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EXPERIMENTAL ARTICLES

The Role of Nitric Oxide in the Regulation of Neurotransmitter Release and Processes of Exo- and Endocytosis of Synaptic Vesicles in Mouse Motor Nerve Endings

O. V. Yakovleva[1], M. U. Shafigullin, and G. F. Sitdikova

Physiology Department, Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia

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Abstract—In experiments with mouse diaphragm muscle, the effects of a nitric oxide (NO) donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP), and an NO-synthase blocker, NG-nitro-L-arginine methyl ester (LNAME), on transmitter release and processes of exo- and endocytosis of synaptic vesicles in the motor-nerve ending were studied using electrophysiological and fluorescence techniques. During single stimulation of the motor nerve, SNAP reduced and LNAME did not change the amplitude of the endplate currents and both of the drugs did not affect spontaneous transmitter release. During high-frequency stimulation (20 Hz, 3 min) SNAP increased and LNAME slowed the depression of the amplitudes of endplate potentials (EPPs) compared to the dynamics of EPPs in the control. In experiments using the fluorescent dye FM 1-43, it was shown that the NO donor induced a decrease and LNAME induced an increase in the fluorescence intensity of motor-nerve endings loaded with dye during stimulation at a frequency of 20 Hz for 30 s compared to the control. At the same time, the rate of dye unloading from the terminals that were preloaded with FM 1-43 was higher after preliminary application of SNAP and lower after preliminary application of LNAME. It was suggested that exogenous and endogenous NO in the mouse neuromuscular synapse caused the depression of neurotransmitter release as a result of the suppression of synaptic-vesicle recycling due to a decrease in endocytosis or/and mobilization of synaptic vesicles from a recycling pool to the exocytosis sites.

Keywords: nitric oxide, neurotransmitter release, motor-nerve ending, FM 1-43, exo- and endocytosis, synaptic vesicles

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INTRODUCTION

Nitric oxide (II) (NO) is the first gaseous mediator whose functions have been fully explored in various systems of the body [1, 2]. In the central nervous system NO can both stimulate and inhibit transmitter release [2–4] and regulate synaptic plasticity, including long-term potentiation and depression; it acts as a retrograde messenger, by synthesis in the postsynaptic neuron, and modulates the function of the presynaptic terminals [2, 5, 6].

In the peripheral nervous system NO reduces the spontaneous and evoked release of acetylcholine from the frog motor-nerve terminal [7–10], whereas data on the role of NO in the regulation of the neuromuscular synapse of warm-blooded animals are contradictory. Current data suggest that NO donors and a substrate for NO synthesis, L-arginine, can both increase and decrease mediator release from rat motor nerve terminals [11–15]. In the neuromuscular junction, a neuronal constitutive NO synthase of the μ-type was detected in the sarcolemma of muscle cells at the endplate region [16], where it is located due to interaction with the dystrophin glycoprotein complex [17]. In addition, neuronal NO synthase was also found in presynaptic Schwann cells and motor nerve terminals [18]. The mechanisms of NO action in the nervous system include a change in the activity second messengers, regulation of ion channels, and a direct effect on the processes of transport and the exo- and endocytosis of synaptic vesicles [2, 19–21]. The processes of synaptic-vesicle recycling, including all stages from vesicle exocytosis to recovery of the ability to perform exocytosis by this vesicle, are crucial for neurotransmitter release [22–24]. The change in the dynamics of these processes underlies the presynaptic forms of plasticity and vesicular cycle disturbances are observed in many mental and neurological diseases [24, 25]. Investigation of the mechanisms of NO action in the neuromuscular junction is a challenging problem because of the participation of this messenger in the pathogenesis of several neuromuscular diseases, including myasthenia gravis and muscular dystrophy [26].

[1] Corresponding author; address: ul. Kremlevskaya, 18, Kazan, 420008 Russia; phone: (843) 233-78-44; e-mail: a-olay@yandex.ru.

Our study was aimed at investigating the influence of NO on transmitter release and the processes of exo- and endocytosis of synaptic vesicles in mouse motor-nerve endings using the electrophysiological and fluorescence approaches.

MATERIALS AND METHODS

Experiments were performed on isolated neuromuscular preparations, *m. diaphragm*, of laboratory white mice. The preparation was placed in a chamber under conditions of continuous perfusion with an oxygenated Krebs solution of the following composition (mM): NaCl 137.0; KCl 5.0; $CaCl_2$ 2.2; $MgCl_2$ 1.0; NaH_2PO_4 1.0; $NaHCO_3$ 16.0; glucose 11.0; ($t = 20°C$, pH 7.2–7.4). To block the contraction of the muscle fibers, we used *d*-tubocurarine (2–2.5 μM). In the experiments, an NO synthase inhibitor, NG-nitro-L-arginine methyl ester (LNAME), at a concentration of 100 μM and an NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP), at a concentration of 100 μM (Sigma, United States) were used.

End-plate currents or potentials (EPCs or EPPs, respectively) were recorded using a standard microelectrode extracellular or intracellular recording technique. The motor nerve was stimulated by rectangular impulses with a superthreshold amplitude and frequencies of 0.2 Hz (single stimulation) or 20 Hz (high-frequency stimulation). The quantal composition was calculated using the method of analysis of variance of the EPPs amplitudes [27, 28]. To amplify and record EPCs and EPPs, we used an automated system that was created on the basis of an L-CARD 1250 digitizer and a computer with a Pentium 4 processor. To analyze the effects of substances on evoked or spontaneous responses, the control signals were recorded for 10–15 minutes; the studied substance was then added into the perfusion solution for 30–40 minutes and washing with a standard Krebs solution was then performed. In the case of high-frequency stimulation SNAP or LNAME were present in the solution for 20–30 minutes prior to stimulation.

To investigate the processes of endo- and exocytosis of synaptic vesicles we used the FM 1-43 fluorescent dye (3 μM) (Biotium, United States), which reversibly binds to the presynaptic membrane; during endocytosis it is taken up by a newly formed nerve terminal (loads into nerve terminal). In this case, the fluorescence of the nerve ending, which reflects the accumulation of synaptic vesicles loaded with the dye, is observed [27]. To analyze the processes of endocytosis of synaptic vesicles, the following loading protocol was used: the motor nerve was incubated in a solution containing FM 1-43 and stimulated for 30 seconds at a frequency of 20 Hz and then washed from the dye for 40–45 minutes with Krebs solution and the fluorescence of nerve terminals was recorded. In each experiment, we recorded the fluorescence of 20–30 terminals. Taking the high rate of recycling of synaptic vesicles in the nerve endings of warm-blooded animals into account [27], the time of dye incubation we used (30 s) allowed us to minimize the unloading of the dye loaded via exocytosis under continued stimulation.

To analyze exocytosis, synaptic vesicles were preliminarily marked with the dye (the dye was loaded into nerve ending). To this aim, the motor nerve was stimulated for 3 min at a frequency of 20 Hz, and FM 1-43 was present in the solution during the stimulation and for 7 min after its termination. After this, the motor nerve was again stimulated at a frequency of 20 Hz for 30 min and we analyzed the decrease in the fluorescence intensity of the nerve ending ("unloading" of the dye).

The fluorescence of the nerve endings was recorded using MIKMED-2 (LOMO, St. Petersburg) equipped with water immersion objectives LUMPLFL 60×/0.9-NA (Olympus, United States). All records were made only from surface nerve terminals. To record the fluorescence, we used an AxioCam MRm rapid black-and-white video camera (Carl Zeiss, Germany). We evaluated the average intensity of the fluorescence at a nerve ending area 10–20 μm in size in arbitrary units (a.u.) taking the maximum pixel intensity, which is 256, as 1. In the analysis of fluorescence images in the case of "loading" of the dye, we measured background fluorescence as average fluorescence intensity in a 50 × 50 square at an image area without a nerve ending, in the case of "unloading" of the dye, we measured the fluorescence of the terminal after 30 minutes of stimulation [27]. Preliminary experiments, where the background fluorescence of muscle fibers under the dye administration in the control and in the presence of SNAP or LNAME was assessed, allowed us to eliminate the interaction of the substances that were studied with the fluorescent marker. All data were analyzed using methods of variation statistics. The quantitative results are shown as mean ± standard deviation; *n* is the number of independent experiments.

RESULTS

The Effects of the NO Donor, SNAP, and the NO Synthase Blocker, LNAME, on Transmitter Release during Single and High-Frequency Stimulation

Under conditions of single stimulation of the motor nerve with extracellular recording of EPCs, application of the NO donor, SNAP, resulted in a significant decrease in the amplitude of EPCs to 81.9 ± 2.5% ($n = 9$, $p < 0.05$) as compared to the control. Administration of the NO synthase blocker, L-NAME, did not lead to significant changes in the amplitude of EPCs (95.0 ± 7.3%) ($n = 10$, $p > 0.05$). To record miniature EPPs (MEPPs) we used intracellular registration. Analysis of the amplitude-temporal characteris-

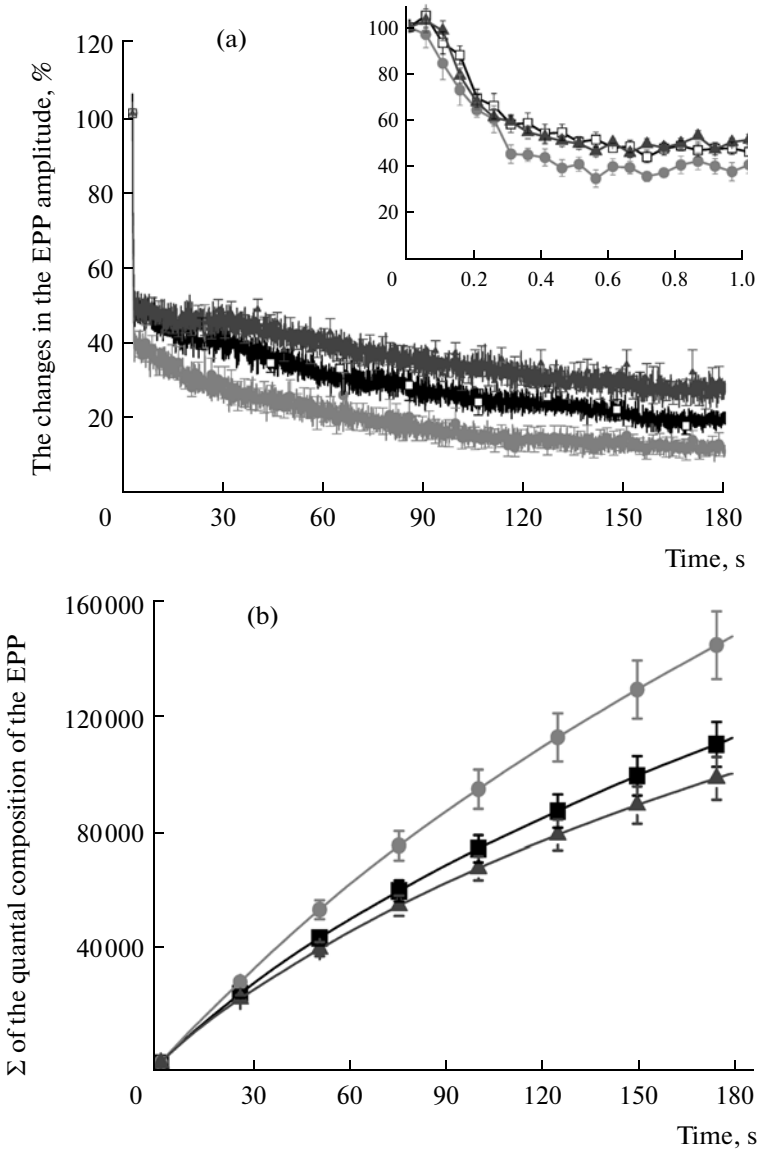

Fig. 1. The effects of an NO donor (SNAP) and an NO-synthase inhibitor (LNAME) on neurotransmitter release from the motor nerve terminal during prolonged high frequency stimulation. (a) The changes in EPP amplitude during stimulation for 3 min at a frequency of 20 Hz in the control (black) and in presence of LNAME (100 μM) (dark gray) and SNAP (100 μM) (gray). The dynamics of EPP amplitude during the first minute of stimulation are shown in the inset. (b) The effects of LNAME and SNAP on the total quantal composition of the EPP. The total quantal content of the EPP of nerve terminals in the control (■, black), in the presence of LNAME (100 μM) (▲, dark gray) and SNAP (100 μM) (●, gray).

tics of MEPCs did not reveal significant changes after both SNAP administration and NO synthase blockade. In the control experiments, the frequency of MEPPs was 1.00 ± 0.16 Hz (n = 8), the amplitude of MEPPs was 0.38 ± 0.07 mV. By the 30th minute of the SNAP application, the frequency of MEPPs was 0.98 ± 0.15 Hz (n = 8, $p > 0.05$), the amplitude of MEPPs was 0.44 ± 0.08 mV (n = 8, $p > 0.05$). By the 30th minute of L-NAME application, the frequency of MEPPs was 0.91 ± 0.07 Hz (n = 8, $p > 0.05$) and the amplitude of MEPPs was 0.34 ± 0.15 mV (n = 8, $p > 0.05$).

To analyze the EPP amplitude during high-frequency activity, the motor nerve was stimulated at a frequency of 20 Hz for 3 min. In the control, we observed a rapid decrease in the EPP amplitude to 46.1±1.7% during the first second of stimulation (Fig. 1a, inset), after which there was a less pronounced decrease to 32.7 ± 2.8% by the end of the first minute and to 19.8 ± 1.9% by the end of the third minute of stimulation as compared to the initial EPP amplitude (n = 13) (Fig. 1a). After preliminary incubation of the neuromuscular preparation in the SNAP-containing solution, the dynamics of the

decrease in EPP amplitude during the first seconds of stimulation did not differ from the dynamics in the control (Fig. 1a, inset); then, the depression became more pronounced and by the end of first minute the EPP amplitude was 21.4 ± 3.9% from the initial level (Fig. 1a); by the end of the third minute it was 10.2 ± 2.3%, which significantly differs from the control ($n = 11$, $p < 0.05$).

In the presence of the NO synthase blocker, LNAME, the initial change in the EPP amplitude during the first second of stimulation did not differ from the amplitude in the control (Fig. 1a, inset). However, when depression of EPP amplitudes developed, the EPP amplitude in the presence of LNAME was 42.7 ± 3.5% by the end of the first minute of stimulation; by the end of the third minute of stimulation it was 28.7 ± 3.1%, which is significantly higher than the amplitude in the control ($n = 13$, $p < 0.05$) (Fig. 1a).

It is well known that the EPP amplitude is proportional to the number of the released quanta of the neurotransmitter and, accordingly, to the number of vesicles that fused with the presynaptic membrane in response to stimulation [29]. In order to evaluate the effect of NO on the total number of quanta of neurotransmitter that were released from the nerve terminal during the entire period of high frequency stimulation, we compared the cumulative curves of the EPP quantal content in the control and in the presence of the substances we studied (Fig. 1b). It was found that the total quantal content in the presence of NO donor decreased by 10.9 ± 5.2% and in the presence of LNAME it increased by 31.2 ± 5.1% as compared to the control (Fig. 1b).

The Effect of SNAP and LNAME on the Intensity of Dye Loading into the Nerve Terminal during Prolonged High Frequency Stimulation

To analyze endocytosis, a neuromuscular preparation was stimulated for 30 s at a frequency of 20 Hz in the presence of FM 1-43 (See Materials and Methods). In the control, the intensity of nerve-terminal fluorescence was 94.4 ± 2.8 a.u. ($n = 7$, Fig. 2a). To analyze the effects of endogenous and exogenous NO on synaptic vesicle endocytosis, the preparation was incubated in Krebs solution containing SNAP and L-NAME for 25 min before the stimulation. In the presence of NO donor, SNAP, the fluorescence of the terminals was 78.2 ± 4.1 a.u. ($n = 6$, $p < 0.05$) and was lower than in the control medium without drug application (Fig. 2a). Under conditions of NO-synthase blockade, the fluorescence intensity of the nerve terminals was 108.4 ± 1.9 a.u. ($n = 6$, $p < 0.05$, Fig. 2a) and was higher than the fluorescence in the control.

The Dynamics of FM 1-43 Unloading from Motor Nerve Terminals during Prolonged High Frequency Stimulation in the Presence of SNAP or LNAME

To determine the dynamics of synaptic vesicle exocytosis, the decrease in the fluorescence intensity of the previously loaded nerve terminals was recorded during stimulation with a frequency of 20 Hz (Fig. 2b) [28, see Materials and Methods]. In the control, the decrease in fluorescence intensity of the nerve terminals by the 40th second and by the end of the 1st, 3rd, and 20th minutes of stimulation was 73.4 ± 4.4%, 66.1 ± 4.7%, 49.1 ± 2.3%, and 26.1 ± 1.3% of the initial value ($n = 8$), respectively. Incubation of the neuromuscular preparation before the stimulation in a solution containing SNAP led to significantly a faster unloading of nerve terminals as compared to the control values. The decrease in fluorescence intensity of the nerve terminals by 40th second and by the end of the 1st, 3rd, and 20th minutes was 59.3 ± 3.9%, 46.4 ± 2.6%, 34.4 ± 4.5%, and 17.3 ± 4.5% of the initial value ($n = 6$, $p < 0.05$), respectively (Fig. 2b). Incubation of neuromuscular preparation before the stimulation in a solution containing LNAME did not affect the decrease in fluorescence intensity during first 40 s (76.8 ± 6.2%); however, further it was slowed and by the end of the 1st, 3rd, and 20th minutes constituted 72.3 ± 2.05%, 65.5 ± 2.4%, and 40.1 ± 2.7%, which significantly differs from the fluorescence intensity in the control (Fig. 2b) ($n = 7$, $p < 0.05$).

DISCUSSION

The investigation revealed that the NO donor reduced the evoked transmitter release from the mouse motor nerve terminal during a single stimulation. We did not observe any changes in amplitude and frequency of MEPP; this indicates that NO does not affect cholinoreceptor sensitivity to acetylcholine. It is known that NO reduces transmitter release in cold-blooded animals [7, 8, 10, 19], while the data that were obtained using neuromuscular preparations of warm-blooded animals are ambiguous. Thus, the early studies using rat phrenic muscle demonstrated that the substrate for NO synthesis, L-arginine (4.7–9.4 mM), increased the amplitude of muscle contractions acting at the presynaptic level [11, 12]. L-arginine and NO donor, 3-morpho-linosydnonimine chloride (SIN-1), reduced the release of [^{3}H]ACh during stimulation of rat phrenic nerves [13], which corresponds to our data. Note that hemoglobin that binds NO prevented a decrease in the release of [^{3}H]ACh induced by SIN-1 and not by L-arginine; this fact supports the idea of NO synthesis in motor nerve terminals [13]. The differences in the effects of L-arginine on the release of acetylcholine may be related to the fact that at high millimolar concentrations [12] L-arginine can inhibit

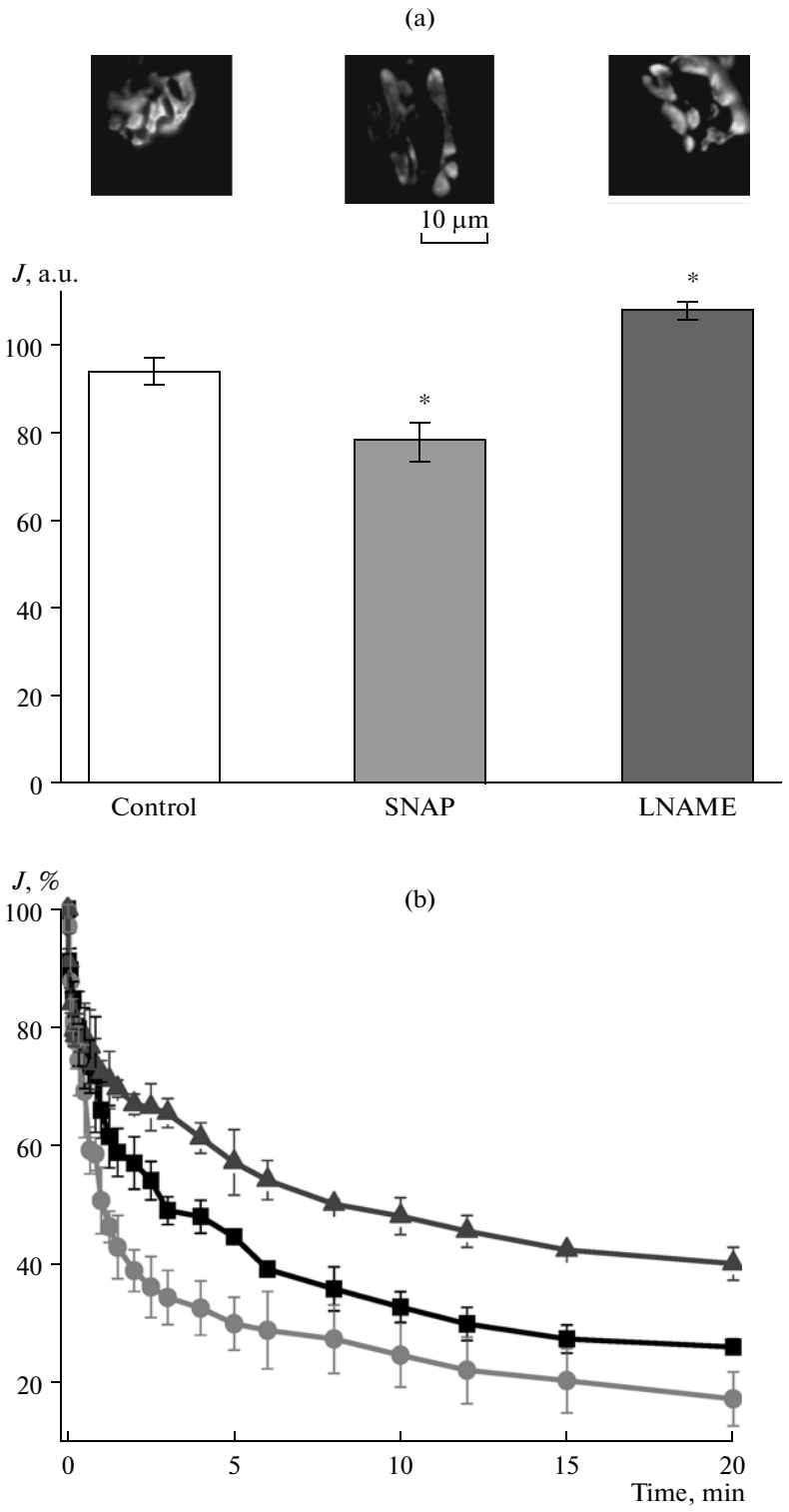

Fig. 2. The effects of NO donor (SNAP) and NO-synthase inhibitor (LNAME) on the exo- and endocytosis of synaptic vesicles in the motor nerve terminal. (a) A fluorescence intensity (*J*) in the control, in the presence of SNAP (100 μM) and LNAME (100 μM) during loading of nerve terminals with dye during stimulation at a frequency of 20 Hz for 30 seconds. *, $p < 0.05$. Examples of stained nerve terminals are shown in the inset. (b) The effects of SNAP and LNAME on the dynamics of unloading of the fluorescent marker, FM 1-43, from nerve terminal during prolonged high-frequency stimulation. The decrease in the fluorescence intensity (*J*) of previously loaded with FM 1-43 nerve terminals during stimulation at a frequency of 20 Hz in the control(■, black), in the presence of LNAME (100 μM) (▲, dark gray), SNAP (100 μM) (●, gray) (see Material and Methods).

NO-synthase [30, 31] or have NO-independent effects [32].

Under conditions of high frequency stimulation of motor nerves (20 Hz, 3 min), an increase in NO by SNAP caused a more pronounced depression of neurotransmitter release and the total amount of neurotransmitter release for 3 minutes of stimulation was ~11% less than in the control (Fig. 1a, 1b). The involvement of endogenous NO in the regulation of synaptic depression has been shown in the frog neuromuscular synapse, where under conditions of high-frequency stimulation, hemoglobin, which binds NO in the synaptic cleft, reduced the degree of depression of EPP amplitudes [7]. In our studies, blockage of the NO-synthesizing enzyme with LNAME also caused a decrease in the depression of EPP amplitudes during prolonged high-frequency stimulation (the total neurotransmitter release increased by 31% as compared to the control) (Fig. 1a, 1b). LNAME did not alter the release of neurotransmitter during single stimulation, which suggests the absence of tonic NO synthesis under conditions of weak synaptic activity. Since the constitutive forms of NO-synthase that are expressed at the neuromuscular synapse of warm-blooded animals are calcium-dependent [17], their activation seems to occur only under conditions of high frequency activity, which leads to an increase in intracellular calcium ion concentration.

Synaptic vesicle recycling in the nerve terminal, including exocytosis, endocytosis, replenishment, and transport to different vesicular pools and release sites, plays a key role in neurotransmitter release under conditions of high frequency activity [23, 29]. According to literature data, the mouse motor nerve terminal has three vesicular pools that differ in their size, localization, and physiological role: a ready releasable pool, a mobilization pool, and a reserve pool [28, 33]. The ready releasable pool is rapidly depleted during high frequency activity and replenished from the mobilization pool. Both pools are recovered mainly by rapid recycling processes and provide exocytosis during high frequency stimulation. Reserve pool vesicles appear not to take part in neurotransmitter release [28]. Under conditions of high frequency stimulation, the initial rapid decrease in the EPP amplitude is associated with the depletion and reduction of the ready releasable pool. Then there is a stabilization of the EPP amplitude, which reflects the replenishment of the ready releasable pool, when rates of depletion and replenishment are equal; this can be provided by the movement of vesicles from the mobilization pool to the active zones and by intensive processes of recycling and reuse of synaptic vesicles. A secondary slower decrease in the EPP amplitude reflects the further depletion of the ready releasable pool, which is not compensated by its replenishment [28]. An increase in the depression of EPP amplitudes in the presence of NO may be caused by the enhancement of the exocytosis during high frequency stimulation, as well as by the slowing of the synaptic vesicle mobilization to the release sites or by reduced synaptic vesicle recycling. To analyze exocytosis, synaptic vesicles were loaded with the FM 1-43 fluorescent dye for 30 s during high-frequency stimulation (20 Hz). NO appears to reduce nerve-terminal fluorescence relative to the control (Fig. 2a), which indicates the slowing of synaptic-vesicle endocytosis, which may enhance the depression of the EPP amplitudes and cause more rapid unloading of the fluorescent dye from previously loaded nerve terminals (Fig. 2b)

Blockage of NO synthesis resulted in effects that were opposite to the influence of the exogenous gas donor. Thus, when the synthesis of endogenous NO was blocked, more intensive loading of nerve terminal with the fluorescent marker was observed during high frequency stimulation (Fig. 2a). The latter is evidence of the enhancement of endocytosis and may lead to the acceleration of synaptic vesicle recycling. This results in slowing the EPP depression, as well as in slower unloading of FM 1-43 during high frequency stimulation (Fig. 2b). In this case, the vesicles that lost the dye were repeatedly involved in the transmitter release along with dye-labeled vesicles, which results in slower destaining of the nerve endings [27, 34, 35].

It is possible to assume that under conditions of high synaptic activity NO synthase is activated and produces NO, which diffuses in a retrograde manner into nerve ending and slows synaptic vesicle recycling; this is accompanied by an increase in the depression of neurotransmitter release. cGMP-dependent or independent mechanisms may underlie the effects of NO, and the activation of certain signaling pathways will determine the final effect of the gas [1, 10, 20]. It is known that in the motor nerve terminal one of the main targets of NO is cAMP which level decreases in response to the activation of cGMP-stimulated phosphodiesterase, which hydrolyzes cAMP [9, 10]. The reduction of the cAMP level in the nerve terminal will have a significant influence on synaptic vesicle recycling during prolonged high frequency stimulation. It has been shown that a decrease in intracellular cAMP by blockage of adenylyl cyclase resulted in changes in the exo- and endocytosis that were similar to the changes induced by exogenous NO, namely, the inhibition of exocytosis of vesicles from the ready releasable pool and the weakening of endocytosis. Under these conditions, prolonged rhythmic stimulation caused a more pronounced depression of EPC relative to the control [36].

Changes in the rate of vesicle cycling may underlie various forms of short-term and long-term synaptic plasticity [25]. Indeed, NO, as a retrograde transmitter, has been demonstrated to enhance endocytosis

and synaptic vesicle recycling via a cGMP-dependent increase in the level of phosphatidylinositol 4,5-biphosphate in a culture of hippocampal neurons [21]. On the other hand, NO has been shown to induce prolonged depression of release of vesicle of the ready releasable pool at the synapses between Schaffer collaterals and CA1 pyramidal neurons [20].

There is also evidence of a direct effect of NO on the activity of proteins that are involved in exo- and endocytosis in various cell types [37]. It has been shown that the neurotransmitter release and exo- and endocytosis are inhibited by S-nitrosylation of the N-ethylmaleimide-sensitive factor (NSF) which implements SNARE complex disassembly and is involved in endocytosis [38, 39]. In chromaffin cells, NO donors caused a significant reduction in the release of secretory granules, whereas inhibitors of NO synthase increased the release of catecholamines [40]. In contrast, there are the data on the enhancement of endocytosis under conditions of NO action, e.g., in endothelial cells NO increased endocytosis by S-nitrosylation of dynamin, which plays a key role in vesicle budding from the membrane [41].

Thus, in our study, it was shown that an NO donor reduces the evoked release of neurotransmitters from the mouse motor nerve terminal under conditions of a single stimulation. In this case, exogenous and endogenous NO are involved in the regulation of high frequency synaptic activity and cause depression of neurotransmitter release. This may result from a decrease in synaptic vesicle recycling caused by endocytosis and/or mobilization of synaptic vesicles from a recycling pool to the release sites.

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