MECHANISMS OF HYDROGEN SULFIDE (H₂S) ACTION ON SYNAPTIC TRANSMISSION AT THE MOUSE NEUROMUSCULAR JUNCTION

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Abstract—Hydrogen sulfide (H₂S) is a widespread gasotransmitter also known as a powerful neuroprotective agent in the central nervous system. However, the action of H₂S in peripheral synapses is much less studied. In the current project we studied the modulatory effects of the H₂S donor sodium hydrosulfide (NaHS) on synaptic transmission in the mouse neuromuscular junction using microelectrode technique. Using focal recordings of presynaptic response and evoked transmitter release we have shown that NaHS (300 μM) increased evoked end-plate currents (EPCs) without changes of presynaptic waveforms which indicated the absence of NaHS effects on sodium and potassium currents of motor nerve endings. Using intracellular recordings it was shown that NaHS increased the frequency of miniature end-plate potentials (MEPPs) without changing their amplitudes indicating a pure presynaptic effect. Furthermore, NaHS increased the amplitude of end-plate potentials (EPPs) without influencing the resting membrane potential of muscle fibers. L-cysteine, a substrate of H₂S synthesis induced, similar to NaHS, an increase of EPC amplitudes whereas inhibitors of H₂S synthesis (β-cyano-L-alanine and aminoxyacetic acid) had the opposite effect. Inhibition of adenylate cyclase using MDL 12,330A hydrochloride (MDL 12,330A) or elevation of cAMP level with 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate (pCPT-cAMP) completely prevented the facilitatory action of NaHS indicating involvement of the cAMP signaling cascade. The facilitatory effect of NaHS was significantly diminished when intracellular calcium (Ca²⁺) was buffered by 1,2-bis(2-aminophenoxo)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM) and ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid acetoxymethyl ester (EGTA-AM). Activation of ryanodine receptors by caffeine or ryodine increased acetylcholine release and prevented further action of NaHS on transmitter release, likely due to an occlusion effect. Inhibition of ryanodine receptors by ryanodine or dantrolene also reduced the action of NaHS on EPC amplitudes. Our results indicate that in mammalian neuromuscular synapses endogenously produced H₂S increases spontaneously and evoked quantal transmitter release from motor nerve endings without changing the response of nerve endings. The presynaptic effect of H₂S appears mediated by intracellular Ca²⁺ and cAMP signaling and involves presynaptic ryanodine receptors.

Key words: hydrogen sulfide, neuromuscular junction, transmitter release, L-cysteine, adenylyl cyclase, ryanodine receptors.

INTRODUCTION

Hydrogen sulfide (H₂S), is a gaseous transmitter along with nitric oxide (NO) and carbon monoxide (CO) (Kimura, 2010, 2011; Hermann et al., 2012; Paul and Snyder, 2012; Wang, 2012, 2014). Several important biological actions of H₂S have been identified including regulation of blood pressure, insulin release, cytoprotection, smooth muscle relaxation and neuronal excitability (Hosoki et al., 1997; Kimura and Kimura, 2004; Kawabata et al., 2007; Sitdikova et al., 2010; Hermann et al., 2012; Wang, 2012, 2014; Kuksis et al., 2014). Endogenous production of H₂S in mammalian tissues occurs mainly through three enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptoppyruvate sulfurtransferase along with an additional contribution of cysteine aminotransferase (Abe and Kimura, 1996; Kamoun, 2004; Shibuya et al., 2009; Kimura, 2011, 2014). Ion channels are a main target of...
H₂S action in excitable cells (Tang et al., 2010). H₂S is reported to increase the NMDA-currents in the rat hippocampus (Abe and Kimura, 1996), inhibits or activates different types of potassium (K⁺) and calcium (Ca²⁺) channels (Kimura et al., 2006; Sittikova et al., 2010, 2014; Zhong et al., 2010; Sekiguchi et al., 2014) or activates sodium channels (Luo et al., 2014). In addition to its function as a signaling molecule, H₂S acts as a protective agent against oxidative stress by increasing levels of glutathione (Kimura and Kimura, 2004). Interestingly, in neurons H₂S directly antagonizes the neurotoxicity of homocysteine, whose level increases during aging and in neurodegenerative diseases (Tang et al., 2010; Veeranki and Tyagi, 2013).

We have previously shown that in the frog neuromuscular junction H₂S enhanced both spontaneous and evoked neurotransmitter release without changing focally recorded presynaptic responses. We also found that the substrate of H₂S synthesis, L-cysteine, increased evoked transmitter release whereas inhibitors of CSE and CBS induced the opposite action (Gerasimova et al., 2008). The analysis of intracellular mechanisms of H₂S action suggested a role of cAMP and ryanodine receptors (RyR) in the action of H₂S (Sittikova et al., 2009; Sittikova and Zefirov, 2012; Gerasimova et al., 2013). H₂S also impairs the processes of exo- and endocytosis of synaptic vesicles during high-frequency stimulation of the motor nerve (Sittikova et al., 2011; Mitrukhina et al., 2013).

However, the action of H₂S has not been studied in mammalian synapses. In the current project we explored the action of this gaseous transmitter on synaptic transmission in the mouse neuromuscular junction using a classical preparation of the diaphragm muscle innervated by the phrenic nerve. We show that H₂S increases both spontaneous and evoked transmitter release in mammalian neuromuscular junctions, provide evidence on the tonic action of endogenous H₂S and describe the molecular mechanisms underlying H₂S stimulatory actions.

**EXPERIMENTAL PROCEDURES**

**Preparation and solutions**

Experiments were performed on isolated phrenic nerve–diaphragm preparations from the white mouse (BALB/c strain) of both sexes of 20–25 g body weight. Animals were anesthetized using 5% isoflurane (Abbott Laboratories, USA) before being decapitated in accordance with the European Communities Council Directive (November 24, 1986: 86/609/EEC). Animal experiments were approved by the Ethics Committee of Kazan Medical University. Efforts were made to minimize the number of animals used for these studies. The neuromuscular preparation was mounted to the recording chamber and constantly superfused with gassed (95% O₂/5% CO₂) Krebs solution containing (in mM): NaCl – 154; KCl – 5; CaCl₂ – 2; HEPES – 5, MgCl₂ – 1, glucose – 11 (t = 20 ± 0.5°C, pH 7.2–7.4). To prevent muscle contractions in response to nerve stimulation D-tubocurarine (2–3 μM) was added to the solution.

**Drugs**

All drugs were dissolved to a final concentration in Krebs solution and applied to preparations via a bath perfusion system (2 ml/min). Sodium hydrosulfide (NaHS) was used as a donor of H₂S, which in aqueous solutions dissociates to sodium ion (Na⁺) and hydrosulphide anion (HS⁻) which forms H₂S through reacting with protons (H⁺). Recent recalculations of the concentration of H₂S produced from NaHS taking pH, temperature, salinity of the perfusate, and evaporation of H₂S into account indicated that only 11–13% is effective as H₂S in solution from the initial concentration of NaHS (Sittikova et al., 2014). Compounds used were: the substrate for endogenous H₂S production – L-cysteine (1 mM) and inhibitors of H₂S synthesis – β-cyano-L-alanine (β-CA) (1 mM), aminooxyacetic acid (AOAA) (1 mM), activators and inhibitors of RyR – ryanodine (0.1 μM and 3 μM), dantrolene (25 μM), caffeine (3 mM), adenylyl cyclase inhibitor – MDL 12,330A hydrochloride (MDL 12,330A) (3 μM) and cAMP analog – 8-(4-chlorophenylthio)-adenosine 3’,5’-cyclic monophosphate (pCPT-cAMP) (100 μM), 1,2-bis(2-ami nophenoxo)ethane-N,N,N’,N’-tetracetic acid tetras acid acetoxymethyl ester (BAPTA-AM) (50 μM), ethylene gly col-bis(2-aminoethylether)-N,N,N’,N’-tetracetic acid acetoxymethyl ester (EGTA-AM) (50 μM). All substances were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Electrophysiological recordings**

Presynaptic response, miniature and evoked end-plate currents (MEPCs and EPCs) were recorded focally using thick-walled, heat-polished glass microelectrodes (2.0–4.0 MΩ filled with 2 M of NaCl). Extracellular current that enters (or leaves) the membrane under the electrode was recorded as a potential drop across the sealing resistance. Positive signals indicated outward current; negative signals, inward current (Brigant and Mallart, 1982). To improve presynaptic signal to noise ratio, twenty to thirty sweeps were sampled in 2-μs steps and averaged. Intracellular recordings of miniature end-plate potentials (MEPPs) and end-plate potentials (EPPs) were performed by using glass microelectrodes (5–10 MΩ) filled with 2.5 M KCl). Experiments were performed on muscle fibers with a membrane potential more negative than –60 mV. Pre- and postsynaptic responses were evoked by motor nerve stimulation by supra-threshold stimuli of 0.1–0.2-ms duration and 0.2-Hz frequency, amplified (amplification coefficient ×400) and acquired by the PC software using an L-CARD 1250 A/D. Analysis of focal currents and intracellular recorded potentials was performed using the original software developed in our laboratory (Zakharov A.). The amplitude, rise time, decay time (τ) and the frequency of MEPPs and MEPCs, the amplitudes of EPPs and EPCs and presynaptic waveforms were calculated.

Data are presented as the mean ± SEM (n = number of animals), with statistical significance assessed by Student’s t-test. Differences were considered significant when p < 0.05.
RESULTS
NaHS effects on spontaneous and evoked acetylcholine release

First, we carried out experiments with focal recording of synaptic events in order to reveal the simultaneous effects of NaHS on presynaptic response and transmitter release. Presynaptic wave form configuration was dependent on electrode position, and triphasic responses in our experiments consisted from positive-negative-positive phases – outward passive current, active Na current and delayed outward K current (Fig. 1A) (Brigant and Mallart, 1982; Van der Kloot and Molgo, 1994). Application of NaHS (300 μM, resulting in effectively approx. 30–40 μM H2S in solution, Sitdikova et al., 2014) induced a reversible increase in the amplitude of EPCs up to 182.3 ± 16.6% (n = 9; p < 0.05) (Fig. 1A, B) without changing the presynaptic response. The analysis of amplitudes of second and third phases revealed the absence of NaHS effect on Na+ and K+ currents of motor nerve endings (Fig. 1C). Also, an increase of MEPCs frequency was observed up to 210.1 ± 42.5% (n = 4; p < 0.05) relative to control (Fig. 1D). The amplitude and time parameters of MEPCs were not changed indicating the absence of postsynaptic effects (not shown).

Next we studied the action of NaHS using intracellular recordings of spontaneous and evoked EPPs. Application of the H2S donor NaHS to the diaphragm muscle induced a rapid and reversible increase of spontaneous and evoked EPPs. NaHS increased the amplitude of EPPs at 15 min to 186.3 ± 15.4% (n = 7; p < 0.05) of control (Fig. 2A, B). At the same time the frequency of MEPPs increased from 1.38 ± 0.4 Hz to 2.78 ± 0.5 Hz (n = 5, p < 0.05; Fig. 2C). Notably, the amplitude and time parameters of MEPP did not change. In control conditions, the amplitude of MEPPs was 0.48 ± 0.09 mV (n = 5) and remained at the same level (0.44 ± 0.09 mV) during NaHS application (n = 5, p > 0.05, Fig. 2D). Likewise, no changes were observed in the rise time of MEPPs (0.69 ± 0.04 ms, n = 5 in control versus 0.81 ± 0.08 ms, n = 5, p > 0.05 under NaHS) and no changes in the decay of MEPPs (τ = 2.57 ± 0.11 ms, n = 5 in control versus 2.34 ± 0.13 ms n = 5, p > 0.05 under NaHS), were observed (Fig. 2E, F). Resting membrane potential measured during experiment in control was −63.08 ± 2.75 mV, and by 15 min of NaHS application −62.41 ± 1.83 mV (n = 13, p > 0.05). The obtained results indicated a pure presynaptic action of the H2S donor at the mouse neuromuscular junction.

Thus, using these two modes of recording we demonstrated that the H2S donor NaHS enhances spontaneous and evoked neurotransmitter release from nerve endings of the mouse diaphragm muscle and has no effect on postsynaptic responses.

Effect of the H2S substrate L-cysteine and inhibitors of H2S synthesis on synaptic responses

In order to test the potential action of endogenous H2S on synaptic transmission we next used the H2S substrate L-cysteine and inhibitors of H2S synthesis. It is known that L-cysteine is a major substrate of H2S endogenous
The application of L-cysteine (1 mM) significantly increased EPCs amplitude up to 112.2 ± 1.5% (n = 4; p < 0.05; Fig. 3A, B). The inhibitor of CBS, AOAA, and the inhibitor of CSE, β-CA, are widely used to test the involvement of endogenous H$_2$S in physiological regulation (Kamoun, 2004). In our experiments the application of β-CA (1 mM) decreased the EPCs amplitude down to 73.1 ± 4.2% (n = 6; p < 0.05; Fig. 3A, B). Similarly, AOAA (1 mM) reduced EPCs to 63 ± 16% (n = 5; p < 0.05; Fig. 3A, B). Both L-cysteine and inhibitors of H$_2$S synthesis did not affect the presynaptic response.

Hence, the H$_2$S substrate increased neurotransmitter release whereas the inhibitors of H$_2$S synthesis exerted the opposite effects. Taken together these data are consistent with the notion of a tonic positive tone mediated by endogenous H$_2$S in the mammalian neuromuscular junction.

**The role of adenylate cyclase in the action of H$_2$S**

Next we tested the potential involvement of intracellular signaling cascades in the action of H$_2$S. Since previous studies suggested the involvement of cAMP pathways (Kimura, 2000; Njie-Mbye et al., 2010) we first tested the role of the adenylate cyclase in the effects of H$_2$S. To this end we used the adenylate cyclase inhibitor – MDL 12,330A (5 μM) which by itself induced a significant decrease of EPCs to 72.2 ± 4.8% (n = 5; p < 0.05; Fig. 4A). Further application of NaHS did not exert any
Effect on the amplitude of EPCs (Fig. 4A). In order to increase intracellular cAMP concentration the membrane-permeable, non-hydrolyzing cAMP analog pCPT-cAMP (100 µM) was added to the bath solution. pCPT-cAMP (100 µM) by itself had no significant effect on EPCs amplitude. Further application of NaHS did not significantly change the amplitude of the EPCs to 103.5 ± 12.5% (n = 7; p > 0.05). In summary these data suggest that the cAMP pathway is involved in the facilitatory action of H₂S.

The role of intracellular calcium and ryanodine receptors

To evaluate the role of intracellular Ca²⁺ in NaHS effects mouse diaphragm was incubated for 1 h in bath solution, containing Ca²⁺ chelators BAPTA-AM (50 µM) and EGTA-AM (50 µM), and then it was washed for 1 hour. Subsequent application of NaHS (300 µM) induced an increase of EPCs amplitude by 113 ± 4% (n = 4, p < 0.05), which is significantly lower than NaHS effect.
in control (Fig. 4B). We next examined whether the Ca\(^{2+}\) released from intracellular stores is responsible for NaHS-induced synaptic facilitation. Previously we obtained evidence of the involvement of RyR in the action of H\(_2\)S in frog neuromuscular junction (Gerasimova et al., 2013). To test the role of RyR in the effects of H\(_2\)S in mammalian junctions the activators of RyR – ryanodine (0.1 \(\mu\)M) and caffeine (3 mM) and the inhibitors – dantrolene (25 \(\mu\)M) and ryanodine (3 \(\mu\)M) were used (Zucchi and Ronca-Testoni, 1997; Zhao et al., 2001; Balezina, 2002). Application of RyR inhibitor dantrolene (25 \(\mu\)M) via bath solution reduced EPCs to 64.2 ± 6.2% (\(n = 4\); \(p < 0.05\); Fig. 4C). In these conditions NaHS increased the amplitude of the EPCs by 24.5 ± 6.3% (\(n = 5\); \(p < 0.05\); Fig. 4C), which was significantly less compared to NaHS effect on EPCs in control (\(p < 0.05\)). Further application of NaHS did not induce any effect on EPCs (Fig. 4C).

The activation of RyR by the nonspecific agent caffeine (3 mM) resulted in an increase of the EPCs amplitude to 153.6 ± 17.8% (\(n = 4\); \(p < 0.05\); Fig. 4D). Similar to the inhibitors, the subsequent application of NaHS did not change significantly the EPCs amplitude (Fig. 4D). Ryanodine (0.1 \(\mu\)M) increased EPCs amplitude to 123.6 ± 5.5% (\(n = 5\); \(p < 0.05\)) versus control (Fig. 4D). Subsequent application of NaHS had no effect on EPCs amplitude (Fig. 4D). Hence, the activation and blocking of the endoplasmic reticulum RyR completely or partially prevented the increase of transmitter release induced by H\(_2\)S.

The obtained data indicate that the effect of H\(_2\)S on evoked neurotransmitter release is mediated by an increase of cAMP and the activation of RyR which are responsible for the increase in the level of intracellular Ca\(^{2+}\) being tightly correlated to transmitter release from motor nerve endings.

**DISCUSSION**

The main finding of our study is that exogenous as well as endogenous H\(_2\)S facilitates both spontaneous and evoked transmitter release at the mammalian neuromuscular synapse without changing the presynaptic response. These effects appear mediated by cAMP signaling and RyR receptors suggesting the involvement of intracellular Ca\(^{2+}\) handling.

**Effects of exogenous and endogenous H\(_2\)S at the mammalian neuromuscular junction**

Gaseous messengers regulate neurotransmitter release and synaptic plasticity acting on pre- or postsynaptic levels (Abe and Kimura, 1996; Kimura, 2000; Eto et al., 2002). In our previous studies we provided evidence that NO and CO are produced at the neuromuscular junction and modulate acetylcholine release from motor nerve endings by changing the cAMP level which was increased or decreased by cGMP-dependent phosphodiesterases (Yakovlev et al., 2002; Shtikova et al., 2007; Valiullina and Shtikova, 2012; Yakovleva et al., 2013). Here we analyzed the effects and mechanisms of H\(_2\)S in the mouse neuromuscular junction. The expression of the H\(_2\)S synthesis enzymes, CBS and CSE, in mammalian skeletal muscles suggests a potential physiological role for H\(_2\)S in this tissue (Chen et al., 2010).

Nerve action potential is responsible for evoked transmitter release by depolarizing motor nerve endings and activating Ca\(^{2+}\)-influx (Van der Kloot and Molgo, 1994). It was shown in various studies that H\(_2\)S could activate different types of K\(^{+}\) and Ca\(^{2+}\) channels (Kimura et al., 2006; Shtikova et al., 2010, 2014; Zhong et al., 2010; Sekiguchi et al., 2014) and increase in Na\(^{+}\)-currents in rat brain slices (Luo et al., 2014). Therefore it was important in our experiments to carefully analyze the changes in presynaptic response during NaHS application. Extracellular focal recordings allow to study simultaneous ion currents of motor nerve endings and evoked transmitter release. Our study shows that NaHS significantly increased EPCs without changing of presynaptic Na\(^{+}\) and K\(^{+}\) currents. Similar effects were previously also observed in frog muscles by application of H\(_2\)S in gaseous form or by the H\(_2\)S donor NaHS (Gerasimova et al., 2008). Moreover, intracellular recordings were performed in order to verify the ability of NaHS to facilitate spontaneous transmitter release and evoked EPPs. These experiments indicate that the H\(_2\)S donor NaHS increased the level of spontaneous and evoked transmitter release from mouse motor nerve ending without changes in the amplitude, rise time or decay time of MEPPs and the resting membrane potential of muscle fibers. From these findings we conclude that the H\(_2\)S action is localized at the presynaptic side without changes in the sensitivity of postsynaptic acetylcholine receptors. Our study suggests a common targets of H\(_2\)S in neuromuscular junctions of different species consistent with the view that H\(_2\)S is a phylogenetically ancient regulatory molecule (Dombkowski et al., 2004).

Modulation of transmitter release by H\(_2\)S was first demonstrated in the rat hippocampus, where NaHS (at concentrations higher than 130 \(\mu\)M) suppressed hippocampal field excitatory postsynaptic potentials evoked by electrical stimulation of the Schafer collaterals (Abe and Kimura, 1996). Endogenous concentrations of H\(_2\)S were initially reported to be 50–160 \(\mu\)M (Goodwin et al., 1989). More recent reports by other authors suggest H\(_2\)S concentrations in the range of 10 nM to 3 \(\mu\)M in whole tissue preparations (Furne et al., 2008; Ishigami et al., 2009). In our experiments NaHS was used in a concentration of 300 \(\mu\)M which appears rather high. However, our recalculations taking pH, temperature, salinity of the perfusate, and evaporation of H\(_2\)S into account indicate that only 11–13%, i.e., 34–41 \(\mu\)M is effective as H\(_2\)S in solution which brings it closer to an apparently more physiological range (Shtikova et al., 2014). In addition, it has been shown that H\(_2\)S rapidly disappears from the solution suggesting that H\(_2\)S may function as a molecular ‘switch’ that activates downstream pathways that persist long after H\(_2\)S has vanished (Hu et al., 2009; Deleon et al., 2012). However, the effective concentrations of H\(_2\)S at target sites are still unknown.
Endogenous synthesis and signaling via H$_2$S in the neuromuscular junction was suggested from our experiments with l-cysteine which increased EPCs similarly to NaHS whereas the inhibition of CSE or CBS decreased EPCs. Despite the various effects of H$_2$S in many tissues, the major cellular sources of H$_2$S and the mechanism of its release are not well understood. H$_2$S, like the other gasotransmitters, can be immediately released after their production (Shibuya et al., 2009; Kimura, 2010) by short-term activation of CBS after neuronal excitation and Ca$^{2+}$ entry (Eto et al., 2002; Perry et al., 2009). CSE produces H$_2$S at steady-state low intracellular Ca$^{2+}$ concentrations (<300 nM) independently from calmodulin and its production is suppressed by high Ca$^{2+}$ concentrations in the 0.3–3 μM range (Mikami et al., 2013). In addition, bound sulfane sulfur may be another source of H$_2$S that can immediately release H$_2$S in response to physiological stimulation (Ishigami et al., 2009; Kimura, 2010).

**H$_2$S effects are mediated by cAMP signaling pathway and activation of RyR**

It has been shown that NaHS increases cAMP production in primary cultures of cerebral cortex cerebellar neurons as well as in glial cells (Kimura, 2000) and in isolated retinas (Njie-Mbye et al., 2010) in a concentration-dependent manner. In our study inhibition of adenylyl cyclase or the increased intracellular concentration of cAMP prevented the increase of acetylcholine release by NaHS. These data indicate the involvement of cAMP-PKA signaling in the action of H$_2$S. In frog motor nerve endings we have previously shown the participation of cAMP-dependent mechanisms in the effects of H$_2$S although the latter had no direct impact on the adenylyl cyclase activity (Skitkova et al., 2009).

It has been shown that PKA-dependent phosphorylation increased the open probability (Po) of RyR leading to calcium-induced calcium release (Zucchi and Ronca-Testoni, 1997). Emerging evidence suggests that RyR-mediated Ca$^{2+}$ release from the endoplasmic reticulum plays an important role in synaptic exocytosis in different preparations (Narita et al., 2000) including the mouse neuromuscular junction (Nishimura et al., 1990; Balezina, 2002). This is consistent with the view that H$_2$S increases acetylcholine release by activating RyR via cAMP-dependent signaling (Gerasimova et al., 2013).

In our experiments caffeine, a non-specific agonist of RyR induced a rapid enlargement of EPCs amplitude and completely prevented a further increase of acetylcholine release by H$_2$S. The same result was observed after application of the specific activator of RyR – ryanodine in low (activating RyR) concentrations. RyR inhibitors – dantrolene and ryanodine (at inhibitory high concentrations) decreased EPCs but the former prevented NaHS effects on transmitter release. In contrast, after dantrolene application we still observed an increase of EPCs amplitudes. This can be explained by the fact that dantrolene is a nonspecific inhibitor of RyR which does not block type 2 RyR (Zhao et al., 2001). Furthermore, the binding sites of dantrolene and H$_2$S may occur at different sites located at different membrane fractions (Palmitkar et al., 1997). It is also possible that the H$_2$S directly modifies RyR by its reducing action on disulfide bonds or by S-sulfhydration of cysteine residues (Sitdikova et al., 2010; Paul and Snyder, 2012). These modifications may also cause a change in the structure of the SNARE complex responsible for exocytosis and endocytosis of synaptic vesicles (LoPachin and Barber, 2006). Participation of intra-terminal Ca$^{2+}$ in the effect of NaHS was also confirmed using EGTA-AM or BAPTA-AM which chelated intracellular Ca$^{2+}$ and decreased facilitatory NaHS action on transmitter release.

**CONCLUSIONS**

In summary, our data indicate that in mammalian neuromuscular synapses H$_2$S endogenously produced by CSE and CBS increases spontaneous and evoked transmitter release from motor nerve endings without changing focally recorded presynaptic Na$^+$ and K$^+$ currents. The increase of acetylcholine release appears mediated by the enhancement of intracellular Ca$^{2+}$ via cAMP cascade and direct modulation of presynaptic RyR. The modulatory effect of H$_2$S should increase the safety factor of neuromuscular transmission and may have a protective effect on nerve terminals known to be inhibited by reactive oxygen species (Giniatullin et al., 2006) induced during various pathological conditions including diseases such as ALS and aging (Kimura and Kimura, 2004; Hu et al., 2009; Tang et al., 2010; Naumenko et al., 2011; Veeranki and Tyagi, 2013).

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest regarding the publication of this paper.

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