Words: *nitric oxide; NO-ergic neurons; nitroprusside; S-nitroso-N-acetylpenicillamine; N-nitro-L-arginine; 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide*

NO regulates several functions of the central nervous system (CNS). Reduced synthesis or overproduction of NO is associated with a variety of pathological processes [1,2,15]. Understanding of the mechanisms of regulation and autoregulation of the NO-ergic system is one of the most important problems of basic and applied neurophysiology.

Despite recent progress in understanding of the mechanisms regulating NO synthesis in CNS [4-6], many problems remain to be solved. The effect of NO on electrical properties of NO-producing neurons and the role of this mechanism in autoregulation of NO synthesis are poorly understood. The type of NO diffusion in the nervous tissue [8] suggests that NO modulates activity of a specific cell in the presence of NO targets in the NO-producing neuron. Previous experiments with sections demonstrated changes in excitability of nerve endings under the effect of endogenous NO [7]. Experiments on isolated cells can provide data on this problem, but it is difficult to preserve the intrinsic properties during isolation of NO-producing neurons from vertebrates.

A large paired NO-producing neuron B2 from the buccal ganglia of pond snails is a convenient model for such studies [11-13]. This neuron is activated during feeding behavior, which results in a significant increase in endogenous NO pool. Rhythmic variations in this NO pool correlate with the rhythm of train activity of B2 cells [11], which is consistent with the important role of free intracellular Ca^{2+} in the regulation of NO synthesis [6]. It remains unclear whether electrical activity of the NO-producing neuron depends on variations in NO concentration in the medium. We studied the effect of increased or decreased NO concentration on isolated neuron B2 and on neuron B2 *in situ* (*i.e.* in the ganglion). The method of neuron isolation previously developed by us allows continuous recording of electrical activity of cells in CNS, as well as during isolation and over the first hours after isolation (when the cells retain properties typical for its *in situ* state) [3].

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MATERIALS AND METHODS

Experiments were performed on adult pond snails (*Lymnaea stagnalis*). The snails were maintained in an aquarium at room temperature. CNS was isolated and treated with protease from *Streptomyces griseus* (2.5 mg/ml, 10-15 min). The membranes were removed. The preparation was maintained in a Sylgard-coated chamber with physiological saline of 50 mM NaCl, 1.6 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, and 10 mM Tris (pH 7.6). Electrical activity of neurons was recorded using glass microelectrodes (20-30 MΩ) filled with 3 M KCl. These neurons were isolated from the ganglion with a microelectrode and placed in a continuous flow chamber [3]. Experiments were performed on standard electrophysiological devices. Electrical signals were digitized and processed with Spike-3 software (D. D. Vorontsov). NO synthase inhibitor N-nitro-L-arginine (L-NNA, 0.5 mM) and specific NO acceptor 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO, 0.25 mM) were used to decrease NO concentration in the medium. Nitroprusside (0.5 mM) and S-nitroso-N-acetylpenicillamine (SNAP, 1 mM) served as NO donors. We also studied the effect of NO-generating compound sodium nitrite (1 mM) [5]. The concentrations of the test substances were chosen from published data [12-13].

A nonhydrolyzable cell membrane-permeable cGMP analogue 8-bromoguanosine 3':5'-cyclic monophosphate (8-Br-cGMP, 1 mM) and guanylate cyclase (GC) inhibitor methylene blue (0.1 mM) were used to evaluate whether the effects of NO on activity of neurons B2 are mediated by GC activation. All reagents were from Sigma. The results were analyzed by Student's *t* test.

RESULTS

Electrical activity of neurons B2 was characterized by 2 specific features. First, this activity included trains of action potentials (AP, 2-8 AP per train). These trains were also typical of isolated neurons B2. The frequency of trains depended on membrane potential (MP), which reflects their endogenous origin. And second, slow hyperpolarization waves (duration, 10-20 sec; amplitude, up to 30 mV) contributed to irregularity of train activity (Fig. 1, *a*). These waves were not revealed in isolated neurons B2, which illustrates their exogenous origin.

NO synthase inhibitor L-NNA *in situ* caused depolarization of neurons B2 (n=16). This compound induced or increased endogenous train activity and completely blocked the hyperpolarization waves (Figs. 1, *a* and 2, *a*). The latency of L-NNA-induced changes did not exceed 1 min. L-NNA had a reversible effect. Washout was accompanied by a decrease or inhibition of endogenous train activity and recovery of slow hyperpolarization (Fig. 1, *a*). Experiments with isolated neurons B2 (n=7) showed that L-NNA caused depolarization, increased total activity, and induced endogenous train activity (n=4; Figs. 1, *b* and 2, *b*). L-NNA had a reversible effect on the isolated neuron.

The effect of NO acceptor PTIO on neurons B2 was similar to that of a NO synthase inhibitor. PTIO *in situ* produced depolarizing and excitatory effects on neurons B2 (n=8), which did not depend on

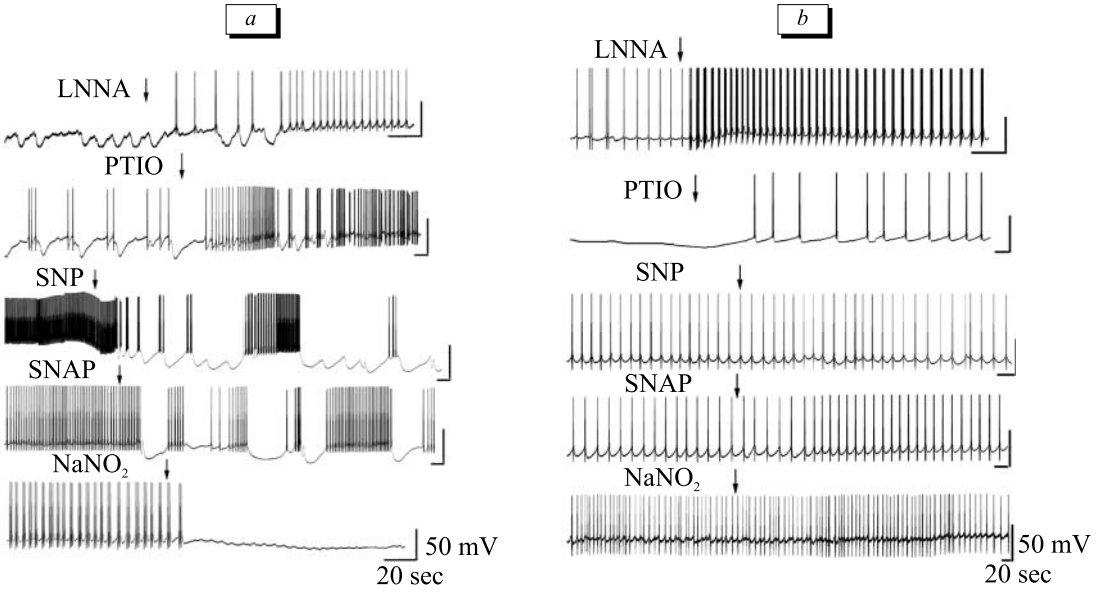

Fig. 1. Effects of NO synthase inhibitor L-NNA (0.5 mM), NO acceptor PTIO (0.25 mM), and NO donors nitroprusside (SNP, 0.5 mM), SNAP (1 mM), and sodium nitrite ($NaNO_2$, 1 mM) on NO-producing neuron B2 under *in situ* conditions (*a*) and after isolation (*b*).

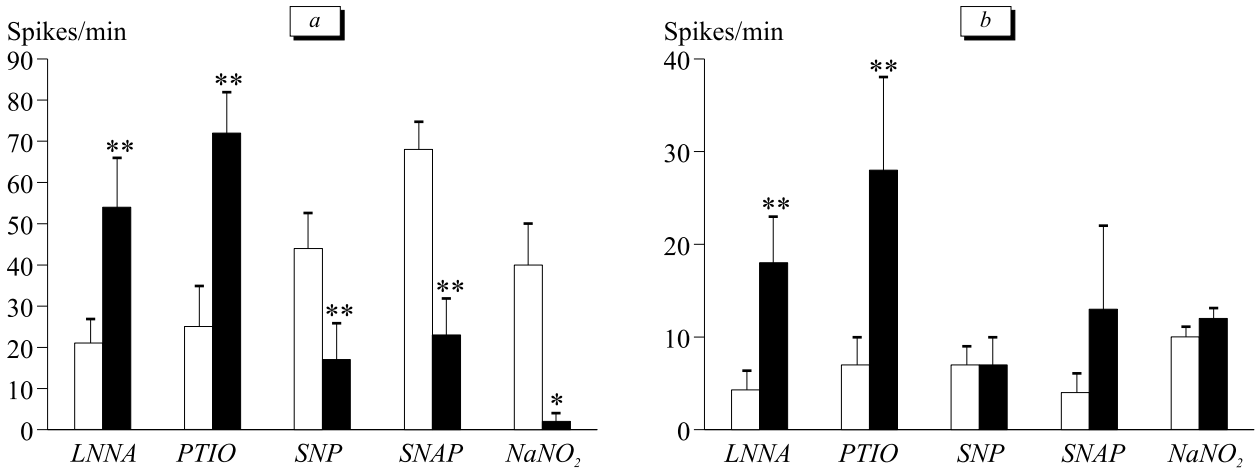

Fig. 2. Average spike frequency in neuron B2 before (light bars) and after application (dark bars) of L-NNA, PTIO, SNP, SNAP (1 mM), and $NaNO_2$ (1 mM). *In situ* conditions (*a*) and after isolation (*b*). *$p<0.05$ and **$p<0.01$ compared to the control (light bars).

basal activity of cells. Hyperpolarization waves were suppressed. PTIO increased the frequency of train activity or induced train activity in silent cells (Figs. 1, *a* and 2, *a*). The effect of PTIO was reversible. Neuronal activity returned to normal after washout with a pure solution for 10-20 min. PTIO also produced a depolarizing effect on isolated neurons B2 (n=10). This compound increased activity of these neurons. Addition of PTIO was followed by train activity of neurons, which did not exhibit activity under basal conditions (Figs. 1, *b* and 2, *b*). PTIO had a reversible effect on isolated neurons.

NO donors *in situ* induced a strong inhibitory effect on neurons B2. Nitroprusside (n=23), SNAP (n=5), and sodium nitrite (n=3) caused hyperpolarization, activated the slow hyperpolarization waves, decreased the frequency of endogenous train activity, or completely abolished this activity (Figs. 1, *a* and 2, *a*). The effect of NO donors was reversible. NO donors in the same concentrations had little effect on isolated neurons (Figs. 1, *b* and 2, *b*). Nitroprusside (n=11) was ineffective in 8 neurons, but produced a small inhibitory effect, caused hyperpolarization, and decreased activity of 3 neurons. SNAP (n=4) and sodium nitrite (n=3) had little effect on the majority of neurons. NO donors did not cause the hyperpolarization waves in isolated neurons B2. Nitroprusside abolished the excitatory effect of L-NNA under *in situ* conditions (n=3) and in experiments with the isolated neuron (n=2),

The *in situ* effect of 8-Br-cGMP (n=5) on neurons B2 was similar to that of NO donors. The decrease in total activity was accompanied by the appearance of slow hyperpolarization waves. Train activity was completely blocked. Otherwise, the period of train activity significantly decreased (Fig. 3). The effect of 8-Br-cGMP persisted over a long time (up to 1.5 h). This effect was observed even after washout. Under these conditions, hyperpolarization waves in the right and left neurons B2 were regular and synchronous. 8-Br-cGMP significantly decreased or completely abolished the excitatory effect of NO synthase inhibitor L-NNA and NO acceptor PTIO.

GC inhibitor methylene blue produced potent depolarizing and excitatory effects under *in situ* conditions (n=3) and in experiments on isolated neuron B2 (n=4; Fig. 3, *b*). Methylene blue *in situ* increased basal activity of neuron B2, blocked the slow hyperpolarization waves, and abolished the inhibitory effect of NO donors (n=3). Administration of nitroprusside or SNAP 15 min after addition of methylene blue did not cause slow hyperpolarization or decrease in activity of neurons B2.

Our results indicate that electrical activity of the NO-producing neuron depends on NO concentration. Neuron B *in situ* exhibits an excitatory response to a decrease in NO concentration caused by NO synthase inhibitor or specific NO acceptor. Addition of NO donors to the medium is followed by inhibition of this neuron. The excitatory effects of NO acceptor and NO synthase inhibitor are preserved, while the inhibitory action of NO donors significantly decreases during neuron isolation. NO-dependent slow waves of hyperpolarization are not revealed in isolated neurons B2, which illustrates their exogenous origin. The potent inhibitory effect of NO donors *in situ* is probably related to the influence on cells B2 in other ganglionic neurons, which exhibit an excitatory response to the increase in NO concentration. The data indicate that NO regulates activity of neurons B2 by the negative feedback mechanism. This regulation includes the following complementary mechanisms: (1) endo-

genous mechanism of cell autoactivation in response to the decrease in NO concentration; and (2) exogenous mechanism of cell-mediated inhibition in an excessive concentration of NO.

Silent cells exhibit the excitatory response to a NO synthase inhibitor or NO acceptor. Therefore, tonic activity of cellular NO synthases does not depend on AP of neurons. This activity probably provides the minimal basal level of intracellular NO. The decrease in intracellular NO concentration is followed by neuronal activation. The hyperpolarizing effect of NO donors on isolated cells was more significant after preincubation with NO synthase inhibitor. The direct measurement of NO concentration and recording of neuronal electrical activity are required to test this hypothesis.

The action of NO synthase inhibitor and NO acceptor on isolated neurons suggests the presence of a specific NO target in NO-producing neurons. Similarly to vertebrates, function of NO in the nervous system of mollusks is associated with activation of the GC—cGMP metabolic pathway [9,10]. Previous studies revealed coexistence of NO synthase and GC in mollusk neuron [9]. We showed that GC inhibitor decreases the effectiveness of NO donors and has an excitatory effect on neuron B2. cGMP analogue caused the slow waves of hyperpolarization in CNS and abolished the effect of NO acceptor PTIO. Our results indicate that the effects of NO on neuron B2 are realized via an increase in cGMP synthesis [6].

The influence of NO on electrical activity of NO-producing neurons is a possible mechanism of autoregulation of NO synthesis. Direct evidence exists for activation of NO synthesis with the increase in train activity of neurons B2 [11], the local synaptic effects of NO are comparable with the effect of SNAP in a concentration of 5 mM [13]. Our results suggest that activity of NO-producing neurons decreases with increasing in NO concentration. The concentration of NO decreases under these conditions. The observed changes are followed by an increase in neuronal activity and activation of NO synthesis. These processes proceed in the following order: increase in activity of NO-producing neurons→increase in NO synthesis→decrease in activity of NO-producing neurons→decrease in NO synthesis→increase in activity of NO-producing neurons→*etc*. Basically, the mechanism of autoregulation of NO synthesis via the inhibitory effect on activity of NO-producing neurons is of considerable importance. This mechanism prevents overproduction of NO in the cell. NO in high concentration serves as a damaging toxic agent and causes death of nerve cells [15]. It remains unclear whether this mechanism of regulation is also typical of other NO-producing neurons.

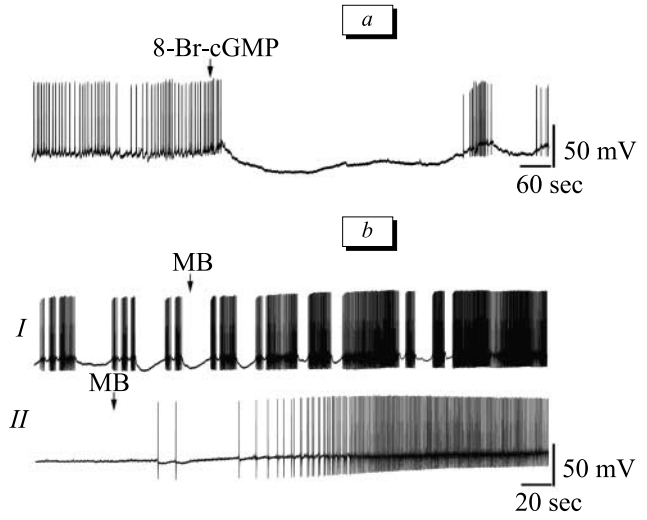

Fig. 3. Involvement of the GC-cGMP pathway into regulation of activity of NO-producing neuron B2. Effect of 8-Br-cGMP (1 mM) on neuron B2 under *in situ* conditions (*a*); effect of a GC inhibitor methylene blue (MB, 0.1 mM) on neuron B2 under *in situ* conditions (*I*) and after isolation (*II*).

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