ISSN 1990-7478, Biochemistry (Moscow) Supplement Series A: Membrane and Cell Biology, 2013, Vol. 7, No. 1, pp. 52–57. © Pleiades Publishing, Ltd., 2013.
Original Russian Text © N.N. Khaertdinov, D.R. Ahmetshina, A.L. Zefirov, G.F. Sitdikova, 2012, published in Biologicheskie Membrany, 2012, Vol. 29, No. 4, pp. 231–237.

ARTICLES

Hydrogen Sulfide in Regulation of Frog Myocardium Contractility[1]

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Received June 25, 2011; in final form, October 19, 2011

Abstract—Hydrogen sulfide (H_2S) is an endogenously synthesized gaseous molecule which, along with nitric oxide and carbon monoxide, induces a number of effects in cardiovascular system under normal and pathological conditions. In the present work, the effects and underlying mechanisms of the H_2S donor sodium hydrosulfide (NaHS) on the isometric force of frog myocardium contraction have been studied. NaHS at the concentration of 100 μM induced negative inotropic effect and reduced the maximum velocity of the contraction and relaxation of the isolated ventricle strips. The substrate of H_2S synthesis, *L*-cysteine (200 μM and 1 mM), induced the same effect, while the inhibitors of cystathionin-γ-lyase, the H_2S-producing enzyme in heart, β-cyanoalanine (500 μM) and propargylglycine (500 μM), increased the amplitude of contraction. Inhibition of cystathionin-γ-lyase by β-cyanoalanine prevented the negative inotropic effect of *L*-cysteine. After the inhibition of adenylate cyclase by MDL-12,330A (3 μM) or phosphodiesterases by IBMX (200 μM), the effect of NaHS was less than that in the control. In the presence of membrane-penetrating analogous of cAMP, 8Br-cAMP (100 μM) and pCPT-cAMP (100 μM), the negative inotropic effect of NaHS was completely retained. The effect of NaHS significantly decreased after preliminary application of the NO donor, SNAP (10 μM), and did not change after the inhibition of NO synthases by L-NAME (100 μM). The results suggest the possibility of endogenous synthesis of H_2S in frog myocardium and regulation of its contractility by the activation of phosphodiesterases hydrolyzing cAMP, which leads to a decrease in the activation of cAMP-dependent protein kinases and phosphorylation of voltage-dependent L-type Ca channels. As a result, the reduction of calcium entry into cardiomyocytes decreases the contractility of frog myocardium.

Keywords: hydrogen sulfide, myocardial contractility, adenylate cyclase, nitric oxide

DOI: 10.1134/S1990747812030117

Hydrogen sulfide (H_2S) is known as a toxic gas [1]; however, the more emerging evidence shows that H_2S is synthesized endogenously and has various physiological effects on the cardiovascular and nervous systems, as well as on gastrointestinal tract [2–7]. It is supposed that H_2S is an endogenous "gasotransmitter", like two other physiologically active gasses: nitric oxide (NO) and carbon monoxide (CO) [8, 9]. In the cardiovascular system, H_2S is synthesized from *L*-cysteine by cystathionin-γ-lyase and 3-mercaptosulfotransferase [5, 10, 11]. It induces vasodilation and regulates proliferation, apoptosis, and angiogenesis. In addition, H_2S has a cardioprotective effect against injuries induced by ischemia–reperfusion [5, 10, 12]. It is interesting that H_2S influences the vascular tone of all classes of vertebrates (fish, amphibian, reptiles), including vasoconstriction and vasodilation, which indicates the phylogenetic antiquity of H_2S as a gasotransmitter and universality of its action [13, 14].

It was shown in the intact rat heart and isolated cardiomyocytes that H_2S reduced the duration of action potential and had a negative inotropic effect [10, 15, 16]. The mechanisms of H_2S action in mammalian myocardium include the adenylate cyclase system, ATP-dependent K channels, and voltage-dependent L-type Ca channels, according to data from various sources and depending on the animal species [11, 15–17]. The regulation of cAMP synthesis by H_2S may play a critical role in cardioprotection, as there is a substantial decrease in H_2S production in different models of ischemia and hyperstimulation of β-adrenoceptors [10, 12]. In the frog myocardium, the hydrogen sulfide donor NaHS had a dose-dependent negative inotropic effect [18], but the mechanisms of H_2S action were not revealed.

The goal of this work was to study the effects of exogenous and endogenous H_2S on frog myocardial contractility and to elucidate the plausible molecular mechanisms of its action.

MATERIALS AND METHODS

The experiments on contractility recording were performed on the strips of frog myocardium in a Pow-

[1] The article was translated by the authors.

erLab device using an isometric transducer with a sensitivity of 0–50 g (AD Instrument, Australia, and Biopac, USA). A ventricular myocardium strip of 4–6 mm in length and 1 mm in diameter was submerged vertically in the chamber with the working volume of 20 ml and perfused with Ringer's solution for cold-blooded animals containing (mM): 118.0 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 Trizma (pH 7.3–7.4, 20°C). The preparation was stimulated by electric pulses at 0.1 Hz via two silver electrodes. The signals were processed with the Chart software; the force of contraction was determined in grams. The contraction amplitude and the maximum rates of contraction and relaxation were estimated. Statistical analysis was performed using the standard methods; statistical significance of the differences was determined by Student's *t*-criterion.

Sodium hydrosulfide (NaHS) was used as a donor of H_2S; it dissociates into Na^+ and HS^- in aqueous solution; then HS^- is bound to H^+ forming H_2S. In the neutral solution, one third of NaHS is present as gaseous H_2S and two thirds are present as HS^- [1]. The following agents were used in the experiments: *L*-cysteine, β-cyanalanine, propargylglycine, IBMX (3-isobutyl-1-methylxantine), 8Br-cAMP, pCPT-cAMP (sodium salt of 8-(4-chlorophenylthio)adenosine-3',5' cyclophosphate), MDL-12,330A (hydrochloride of *cis*-N-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine), L-NAME (N^G-nitro-*L*-arginine methyl ester), SNAP (S-nitroso-N-acetylpenicillamine). Water-insoluble substances were dissolved in dimethylsulfoxide (DMSO). DMSO concentration in the solution did not exceed 0.01%. At this concentration, DMSO in the control experiments had no substantial effect on the force of myocardial contraction. All substances were produced by Sigma (USA).

RESULTS

The effects of the donor, substrate and inhibitors of H_2S synthesis on the force of myocardial contraction. The H_2S donor NaHS at the concentration of 100 μM caused a reversible decrease in the amplitude of contraction of the frog heart ventricle strip to 66 ± 6% ($n = 14$, $p < 0.05$) by the 20th min of application, which is in agreement with our previous data [18]. At the same time, NaHS reduced the maximum rates of the myocardium strip contraction and relaxation to 57 ± 8 and 53 ± 7% ($n = 5$, $p < 0.05$) compared to the control, respectively. The substrate of the H_2S synthesis, *L*-cysteine, by the 20th min of application caused, like the gas donor, the decrease in the amplitude of myocardial strip contraction to 83 ± 6% ($n = 6$, $p < 0.05$) at a concentration of 200 μM and to 58 ± 4% ($n = 7$, $p < 0.05$) at a concentration of 1 mM (Fig. 1a, b).

The possibility of endogenous gas synthesis was revealed using cystathionin-γ-lyase inhibitors (β-cyanoalanine and propargylglycine) at the concentration of 500 μM. β-Cyanoalanine and propargylglycine increased the force of myocardial contraction to 117 ± 6% ($n = 8$, $p < 0.05$) and 112 ± 3% ($n = 8$, $p < 0.05$), respectively, by the 15th minute of application (Fig. 1b). Thus, the endogenously synthesized H_2S induced the same effects as the exogenously applied H_2S, while cystathionin-γ-lyase inhibitors exerted an opposite effect. Under the conditions of cystathionin-γ-lyase inhibition by β-cyanoalanine, the application of *L*-cysteine (200 μM or 1 mM) did not lead to the decrease in the contraction amplitude.

Adenylate cyclase system and H_2S effects in myocardium. In the nervous system and cardiomyocytes of warm-blooded animals, H_2S effects are probably mediated by the changes in cAMP level [11, 19]; therefore, the effect of the gas was studied under the increase and decrease in the level of this cyclic nucleotide. Adenylate cyclase inhibition by MDL-12,330A (3 μM) reduced the force of contraction to 81 ± 3% ($n = 11$, $p < 0.05$) by the 25–30th minute of application (Fig. 2), which is probably associated with the decrease of cAMP concentration in cardiomyocytes. Under these conditions, the effect of H_2S was less pronounced than in the control: 87 ± 2% ($n = 13$, $p < 0.05$) (Fig. 2).

cAMP concentration was increased by using the membrane penetrating analogs: 8Br-cAMP and pCPT-cAMP, at a concentration of 100 μM. The application of 8Br-cAMP or pCPT-cAMP did not cause significant changes in the amplitude of contraction. By the 20th minute of pCPT-cAMP or 8Br-cAMP action, the force of myocardium strip contraction was 110 ± 11% ($n = 5$, $p > 0.05$) or 101 ± 5% ($n = 5$, $p > 0.05$), respectively, compared to the control values. The addition of NaHS reduced the strip contractility to 70 ± 6% ($n = 5$, $p < 0.05$) in the presence of 8Br-cAMP and to 57 ± 11% ($n = 5$, $p < 0.05$) in case of pCPT-cAMP (Fig. 2). Thus, the effect of NaHS under the influence of cAMP analogs was maintained to the same extent as in the control conditions.

Phosphodiesterase inhibition is another way to increase the level of cAMP in the cells. IBMX, a non-specific blocker of phosphodiesterases of the cyclic nucleotides, was used in our experiments at the concentration of 200 μM. IBMX increased the force of contraction by 120 ± 4% ($n = 7$, $p < 0.05$) by the 8th minute of application, probably due to the accumulation of cAMP in cardiomyocytes (Fig. 2). At the enhanced level of endogenous cyclic nucleotides, NaHS reduced the force of myocardium strip contraction to 82 ± 4% ($n = 5$), and this effect was less than the effect of H_2S donor in the control ($p < 0.05$) (Fig. 2).

Thus, the effect of H_2S was partially eliminated under the inhibition of adenylate cyclase or phosphodiesterases but maintained under the action of cAMP analogs.

The nitric oxide system and the effects of hydrogen sulfide. It is known that NO is an important regulator of the force of myocardial contraction in frog [20]. Based on the data on interaction between the systems of gaseous mediators, we analyzed the effects of NaHS

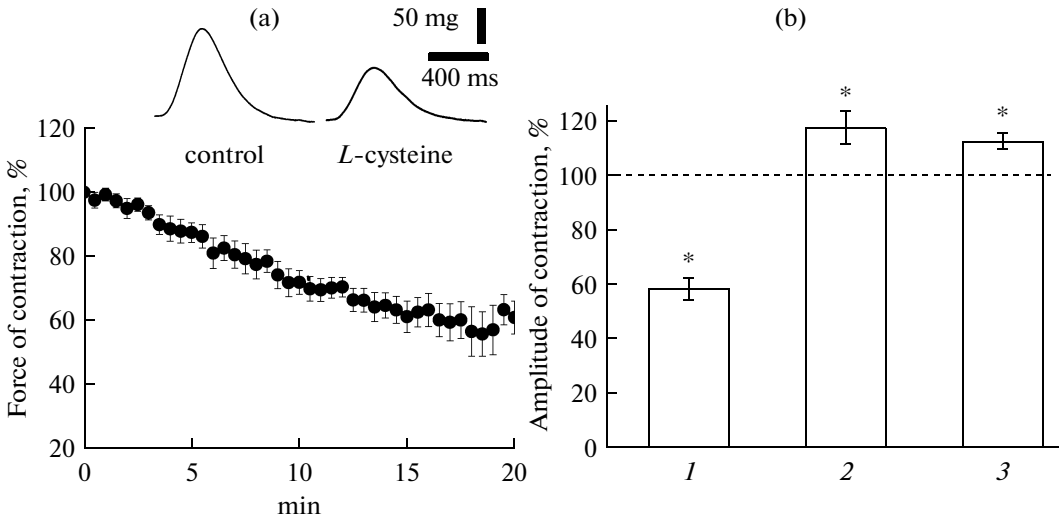

Fig. 1. The effects of the substrate and inhibitors of hydrogen sulfide synthesis on myocardial contractility. (a) The dynamics of contraction force under the action of the H_2S synthesis substrate, *L*-cysteine, at a concentration of 1 mM. On top, the original recordings of contraction in the control and by the 20th minute of *L*-cysteine application are shown. (b) The effects of 1 mM *L*-cysteine (*1*) and cystathionin-γ-lyase blockers: 500 μM β-cyanalanine (*2*) and 500 μM propargylglycine (*3*) on the force of myocardial contraction. $*p < 0.05$.

under the conditions of NO synthesis inhibition and increase of its endogenous concentration. The application of L-NAME, the blocker of different forms of NO synthases (100 μM), resulted in a slight increase in the amplitude of contractions. By the 15th min of L-NAME application, the force of contraction was $115 \pm 4\%$ ($n = 7, p < 0.05$). Under the inhibition of NO synthesis, NaHS reduced the force of myocardial contraction to $65 \pm 8\%$ ($n = 7$), which was not different from the effect of H_2S in the control. Thus, the effect of H_2S is not associated with the changes in NO synthesis. The NO donor (SNAP) at the concentration of 10 μM reduced myocardial contractility to $86 \pm 5\%$ ($n = 10$, $p < 0.05$). Under the influence of SNAP, the negative inotropic effect of NaHS was much less pronounced than in the control: $89 \pm 2\%$ ($n = 11, p < 0.05$).

DISCUSSION

The negative inotropic effect of exogenous and endogenous H_2S. We have shown that both exogenous and endogenously synthesized H_2S has a negative inotropic effect on the frog myocardium. The substrate of H_2S synthesis *L*-cysteine caused the same decrease in the amplitude of myocardial contractions as the H_2S donor NaHS, whereas the cystathionin-γ-lyase inhibitors had an opposite effect: increased the amplitude of the contraction. It is known that *L*-cysteine is present in mammalian plasma in micromolar concentrations, but the enzymes of H_2S synthesis have low affinity to cysteine. Therefore, H_2S synthesis is usually enhanced by using high substrate concentrations: 1 mM and more [21–23]. In our experiments, the force of myocardial contraction decreased already at 200 μM and was well pronounced at 1 mM of the substrate, probably due to intensification of H_2S synthesis. In addition, the effect of *L*-cysteine was not manifested during the inhibition of cystathionin-γ-lyase (Fig. 1). The findings demonstrate the possibility of H_2S synthesis in the myocardium of cold-blooded animals by cystathionin-γ-lyase, with *L*-cysteine as a substrate. The negative inotropic effect of NaHS in the myocardium was observed in warm-blooded animals [10, 11, 16] and in our previous works with frog heart [18]. There are no data on gas concentration in frog myocardium. However, H_2S is synthesized in the vessels of lower vertebrates by cystathionin-γ-lyase at concentrations comparable with the H_2S concentration in rat vessels. The H_2S concentration in the plasma of trout is approximately 40 μM, i.e., higher than in mammals [13, 14]. It seems that the myocardium of frog, similar to the vertebrates of other classes, contains a H_2S synthesis system, which implies its tonic effect on the myocardium.

cAMP as a factor mediating the effect of H_2S on myocardial contractility. The main factor that triggers and determines the force of myocardial contractility is intracellular concentration of calcium ions. The voltage-dependent L-type Ca channels open in response to the cardiomyocyte membrane depolarization followed by the entry of Ca^{2+}, which induces Ca^{2+} release from the intracellular Ca stores via ryanodine receptors and triggers the process of muscular contraction [24, 25]. In the ventricular cardiomyocytes of frogs, as a result of poor development of sarcoplasmic reticulum, the main calcium source is Ca^{2+} entering via the Ca channels of the plasma membrane [26]. The negative inotropic effect of NaHS is probably due to the

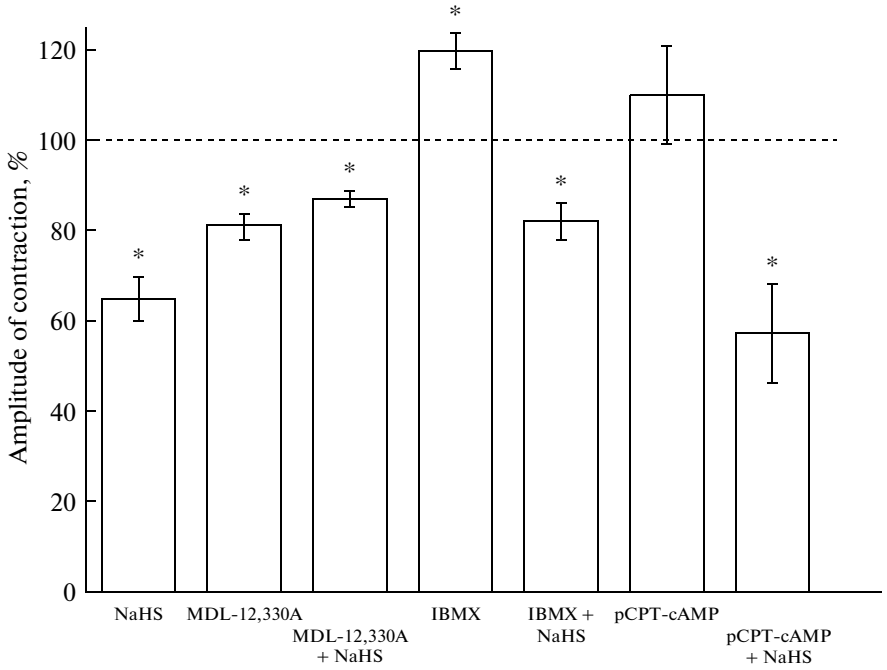

Fig. 2. The effect of sodium hydrosulfide at a decreasing and increasing level of cyclic nucleotides. The amplitudes of myocardial contraction are shown under the action of NaHS (100 μM) in the control, MDL-12,330A (3 μM), IBMX (200 μM), pCPT-cAMP (100 μM) and NaHS under the action of the above reagents. During the analysis of NaHS effects, the values obtained under the action of MDL-12,330A, IBMX, and pCPT-cAMP were taken as 100%, respectively. $*p < 0.05$.

decrease in the entry of calcium ions into cardiomyocytes. In our previous studies, the NaHS effect was completely preserved under the conditions of Ca channels inhibition by nifedipine [18], which indicates the absence of direct inhibitory effect of the gas on Ca-channel subunits. The Ca current may be regulated through phosphorylation by cAMP-dependent kinases at an enhanced level of cAMP in response to the activation of β-adrenoceptors [27]. It is known that the Ca channel in phosphorylation state is characterized by the higher conductivity and open probability and activated at more negative values of membrane potential [25].

In our experiments, both the decrease and the increase in the endogenous level of cAMP reduced the manifestation of H_2S effect, demonstrating the involvement of adenylate cyclase system in these effects (Fig. 2). At the same time, the adenylate cyclase inhibition reduced the force of contraction, while the increase in the endogenous level of cAMP by the nonspecific phosphodiesterase inhibitor IBMX intensified the response, which confirms the involvement of cAMP-dependent pathways in contractility regulation. However, the application of membrane-penetrating cAMP analogs (pCPT-cAMP or 8Br-cAMP) did not result in any substantial changes in contraction intensity nor influenced the effect of NaHS (Fig. 2). The analogs used are relatively stable, though they are known to be subject to hydrolysis by Ca/calmodulin-dependent, cGMP-inhibited and cGMP-stimulated phosphodiesterases [28] expressed in frog cardiomyocytes. In addition, the absence of the effect of pCPT-cAMP or 8Br-cAMP on the force of myocardial contraction indicates that they cannot simulate the situation when the endogenous level of cAMP increases (like in the case with IBMX). It may be due to the fact that cAMP analogs activate not only protein kinase A but also protein kinase G [28, 29] and inhibit the cGMP-specific phosphodiesterase, which results in enhancement of the cGMP level [28, 30]. The activation of protein kinase G, in turn, will lead to a decrease in the Ca current amplitude [31]. In addition, an important factor of cAMP influence on contractility is its localization close to the targets, while mere increase in the total level of cAMP is insufficient for specific regulation of protein target [32]. In the ventricular cardiomyocytes of frog, Ca current is regulated by the local increase in cAMP level close to the cytoplasmic membrane. Phosphodiesterases provide cAMP compartmentalization, preventing its diffusion along the cardiomyocyte. The location of A-kinase anchoring protein close to the Ca channels provides the local control of Ca current by cAMP-dependent processes [33].

In accordance with the published data, adenylate cyclase may be a target for NaHS. In the central nervous system, NaHS enhanced the conductance of glutamate NMDA-receptors and increased the cAMP level [19]. In rat cardiomyocytes, H_2S reduced the positive inotropic effect of isoproterenol due to the

inhibition of cAMP synthesis [11]. The involvement of the cAMP system in H_2S effects was noted also in the motor nerve ending of frog, where the effect of the gas decreased at an enhanced level of cAMP [34].

The role of nitric oxide in H_2S effects. Our data show the interaction between the two systems of gaseous mediators: H_2S and NO. So, after preliminary application of the NO donor (SNAP), the effect of NaHS was less pronounced than in the control. At the same time, SNAP caused a decrease in the contraction amplitude, which is in agreement with the known data [20]. Such interaction may be at a level of regulation of both the activities of NO- and H_2S-synthesizing enzymes and the signaling pathways triggered by these gasses. In vascular tissue, H_2S reduced the activity of NO synthase [35], while the NO donor, sodium nitroprusside, increased the expression of cystathionin-γ-lyase and cystathionin-β-synthase [36]. In our studies, the effect of NaHS was completely preserved under the conditions of NO synthase inhibition by the nonspecific blocker L-NAME, indicating the absence of NaHS effect on NO synthesis. The interrelationship between H_2S and NO functions has been shown in the vascular tissues of warm-blooded animals, where sodium nitroprusside increased the vasorelaxation induced by H_2S [37], while H_2S reduced the relaxation effect of NO [38].

The negative inotropic effect of NO in frog myocardium is mediated by increase in the level of cGMP, the target of which is cGMP-dependent phosphodiesterase (phosphodiesterase 2). The activation of phosphodiesterase 2 leads to a decrease in the cAMP level, depression of the Ca current, and reduction of the force of contraction [39]. Owing to co-localization of Ca channels and phosphodiesterase 2, which is present in frog mainly in the membrane fraction, a drastic decrease in cAMP concentration occurs only near the Ca channels [40], ensuring cAMP compartmentalization and regulating the phosphorylation of Ca channels separately from other substrates of protein kinase A [39]. It seems that preliminary application of the NO donor results in the activation of phosphodiesterase 2 and decrease in the cAMP level. Under these conditions, the effect of NaHS was reduced, once again demonstrating the potential involvement of phosphodiesterases in the effect of the gas. In addition, one cannot rule out the possibility of chemical interaction between NO and H_2S with the formation of nitrosothiols, which will have influence on the concentration and effects of both gasses [41].

Thus, our data show the possibility of endogenous synthesis of H_2S by cystathionin-γ-lyase in frog myocardium. Both endogenous and exogenous H_2S has a negative inotropic effect. The effect of H_2S is based on the decrease in cAMP level as a result of reduction of its synthesis or increase of its hydrolysis. As a result, the activity of cAMP-dependent protein kinases and phosphorylation of voltage-dependent L-type Ca channels, the Ca entry into cells and the force of myocardial contraction decrease. This effect may play a key role in regulation of the inotropic function of the heart during the activation of β-adrenoceptors involved in the adrenergic regulation of myocardial contractility.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (project no. 12-04-00960) and the Support Program for the Leading Scientific Schools.

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