Neuroscience 303 (2015) 577–585

MECHANISMS OF HYDROGEN SULFIDE (H_2S) ACTION ON SYNAPTIC TRANSMISSION AT THE MOUSE NEUROMUSCULAR JUNCTION

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Abstract—Hydrogen sulfide (H_2S) is a widespread gasotransmitter also known as a powerful neuroprotective agent in the central nervous system. However, the action of H_2S in peripheral synapses is much less studied. In the current project we studied the modulatory effects of the H_2S 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 H_2S synthesis induced, similar to NaHS, an increase of EPC amplitudes whereas inhibitors of H_2S synthesis (β-cyano-L-alanine and aminooxyacetic 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 (Ca^{2+}) was buffered by 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis acetoxymethyl ester (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 ryanodine 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 H_2S increases spontaneously and evoked quantal transmitter release from motor nerve endings without changing the response of nerve endings. The presynaptic effect of H_2S appears mediated by intracellular Ca^{2+} and cAMP signaling and involves presynaptic ryanodine receptors.

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

INTRODUCTION

Hydrogen sulfide (H_2S), 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 H_2S 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 H_2S in mammalian tissues occurs mainly through three enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate 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

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Abbreviations: AOAA, aminooxyacetic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis acetoxymethyl ester; CBS, cystathionine β-synthase; CO, carbon monoxide; CSE, cystathionine γ-lyase; EGTA-AM, ethylene glycol-bis(2-aminoethyle ther)-N,N,N′,N′-tetraacetic acid acetoxymethyl ester; EPCs, end-plate currents; EPPs, end-plate potentials; H_2S, hydrogen sulfide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MDL 12330A, MDL 12,330A hydrochloride; MEPCs, miniature end-plate currents; MEPPs, miniature end-plate potentials; NaHS, sodium hydrosulfide; NO, nitric oxide; pCPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate; RyR, ryanodine receptors; β-CA, β-cyano-l-alanine.

http://dx.doi.org/10.1016/j.neuroscience.2015.07.036
0306-4522/

H_2S action in excitable cells (Tang et al., 2010). H_2S 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 (Ca^{2+}) channels (Kimura et al., 2006; Sitdikova 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, H_2S acts as a protective agent against oxidative stress by increasing levels of glutathione (Kimura and Kimura, 2004). Interestingly, in neurons H_2S 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 H_2S enhanced both spontaneous and evoked neurotransmitter release without changing focally recorded presynaptic responses. We also found that the substrate of H_2S 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 H_2S action suggested a role of cAMP and ryanodine receptors (RyR) in the action of H_2S (Sitdikova et al., 2009; Sitdikova and Zefirov, 2012; Gerasimova et al., 2013). H_2S also impairs the processes of exo- and endocytosis of synaptic vesicles during high-frequency stimulation of the motor nerve (Sitdikova et al., 2011; Mitrukhina et al., 2013).

However, the action of H_2S 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 H_2S increases both spontaneous and evoked transmitter release in mammalian neuromuscular junctions, provide evidence on the tonic action of endogenous H_2S and describe the molecular mechanisms underlying H_2S 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% O_2/5% CO_2) Krebs solution containing (in mM): NaCl – 154; KCl – 5; $CaCl_2$ – 2; HEPES – 5, $MgCl_2$ – 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 H_2S, which in aqueous solutions dissociates to sodium ion (Na^+) and hydrosulphide anion (HS^-) which forms H_2S through reacting with protons (H^+). Recent recalculations of the concentration of H_2S produced from NaHS taking pH, temperature, salinity of the perfusate, and evaporation of H_2S into account indicated that only 11–13% is effective as H_2S in solution from the initial concentration of NaHS (Sitdikova et al., 2014). Compounds used were: the substrate for endogenous H_2S production – L-cysteine (1 mM) and inhibitors of H_2S 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), adenylate 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-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis acetoxymethyl ester (BAPTA-AM) (50 μM), ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic 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 suprathreshold 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 Molgó, 1994). Application of NaHS (300 μM, resulting in effectively approx. 30–40 μM H_2S in solution, Sitdikova et al., 2014) induced a reversible increase in the amplitude of the 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. 1 D). The amplitude and time parameters of MEPCs were not changed indicating the absence of post-synaptic effects (not shown).

Next we studied the action of NaHS using intracellular recordings of spontaneous and evoked EPPs. Application of the H_2S 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 H_2S donor at the mouse neuromuscular junction.

Thus, using these two modes of recording we demonstrated that the H_2S donor NaHS enhances spontaneous and evoked neurotransmitter release from nerve endings of the mouse diaphragm muscle and has no effect on presynaptic responses.

Effect of the H_2S substrate L-cysteine and inhibitors of H_2S synthesis on synaptic responses

In order to test the potential action of endogenous H_2S on synaptic transmission we next used the H_2S substrate L-cysteine and inhibitors of H_2S synthesis. It is known that L-cysteine is a major substrate of H_2S endogenous

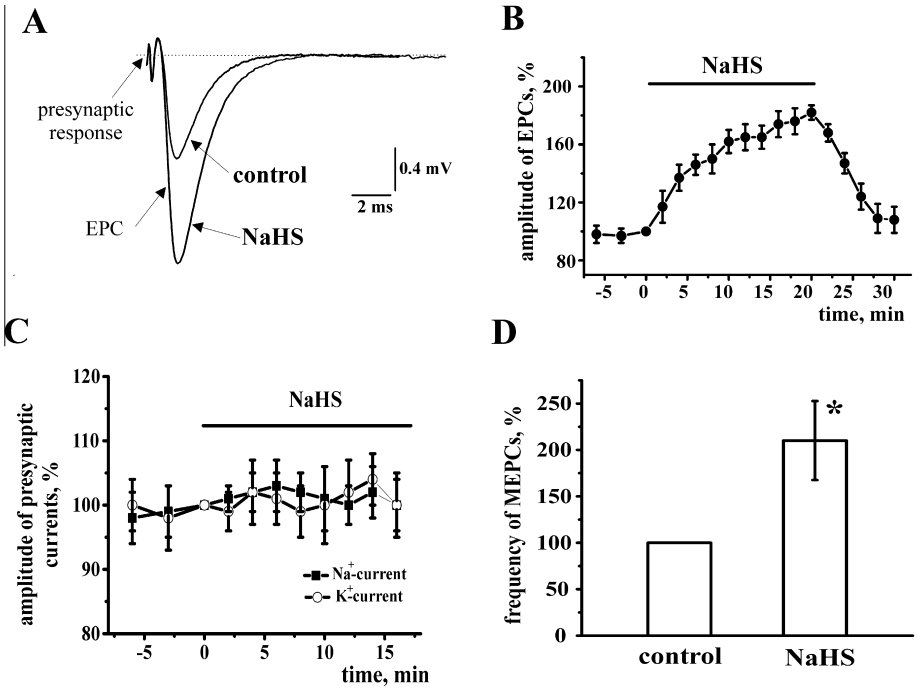

Fig. 1. NaHS effects on presynaptic response, EPCs and spontaneous transmitter release (extracellular recordings). (A) Representative presynaptic response and EPC (indicated by arrows) in control and after application of 300 μM NaHS. (B) Time course of EPCs amplitude during NaHS application (bar). (C) Time course of the amplitude of the second (filled squares) and third phase (open circles) of nerve ending response, reflecting Na^+ and K^+-currents respectively during NaHS application (bar). (D) MEPCs frequency significantly increased after 15 min of NaHS application. $^*p$ < 0.05.

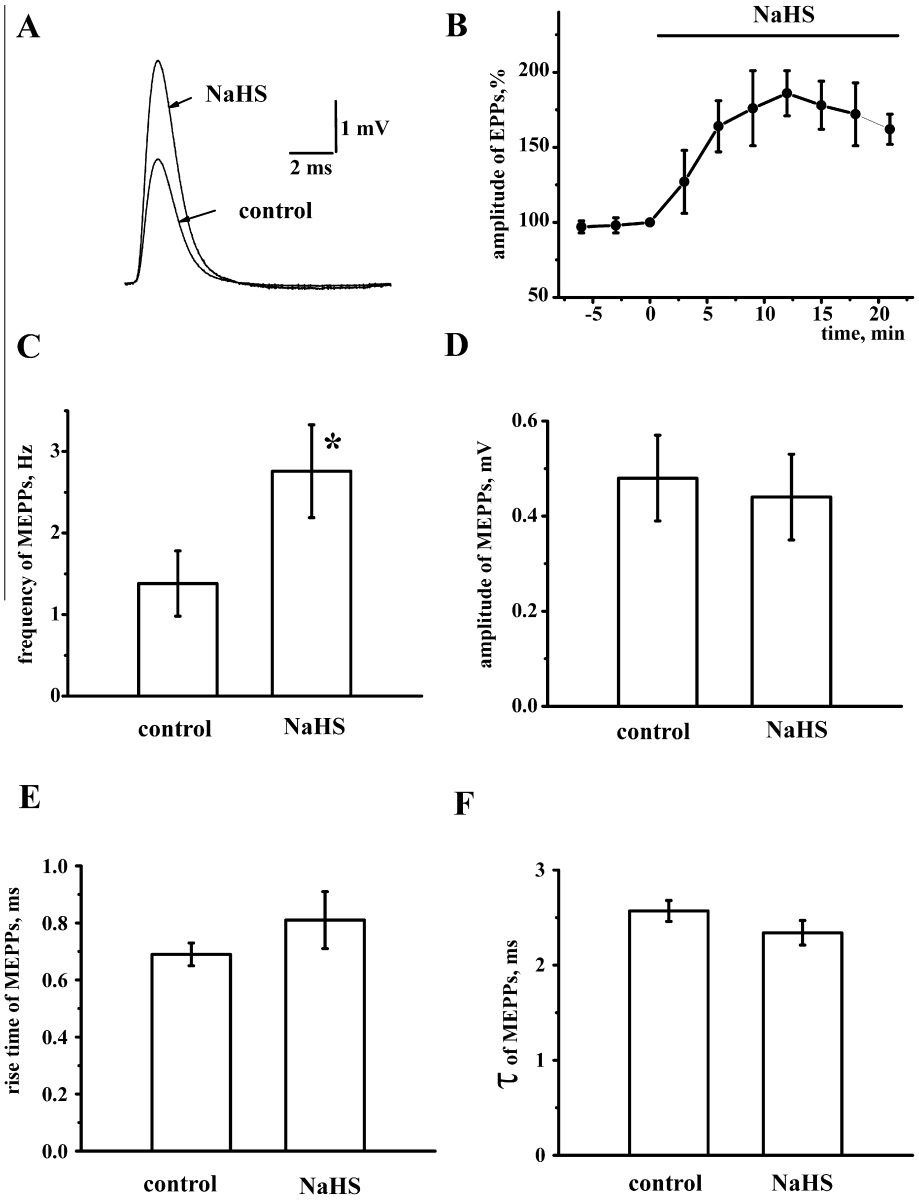

Fig. 2. NaHS increases the amplitude of EPPs and the rate of spontaneous quantal transmitter release in the mouse neuromuscular junction (intracellular recordings). (A) Representative EPPs in control and after application of 300 μM NaHS (effective H_2S concentration approx. 30–40 μM) which significantly increased EPPs amplitude. (B) Time profile of EPPs changes during NaHS application (bar) Mean MEPPs frequencies (C), amplitude (D), rise time (E) and decay time (τ, F) in control and after 15 min of NaHS application. Mean MEPPs frequencies significantly increased whereas other parameters did not change. $^{*}p < 0.05$ compared to control.

synthesis in tissues (Kamoun, 2004). 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_2S 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_2S synthesis did not affect the presynaptic response.

Hence, the H_2S substrate increased neurotransmitter release whereas the inhibitors of H_2S synthesis exerted the opposite effects. Taken together these data are consistent with the notion of a tonic positive tone mediated by endogenous H_2S in the mammalian neuromuscular junction.

The role of adenylate cyclase in the action of H_2S

Next we tested the potential involvement of intracellular signaling cascades in the action of H_2S. 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_2S. 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

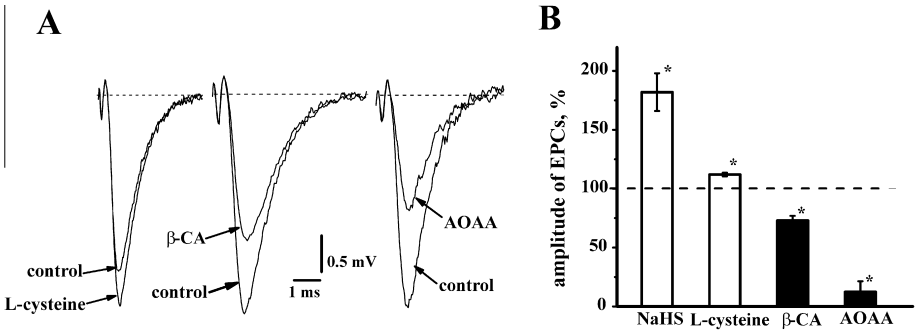

Fig. 3. Effects of substrate and inhibitors of H_2S synthesis on EPCs. (A) Representative EPCs in control and after 35 min application of substrate L-cysteine (1 mM) or H_2S synthesis inhibitors – β-cyano-L-alanine (β-CA, 1 mM) and amino-oxyacetic acid (AOAA, 1 mM). (B). Histograms showing effects of L-cysteine, β-cyano-L-alanine (β-CA) and amino-oxyacetic acid (AOAA) on EPCs amplitudes. Stippled bar shows the amplitude of EPCs in control before application of substances. $^{*}p < 0.05$ compared to control.

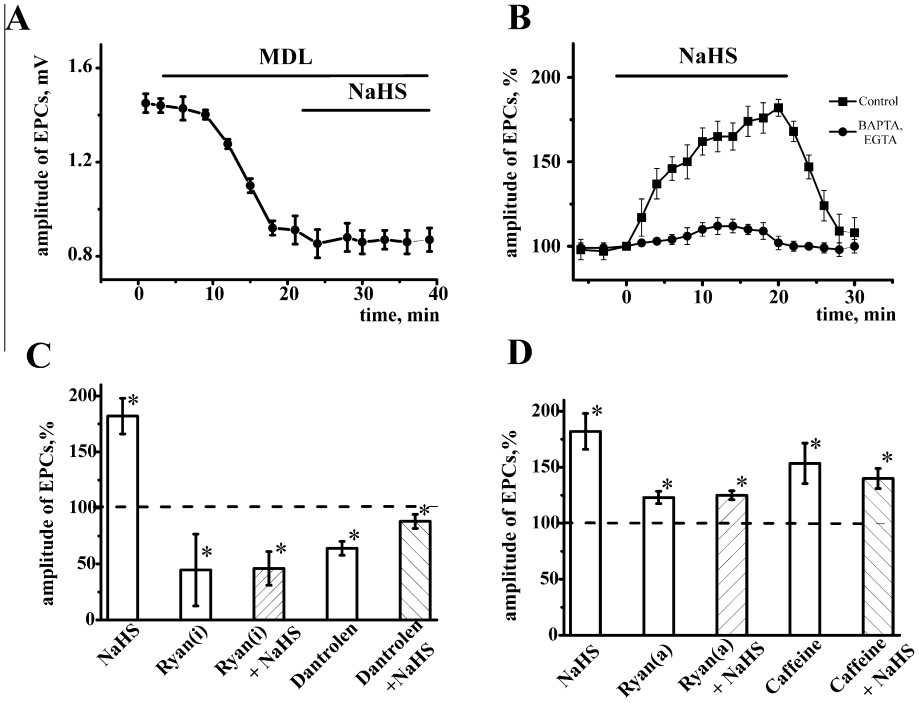

Fig. 4. Intracellular mechanisms of NaHS action in the mouse motor nerve endings. (A) Time-course of NaHS (300 μM) action on EPCs amplitudes in the presence of the adenylate cyclase inhibitor MDL-12330A (MDL, 5 μM) plus application of NaHS (bars). (B) Time-course of 300 μM NaHS action of EPCs in control (filled squares) and after preliminary incubation of mouse diaphragm in bath solution, containing Ca^{2+} chelators BAPTA-AM (50 μM) and EGTA-AM (filled circles). Bar indicates on NaHS application. (C) Histograms showing EPCs amplitudes after applications of NaHS (300 μM); inhibitory concentrations of ryanodine (Ryan(i), 3 μM); ryanodine + NaHS; dantrolene (25 μM); dantrolene + NaHS. (D) Histograms showing EPCs amplitudes after applications of NaHS (300 μM); activating concentration of ryanodine (Ryan(a), 100 nM); ryanodine + NaHS; caffeine (3 mM); caffeine + NaHS. Stippled lines in B and D indicate the amplitude of EPCs in control conditions. $^{*}p < 0.05$ – compared to control.

effect on the amplitude of EPCs (Fig. 4 A). 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 H_2S.

The role of intracellular calcium and ryanodine receptors

To evaluate the role of intracellular Ca^{2+} in NaHS effects mouse diaphragm was incubated for 1 h in bath solution, containing Ca^{2+} 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_2S in frog neuromuscular junction (Gerasimova et al., 2013). To test the role of RyR in the effects of H_2S in mammalian junctions the activators of RyR – ryanodine (0.1 μM) and caffeine (3 mM) and the inhibitors – dantrolene (25 μM) and ryanodine (3 μM) were used (Zucchi and Ronca-Testoni, 1997; Zhao et al., 2001; Balezina, 2002). Application of RyR inhibitor dantrolene (25 μM) 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 ($p < 0.05$). The specific inhibitor of RyR receptors ryanodine (3 μM) decreased EPCs to 55.4 ± 3.2% ($n = 5$, $p < 0.05$). 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% ($n = 4$, $p < 0.05$) compared to control (Fig. 4D). Similar to the inhibitors, the subsequent application of NaHS did not change significantly the EPCs amplitude (Fig. 4D). Ryanodine (0.1 μM) 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_2S.

The obtained data indicate that the effect of H_2S 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_2S 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_2S 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; Sitdikova et al., 2007; Valiullina and Sitdikova, 2012; Yakovleva et al., 2013). Here we analyzed the effects and mechanisms of H_2S in the mouse neuromuscular junction. The expression of the H_2S synthesis enzymes, CBS and CSE, in mammalian skeletal muscles suggests a potential physiological role for H_2S 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 Molgó, 1994). It was shown in various studies that H_2S could activate different types of K^+ and Ca^{2+} channels (Kimura et al., 2006; Sitdikova 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_2S in gaseous form or by the H_2S 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_2S 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_2S action is localized at the presynaptic side without changes in the sensitivity of postsynaptic acetylcholine receptors. Our study suggests a common targets of H_2S in neuromuscular junctions of different species consistent with the view that H_2S is a phylogenetically ancient regulatory molecule (Dombkowski et al., 2004).

Modulation of transmitter release by H_2S was first demonstrated in the rat hippocampus, where NaHS (at concentrations higher than 130 μM) suppressed hippocampal field excitatory postsynaptic potentials evoked by electrical stimulation of the Schafer collaterals (Abe and Kimura, 1996). Endogenous concentrations of H_2S were initially reported to be 50–160 μM (Goodwin et al., 1989). More recent reports by other authors suggest H_2S concentrations in the range of 10 nM to 3 μM in whole tissue preparations (Furne et al., 2008; Ishigami et al., 2009). In our experiments NaHS was used in a concentration of 300 μM which appears rather high. However, our recalculations taking pH, temperature, salinity of the perfusate, and evaporation of H_2S into account indicate that only 11–13%, i.e., 34–41 μM is effective as H_2S in solution which brings it closer to an apparently more physiological range (Sitdikova et al., 2014). In addition, it has been shown that H_2S rapidly disappears from the solution suggesting that H_2S may function as a molecular 'switch' that activates downstream pathways that persist long after H_2S has vanished (Hu et al., 2009; Deleon et al., 2012). However, the effective concentrations of H_2S at target sites are still unknown.

Endogenous synthesis and signaling via H_2S 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_2S in many tissues, the major cellular sources of H_2S and the mechanism of its release are not well understood. H_2S, 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_2S 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_2S that can immediately release H_2S in response to physiological stimulation (Ishigami et al., 2009; Kimura, 2010).

H_2S 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 adenylate 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_2S. In frog motor nerve endings we have previously shown the participation of cAMP-dependent mechanisms in the effects of H_2S although the latter had no direct impact on the adenylate cyclase activity (Sitdikova 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_2S 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_2S. 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 inihibitory 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 cites of dantrolene and H_2S may occur at different sites located at different membrane fractions (Palnitkar et al., 1997). It is also possible that the H_2S 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 exo- 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_2S 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_2S 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.

Acknowledgments—This project was supported by the Russian Scientific Fund (Grant No. 14-15-00618). The authors are grateful to Anton Hermann for useful suggestions.

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(Accepted 11 July 2015)
(Available online 17 July 2015)