ISSN 1990-7478, Biochemistry (Moscow) Supplement Series A: Membrane and Cell Biology, 2013, Vol. 7, No. 2, pp. 170–173. © Pleiades Publishing, Ltd., 2013.
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ARTICLES

# The Effects of Hydrogen Sulfide on the Processes of Exo- and Endocytosis of Synaptic Vesicles in the Mouse Motor Nerve Endings

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Received June 24, 2011

**Abstract**—The effects of sodium hydrosulfide (NaHS), the donor of hydrogen sulfide ($H_2S$), on the exo/endocytosis cycle of synaptic vesicles in the motor nerve ending of the mouse diaphragm were studied using intracellular microelectrode technique and fluorescent microscopy. NaHS increased the frequency of miniature end-plate potentials (MEPPs), without changing their amplitude–time parameters. NaHS also increased the amplitude of the evoked postsynaptic responses during single stimulation (0.3 Hz), which was the evidence of the enhanced synaptic vesicle exocytosis. During high-frequency stimulation (50 Hz), NaHS induced more significant decline of neurotransmitter release, probably due to the lower rate of synaptic vesicle mobilization from recycling pool to exocytic sites. NaHS also decreased the uptake of the fluorescent endocytic dye FM 1-43, which indicated the reduced endocytosis of synaptic vesicles. Thus, the $H_2S$ donor increases exocytosis and decreases the processes of synaptic vesicle endocytosis and mobilization in the mouse motor nerve ending.

*Keywords*: hydrogen sulfide, motor nerve ending, neurotransmitter release, exo- and endocytosis.
**DOI:** 10.1134/S1990747812050121

Hydrogen sulfide ($H_2S$), as well as carbon monoxide and nitric oxide, belongs to a novel class of the mediators called gasotransmitters [1–4]. $H_2S$ is endogenously produced from *L*-cysteine by cystathionine-γ-lyase, cystathionine-β-synthase, and mercaptosulfotransferase, which are specifically expressed in different tissues [2, 3]. $H_2S$ facilitates the induction of long-term potentiation in response to a weak tetanic stimulation in the hippocampus [5] and intensifies neurotransmitter release from the frog and mouse motor nerve endings in the peripheral nervous system [6, 7]. The targets of $H_2S$ in various tissues are the ATP-dependent and calcium-activated potassium channels, the L- and T-type voltage-dependent calcium channels [8, 9], and the adenylate cyclase system [10, 11].

In synaptic structures, the key stages of neurotransmitter release are exo- and endocytosis of synaptic vesicles [12, 13]. The processes from the moment of the exocytosis of a vesicle till it acquires again the ability for exocytosis are referred to as a recycling and the continuous turnover of vesicles in nerve endings is referred to as a vesicle cycle [13]. There are no data on the influence of $H_2S$ on the processes of synaptic vesicle recycling under the conditions of high-frequency synaptic activity. The goal of this work was to investigate the effect of $H_2S$ donor (NaHS) on the dynamics of neurotransmitter release and on the processes of exo- and endocytosis of synaptic vesicles in the mouse motor nerve ending during long-term high-frequency stimulation using electrophysiological and optical (fluorescent) methods.

## MATERIALS AND METHODS

Isolated nerve-muscle preparations of mouse diaphragm were used in the experiments. Neuromuscular preparations were isolated and placed into a bath filled with the standard Krebs solution containing (mM): NaCl, 137; KCl, 5; $CaCl_2$, 2.2; $MgCl_2$, 1; $NaHCO_3$, 11; $NaH_2PO_4$, 1; glucose, 11. Before the experiment, the solution was saturated with carbogene (95% $O_2$, 5% $CO_2$) at 20°C, pH 7.2–7.4. The muscle fiber contractions and action potentials were blocked with d-tubocurarine at a concentration of 2–5 μM. Sodium hydrosulfide (NaHS) (Sigma, USA), the $H_2S$ donor producing $H_2S$ in aqueous solutions [5], was used in the experiments at a concentration of 200 μM.

Spontaneous and evoked end plate potentials (EPPs) were recorded intracellularly using glass microelectrodes filled with the KCl solution (2.5 M) and an amplifier with 0–10 kHz bandpass and an amplification coefficient of 400. The recordings were made by an automated system consisting of an L-CARD 1250 A/D converter and a Pentium 4 computer. The motor nerve was stimulated with rectangular pulses of suprathresh-

old amplitude: 0.3 Hz (single stimulation) and 50 Hz (high-frequency stimulation). The effects of NaHS on EPPs or miniature EPPs (MEPPs) were analyzed as follows: after recording of the control signals for 10–15 min, the substance was added to the perfusion solution for 20 min and washed out subsequently with the standard Krebs solution. In case of high-frequency stimulation, NaHS-containing solution was applied to the neuromuscular preparation during 10–15 min before the stimulation onset.

Fluorescent dye FM 1-43 (Biotium, USA) was used in the experiments at a concentration of 2 μM. The dye reversibly binds to the presynaptic membrane and during endocytosis gets into the newly formed synaptic vesicles. Dye loading is accompanied by the fluorescence of the nerve ending, which reflects the aggregates of the vesicles that have captured the dye [13, 14]. Endocytosis processes were investigated using two protocols. In the first one, FM 1-43 was present in the solution for 1 min during the 50-Hz stimulation of the motor nerve and for 7 min after its termination ("full loading"); in the second protocol, the dye was present in the solution only for 1 min during the 50-Hz stimulation of the nerve ("loading during stimulation"). After the dye loading, the preparation was washed out with the Krebs solution for 40–60 min and the fluorescence of nerve endings was recorded. The effects of $H_2S$ on the endocytosis of synaptic vesicles were studied by incubating the neuromuscular preparation in the presence of NaHS donor for 10 min before stimulation onset.

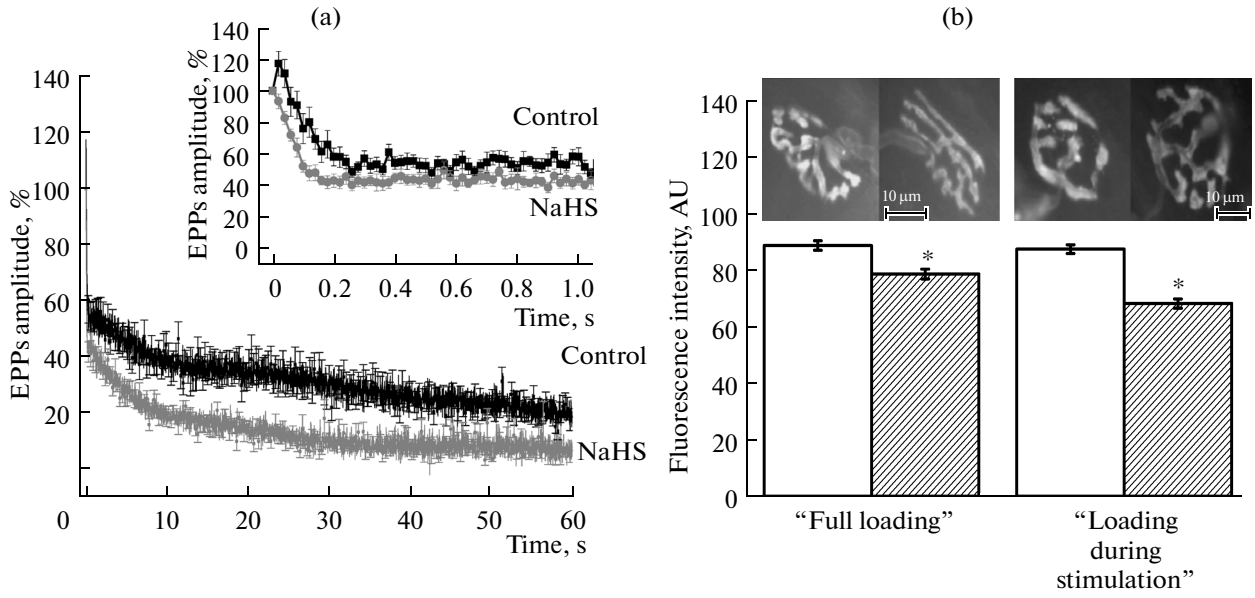
The fluorescence of nerve endings was visulalised using microscope MIKMED-2 (LOMO, Russia) or AxioScope A1 (Carl Zeiss, Germany) equipped with water immersion lens LUMPLFL 60×/0.9-NA (Olympus, USA) and/or Plan-Neofluar 63×/0.9 (Carl Zeiss, Germany). All observations were made only for nerve endings in the most superficial layers of tissues. Fluorescence patterns were recorded with high-speed black-and-white video cameras AxioCam MRm (Carl Zeiss, Germany). The mean value of the fluorescence intensity was estimated in arbitrary units (AU) at a nerve ending segment of 10–20 μm in length, taking the maximum pixel fluorescence of 256 as 1. Then background fluorescence was determined as the mean fluorescence intensity in a 50 × 50 square of pixels in the image area without nerve ending. The background value was then subtracted from each pixel obtained after image averaging. All data were processed by the methods of variation statistics. The quantitative results are presented as the mean value ± standard error; $n$ is the number of independent experiments.

## RESULTS AND DISCUSSION

The application of NaHS (200 μM) increased the EPPs amplitude to 146 ± 7% ($n = 7$; $p < 0.05$) under the conditions of single stimulation and increased the MEPPs frequency to 200 ± 42% ($n = 4$; $p < 0.05$) by the 6–10th min of the application. The time-amplitude parameters of MEPP did not change. The MEPPs amplitude was 0.48 ± 0.09 mV ($n = 4$) in the control and 0.44 ± 0.09 mV ($n = 4$, $p > 0.05$) during NaHS application. The rise time varied insignificantly: 0.69 ± 0.04 ms ($n = 4$) in the control and 0.81 ± 0.08 ms ($n = 4$, $p > 0.05$) in the presence of the $H_2S$ donor; the decay time was 2.57 ± 0.11 ms and 2.34 ± 0.13 ms ($n = 4$, $p > 0.05$), respectively. These data are in agreement with the results of our previous studies and demonstrate that NaHS acts at a presynaptic level, intensifying the release of acetylcholine from the nerve ending [7].

The dynamics of neurotransmitter release under the conditions of high-frequency stimulation was studied during 1 min of 50-Hz stimulation. In the control, high-frequency stimulation of the motor nerve was accompanied by characteristic changes in the EPPs amplitude. The initial abrupt decrease in the EPPs amplitude developed during the first 10–15 impulses and reached 48 ± 3% by the end of the first second of stimulation ($n = 10$, $p < 0.05$) relative to the first signal (Figure (a), *insert*). Then there was a period of plateau at the level of 32 ± 5% ($n = 10$, $p < 0.05$) during 25 s of stimulation, after which the EPPs amplitude slowly decreased to 19 ± 3% ($n = 10$, $p < 0.05$) at the end of stimulation (Figure (a)). With preliminary application of NaHS at a concentration of 200 μM, the EPPs amplitude was 43 ± 6% ($n = 9$, $p > 0.05$) by the first second of stimulation, which did not differ from the control value (Figure (a), *insert*). However, a more pronounced marked decrease of EPPs was observed later and the EPPs amplitude reached 16 ± 2% ($n = 9$, $p < 0.05$) by 25 s and 10 ± 2% ($n = 9$, $p < 0.05$) relative to the control by 60 s of high-frequency stimulation (Figure (a)).

According to the published data, the mouse motor nerve ending has three functioning vesicular pools different in size, localization, and physiological role: readily releasable pool (RRP), mobilization pool, and the reserve one [14, 15]. The RRP is quickly used up during high-frequency stimulation and replenished from the mobilization pool. It seems that both of these pools are replenished mainly due to the fast recycling and provide for exocytosis during high-frequency stimulation. The vesicles of the reserve pool seem not to be involved in neurotransmitter release at all [14]. The initial fast decay of the EPPs amplitude during high-frequency stimulation is associated with the uptake and diminution of the RRP. It is followed by stabilization of the EPPs amplitude, indicating replenishment of the RRP, when the rates of its usage and replenishment are equal due to the movement of vesicles from the mobilization pool to the active zone and intensive recycling of synaptic vesicles. The secondary slower decay of the EPPs amplitude reflects further diminution of the RRP in case of its insufficient replenishment [14]. A more dramatic decline in EPPs amplitudes under the influence of $H_2S$ may be




Effects of NaHS on the processes of exo- and endocytosis of synaptic vesicles at the motor nerve ending. (a) The change in the EPPs amplitude during high-frequency stimulation (50 Hz, 1 min) in the control (*black*) and in the presence of NaHS (200 μM) (*gray*). The *insert* shows the dynamics of the EPPs amplitude in the first second of stimulation. (b) Fluorescence intensity of nerve endings in the control (*white bars*) and in the presence of NaHS (200 μM) (*cross-hatched bars*) after loading of the nerve endings with FM 1-43 using "full loading" or "loading during stimulation" protocol (see Materials and Methods). Above the bars are the examples of black-and-white images of dye-loaded nerve endings. $^*p < 0.05$.

due to both intensification of exocytosis and slowdown of mobilization of synaptic vesicles to the sites of secretion.

In addition, one cannot exclude possible slowdown of endocytosis and recycling produced by NaHS. It is also confirmed by experiments with the loading of endocytic marker into the nerve ending during high-frequency stimulation [16]. The average fluorescence intensity of the nerve ending during full dye loading (see Materials and Methods) after the incubation in the presence of NaHS (200 μM) for 10 min was 78 ± 2 AU ($n = 3$, $p < 0.05$), which is lower by 11% than in the control (89 ± 2 AU, $n = 4$) (Figure (b)). In the course of loading during the stimulation, which displayed only endocytosis that occurred simultaneously with induced exocytosis, the nerve ending fluorescence intensity under the influence of $H_2S$ was 67 ± 1 AU ($n = 3$; $p < 0.05$), i.e., also below the control values (87 ± 2 AU, $n = 3$) (Figure (b)). Thus, as a result of NaHS action on neuromuscular junction, the nerve ending fluorescence intensity significantly decreased both in the case of full loading of the nerve ending with the fluorescent marker and in the case of loading only during the stimulation, demonstrating the slowdown of synaptic vesicle endocytosis under the influence of the gas.

What are possible mechanisms of the $H_2S$ effects? The increase of neurotransmitter release in response to single stimulations under the influence of $H_2S$ may be due to enhanced intracellular concentration of calcium ions. In astrocytes and neuronal cells, $H_2S$ increases the intracellular calcium concentration on account of intensification of Ca entry through L- and T-type voltage-dependent Ca-channels or Ca release from intracellular stores [8, 17]. In addition, the increase of neurotransmitter release may be due to the increase in cAMP intracellular concentration [10], which leads to the activation of protein kinase A and intensification of neurotransmitter release as a result of phosphorylation of exo- and endocytosis proteins, the subunits of voltage-dependent Ca channels, K channels, and ryanodine receptors [18]. The increase in calcium level or protein phosphorylation under the influence of $H_2S$ may also underlie the changes in mobilization and/or endocytosis of synaptic vesicles [19–21]. In addition, $H_2S$ can directly interfere with the mechanisms of exo- and endocytosis of synaptic vesicles through the transformation of SNARE-complex proteins, which is demonstrated by the increase in MEPPs frequency. It is known that $H_2S$ in aqueous solutions has the reducing properties: it can reduce disulfide bonds and modify thiol groups in protein molecules [3]. Thus, for example, the proteins of the SNARE complex, which performs the vesicle and nerve ending membrane fusion, and the factor sensitive to N-ethylmaleimide, which performs disassembly of the SNARE complex and is involved in endocytosis, are enriched in cysteine residues and are potential sites for such modification [22, 23].

The expression of the enzymes of $H_2S$ synthesis has been revealed in the region of the neuromuscular synapse of mouse diaphragm. At the same time, the sub-

strate of $H_2S$ synthesis, *L*-cysteine, increased neurotransmitter release, while the inhibition of the enzyme of $H_2S$ synthesis induced the effects opposite to the effect of NaHS, suggesting the possibility of endogenous synthesis of the gas [7]. It is known that $H_2S$ synthesis is regulated both at the level of cystathionine-γ-lyase and cystathionine-β-synthase expression in tissues and through modification of their activity [24, 25]. Thus, for example, nitric oxide can regulate the level of $H_2S$ in smooth muscle cells by enhancing the activity of cystathionine-γ-lyase through nitrosylation of cysteine residues in the protein molecule [25]. In addition, the Ca/calmodulin-dependent regulation of the enzymes of $H_2S$ synthesis may occur during high-frequency stimulation and calcium accumulation in nerve endings or in muscle fibers [24].

In conclusion, our results demonstrate that exogenous and probably endogenous $H_2S$ enhances neurotransmitter release during a single stimulation and intensifies EPPs depression during high-frequency activity. The effect of $H_2S$ is based on the suppression of mobilization and endocytosis of synaptic vesicles resulting in the decrease of recycling processes.

## ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research and the *Support Program for the Leading Scientific Schools* (project NS-4670.2012.4.).

## REFERENCES

1. Sitdikova, G.F., Zefirov, A.L. 2006. Gaseous mediators in the nervous system. *Ross. Fiziologicheskiy Zh. im. I.M. Sechenova* (Rus.). **92**(7), 872–882.
2. Sitdikova, G.F., Zefirov, A.L. 2010. Hydrogen sulfide: From the Parisian sewer system to a signaling molecule. *Priroda* (Rus.). **9**, 29–37.
3. Gadalla M.M., Snyder S.H. 2010. Hydrogen sulfide as a gasotransmitter. *J. Neurochem.* **113**, 14–26.
4. Hermann A., Sitdikova G.F., Weiger T. 2010. Gase als zelluläre Signalstoffe. Gasotransmitter. *Biol. Unserer Zeit*. **40** (3), 185–193.
5. Abe K., Kimura H. 1996. The possible role of hydrogen sulfide as an endogenous neuromodulator. *J. Neurosci.* **16**, 1066–1071.
6. Gerasimova E.V., Sitdikova, G.F., Zefirov, A.L. 2008. Hydrogen sulfide as an endogenous modulator of mediator release at the neuromuscular synapse of the frog. *Neyrokhimiya* (Rus.). **25**(1–2), 138–145.
7. Gerasimova E.V., Vologin S.G., Mukhacheva Yu.A., Sitdikova G.F. 2010. The effect of hydrogen sulfide on mediator release and detection of the expression of cystathionone-gamma-lyase in the diaphargmal muscle of mouse. *Uchenye zapiski KGU* (Rus.). **152**(2), 41–50.
8. Nagai, Y., Tsugane M., Oka J., Kimura H. 2004. Hydrogen sulfide induces calcium waves in astrocytes. *FASEB J.* **18**, 557–559.
9. Sitdikova G.F., Weiger T.M., Hermann A. 2010. Hydrogen sulfide increases calcium-activated potassium (BK) channel activity of rat pituitary tumor cells. *Pflügers Arch – Eur. J. Physiol.* **459**, 389–397.
10. Kimura H. 2000. Hydrogen sulfide induces cyclic AMP and modulates the NMDA receptor. *Biochem. Biophys. Res. Commun.* **267**, 129–133.
11. Sitdikova G.F., Gerasimova E.V., Khaertdinov N.N., Zefirov A.L. 2009. The role of cyclic nucleotides in the effects of hydrogen sulfide on mediator release in the neuromuscular synapse of frog. *Neyrokhimiya* (Rus.). **26**(4), 1–7.
12. Sudhof T.C. 2004. The synaptic vesicle cycle. *Annu. Rev. Neurosci.* **27**, 509–547.
13. Zefirov A.L., Petrov A.M. 2010. *Sinapticheskaya vezikula i mekhanizm osvobozhdeniya mediatora (ekzo-endotsitozny vezikulyarny tsikl)* (Synaptic Vesicle and the Mechanism of Mediator Release (Exo-Endocytotic Vesicular Cycle)), Kazan: Art-Kafe.
14. Zefirov A L., Zakharov A.M., Mukhametzyanov R.D., Petrov A.M. 2008. Vesicular cycle in the motor nerve endings of the mouse diaphragm. *Zh. Evolyutsionnoy Biokhimii i Fiziologii* (Rus.). **44**(6), 603–612.
15. Rizzoli S.O., Betz W.J. 2005. Synaptic vesicle pools. *Neurosci.* **6**, 57–69.
16. Betz W.J., Bewick G.S. 1992. Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. *Science*. **255**, 200–203.
17. Garcia-Bereguiain M.A., Samhan-Arias A.K., Martin-Romero F.J., Gutierrez-Merino C. 2008. Hydrogen sulfide raises cytosolic calcium in neurons through activation of L-type $Ca^{2+}$ channels. *Antiox. Redox Signal.* **10**, 31–41.
18. Seino S., Shibasaki T. 2005. PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. *Physiol. Rev.* **85**, 1303–1342.
19. Zefirov A.L., Abdrakhmanov M.M., Grigoryev P.N., Petrov A.M. 2006. Intracellular calcium and the mechanisms of endocytosis of synaptic vesicles in the motor nerve ending of frog. *Tsitologiya* (Rus.). **48**(1), 3441.
20. Petrov A.M., Giniatullin A.R., Zefirov A.L. 2008. The role of cAMP-dependent signaling pathway in the synaptic vesicle cycle of motor nerve ending. *Neyrokhimiya* (Rus.). **25**(3), 202–210.
21. Petrov A.M., Giniatullin A.R., Sitdikova G.F., Zefirov A.L. 2008. The role of cGMP-dependent signaling pathway in synaptic vesicle cycle at the frog motor nerve terminals. *J. Neurosci.* **28**(49), 13216–13222.
22. Duman J.G., Forte J.G. 2003. What is the role of SNARE proteins in membrane fusion? *Am. J. Physiol. Cell Physiol.* **285**, C237–C249.
23. LoPachin R.M., Barber D.S. 2006. Synaptic cysteine sulfhydryl groups as targets of electrophilic neurotoxicants. *Toxicol. Sci.* **94**(2), 240–255.
24. Eto K., Ogasawara M., Umemura K., Nagai Y., Kimura H. 2002. Hydrogen sulfide is produced in response to neu- 2
ronal excitation. *J. Neurosci.* **22**, 3386–3391.
25. Fiorucci S., Distrutti E., Cirino G., Wallace J.L. 2006. The emerging roles of hydrogen sulfide in the gastrointestinal tract and liver. *Gastroenterology*. **131**, 259–271.

*Translated by E. Makeeva*



SPELL: 1. bandpass, 2. neuronal