doi:10.4149/gpb_2009_02_190

H_2S and HS^- donor NaHS inhibits intracellular chloride channels

Lubica Malekova, Olga Krizanova and Karol Ondrias

Institute of Molecular Physiology and Genetics, Centre of Excellence for Cardiovascular Research, Slovak Academy of Sciences, Bratislava, Slovakia

Abstract. We have characterized the effect of H_2S on single channel properties of the chloride channels derived from the rat heart lysosomal vesicles incorporated into a bilayer lipid membrane. The single chloride channel currents were measured in 250 : 50 mmol/l KCl *cis*/*trans* solutions. H_2S inhibited the chloride channels by decreasing the channel open probability in a concentration-dependent manner. The inhibitory effect of H_2S was side-dependent with the IC_{50} values of 42 and 75 μmol/l for the *trans* and the *cis* sides, respectively. The mixture of H_2S with the NO donor S-nitroso-N-acetyl-DL-penicillamine had smaller effect (IC_{50} = 180 μmol/l) than H_2S alone. We assume that the inhibitory effect of H_2S on chloride channels may be responsible for some of its numerous biological effects.

Key words: Chloride channels — H_2S — Bilayer lipid membrane — Single channel properties — Heart

Abbreviations: BLM, bilayer lipid membrane; P-open, open probability of single channel; SNAP, S-nitroso-N-acetyl-DL-penicillamine.

Introduction

In water or blood plasma, H_2S dissociates to $H_2S \leftrightarrow HS^- + H^+$ and a trace of S^{2-} (Dombkowski et al. 2004). Since it is not known, which is the active form of H_2S *in vivo,* we used term H_2S for a total mixture. Endogenously produced H_2S is a newly-found gas transmitter, which influences numerous biological processes. It is involved in the regulation of cardiac function and cardioprotection, vasorelaxation, hypertension, proliferation, apoptosis, septis, endotoxin and haemorrhagic shocks, and also in inflammation processes (Hosoki et al. 1997; Zhao et al. 2001; Geng et al. 2004; Johansen et al. 2006; Sivarajah et al. 2006; Chen et al. 2007; Lowicka and Beltowski 2007).

Molecular mechanism of these numerous effects of H_2S is not fully understood. Vasorelaxant effects of H_2S are supposed to be caused by opening of vascular smooth muscle cells K_{ATP} channels, which leads to membrane hyperpolarization and reduces extracellular Ca^{2+} entry and relaxes vascular tissues (Zhao et al. 2001; Chen et al. 2007). In our previous work, we observed that H_2S can release nitric oxide (NO) from nitrosothiols, metal nitrosyl complex, brain homogenate and murine L1210 leukaemia cells (Ondrias et al. 2008), what could be responsible for some of its biological activities. MAPKs pathway, cell cycle-related proteins and cell death-related genes were reported to be involved in H_2S-induced effect on proliferation and apoptosis (Yang and Wang 2007). A decrease in calcium influx and increase in calcium release by H_2S has been also reported (Lee et al. 2006; Sivarajah et al. 2006; Xu et al. 2007).

Chloride channels are involved in the blood pressure regulation, apoptosis, reperfusion injury and cardioprotection (Nilius and Droogmans 2003; Miller 2006; Puljak and Kilic 2006). This biological processes are also influenced by H_2S (Zhao et al. 2001; Johansen et al 2006; Sivarajah et al. 2006; Chen et al. 2007; Lowicka and Beltowski 2007; Yang and Wang 2007). Therefore, in order to contribute to the understanding of the numerous biological effects of H_2S, we tested whether H_2S can influence intracellular chloride channels. We have found that H_2S inhibited chloride channels.

Correspondence to: Karol Ondrias, Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárska 5, 833 34 Bratislava, Slovakia
E-mail: karol.ondrias@savba.sk

Materials and Methods

Chemicals

Lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Protease inhibitors were purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals were purchased from Sigma-Aldrich (USA).

Isolation of submitochondrial particles (membrane vesicles)

A crude rat heart mitochondria and submitochondrial particles (membrane vesicles) from the crude mitochondria were isolated essentially as described previously (Malekova et al. 2007a,b). In our previous study (Malekova et al. 2007b) we have found that a number of observed chloride channels from the submitochondrial particles isolated from the crude mitochondria was about 10–20-times higher than from the submitochondrial particles obtained after the purification of the crude mitochondria on Percoll gradient. Since the crude mitochondria contained significant fraction of lysosomal membranes and the Percoll gradient isolated mitochondria did not contain lysosomal membranes (Malekova et al. 2007b), we assume that most of the channels presented in this study and obtained from the membrane vesicles were derived from lysosomal membranes. However, we did not exclude presence of chloride channel derived also from mitochondria. All procedures were approved by the State VET and Nutritive Administration of Slovak Republic.

Bilayer lipid membrane formation and measurements of conductivity

A formation of bilayer lipid membrane (BLM), fusion of membrane vesicles and measurement of single channel currents were done as described previously (Malekova et al. 2007b). BLM was formed across an aperture (diameter ≤0.1 mm), separating the *cis* and *trans* chambers using a mixture of dioleoyl-glycero-phosphatidylcholine and dioleoyl-glycero-phosphoethanolamine at a molar ratio of 3 : 2 in n-decane (20 mg/ml). Compositions of the *cis* and *trans* solutions were the same (in mmol/l): 0.1 $CaCl_2$, 0.3 EGTA, 1 $MgCl_2$, 10/5 Hepes/Tris, pH 7.4, except of *cis*/*trans* KCl, which was 250/50 mmol/l. The free Ca^{2+} concentration was ~40 nmol/l as calculated by WinMaxc32 program, version 2.50 (http://www.stanford.edu/~cpatton/maxc.html). Ionic permeability Cl^-/K^+ ratio was conventionally defined from the measured reversal potential according to the modified GHK equation (Hayman et al. 1993). Single channel currents were measured by the bilayer clamp amplifier BC-525C (Warner Instrument, Hamden, CT, USA). They were filtered at low pass filter of 1 kHz and were digitized at a sampling rate of 4 kHz using a DigiData 1200 digitizer (Axon Instruments, Foster City, CA, USA). Data were stored in a computer by means of pClamp5 software (Axon Instruments), which was also used for processing of the data. The single channel open probability (P-open) was determined from recordings of ≥2 min before and after addition of drugs and calculated from the ratio of the open time/total time intervals. For present study, we used channels which had regular single channel stable and constant opening and closing currents amplitude in the range of –30 to +30 mV.

Preparation of NaHS solution

NaHS was used in our study as a H_2S and HS^- donor, which dissociates to Na^+ and HS^-, which then reacts with H^+ to yield H_2S. It is reasonable to neglect the third species, S_2^-, due to the high pK_{a2} (11.96). Free H_2S gas and HS^- account for approximately 30–33% and 67–70% of a molar concentration of NaHS at 20°C, respectively (Zhao and Wang 2002; Dombkowski et al. 2004). Thus, we used term "NaHS" for mixture of H_2S, HS^-, and S_2^-.

Stock solution of NaHS (100 mmol/l) was prepared only at the time of measurement and were used within a few hours. Mixture solutions of NaHS and S-nitroso-N-acetyl-DL-penicillamine (SNAP) were prepared as follows: stock 5 mmol/l NaHS solution was prepared in the *trans* BLM buffer, SNAP was dissolved in DMSO (100 μmol/l) diluted with the *trans* BLM buffer to the final concentration 5 mmol/l and final mixture solution containing NaHS (5 mmol/l) and SNAP (5 mmol/l) was incubated for 20 min at 22°C, stored at –70°C and melted just before application.

Results

Properties of chloride channels

After an incorporation of the membrane vesicles into BLM, we observed chloride channels (n = 32) having conductance of 132 ± 16 pS (±SD), reversal potential 25 ± 5 mV, single channel amplitude at 0 mV 3.0 ± 0.6 pA and Cl^-/K^+ selectivity within the range of 3.5–8.5 in the 250/50 mmol/l KCl *cis*/*trans* BLM solutions. The broad range of the single channel parameters may indicate that different intracellular chloride channels were studied.

Effect of H_2S on chloride channels

An example of the single chloride channel current before and after application of 100 μmol/l of NaHS into the *cis* side is shown in Fig. 1a. NaHS decreased P-open of the chloride channels by increasing channel close time. It did not have effect on single channel amplitude, conductance or mean open time of the channel. It decreased average channel activ-

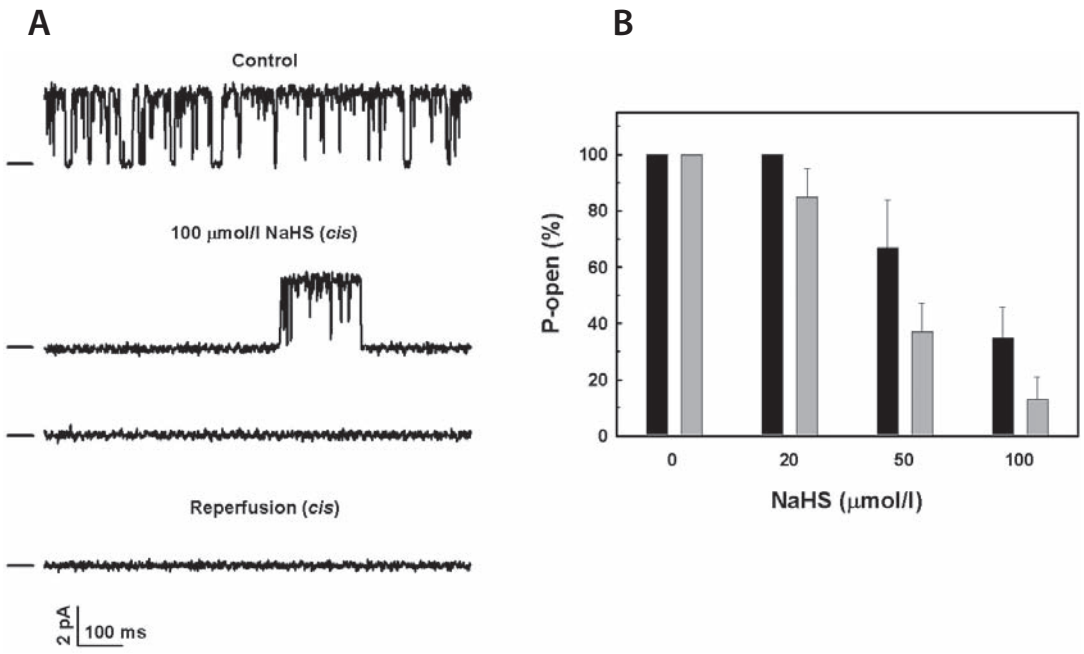

Figure 1. A. Effect of the NaHS on the single chloride channel current. An application of NaHS at the *cis* side of BLM at 100 μmol/l concentrations and reperfusion of the *cis* solution. The first current trace after the application of 100 μmol/l NaHS to *cis* shows closed channel, which is opened for a short period of time only. The second current trace shows a closed channel after 2 min of the application of 100 μmol/l NaHS. The bottom current trace shows the closed channel after the *cis* reperfusion. The voltage is 0 mV. The lines on the left mark the closed state of the channels. **B.** Concentration-dependent effect of NaHS on the single channel open probability (P-open) of the single chloride channel at 0 mV (n = 13, data represent mean ± SEM). Black and gray bars indicate the application of NaHS to the *cis* or *trans* side of BLM, respectively.

ity (open probability) in a concentration-dependent manner (Fig. 1b). Inhibitory effect of the NaSH was side-dependent, with the IC_{50} values of 42 and 75 μmol/l for the *trans* and the *cis* sides, respectively. The inhibition effect of 100 μmol/l NaSH in *cis* was reversible in 3/9 experiments.

Effect of H_2S-NO mixture on chloride channels

We compared the effect of NaHS-SNAP mixture with NaHS having the same history of sample preparation. An equimolar mixture of NaHS-SNAP had smaller inhibitory effect (IC_{50} = 180 μmol/l) than NaHS alone (IC_{50} = 63 μmol/l) (Fig. 2). An effect of a freshly prepared SNAP was complex. At 100–200 μmol/l, it inhibited or transiently inhibited 5/9 chloride channels.

Discussion

So far, K_{ATP} and calcium channels were mostly found to explain some of the numerous H_2S biological effects (Zhao et al. 2001; Lee et al. 2006; Sivarajah et al. 2006; Xu et al. 2007).

We have found that NaHS inhibited intracellular chloride channels. Since in our experimental conditions, NaHS forms 30–33% H_2S, 67–70% HS^-, and also negligible amount of S_2^- in the solution occurs (Dombkowski et al. 2004), we do not know, which form is responsible for the inhibitory effect. The concentrations of NaHS, which

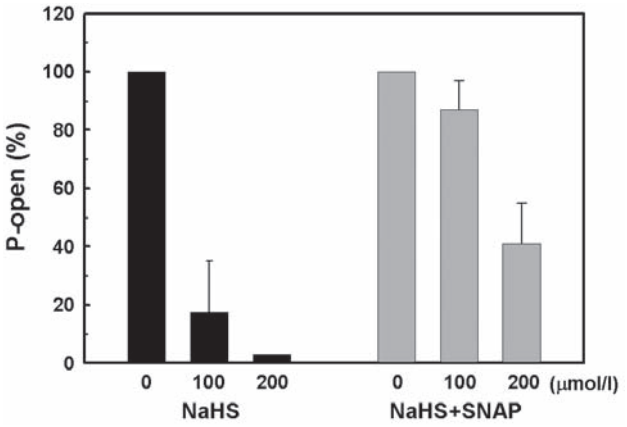

Figure 2. Effect of the NaHS and the mixture of NaHS with SNAP on the single channel open probability (P-open) of the single chloride channel at 0 mV (n = 11, data represent mean ± SEM). The compounds were applied to the *cis* and the *trans* sides of BLM.

inhibited chloride channels, were in the range of its endogenous physiological concentrations. H_2S levels in the circulation have been reported to be 10–50 μmol/l in rats, 10–100 μmol/l in humans, and in rat, human and bovine brain tissues in the range of 50–160 μmol/l (Yang and Wang 2007). On the other hand, recent report have found that sulfide does not circulate in the plasma at measurable concentrations (Whitfield et al. 2008). This controversy is not resolved at the present time.

Inhibitory effect of the NaHS was side-dependent, and its target on chloride channel was better accessible from the *trans* side than from the *cis* side, similarly as we observed in our previous study for bongkrekic acid (BKA) and atractyloside (CAT; Malekova et al. 2007a). On the other hand, the inhibitory effect of 5-nitro-2-(phenylpropylamino)-benzoate (NPPB) and dihydro-4,4′ diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) was reported to be from the *cis* side (Malekova et al. 2007b), opposite than the effect of NaHS. A mode of the chloride channel inhibition by NaHS was different than reported for other compounds, BKA, CAT, DIDS or NPPB (Malekova et al. 2007a,b). NaHS, differently from the compounds, did not change single channel amplitude, conductance or mean open time of the channel. It increased channel close time.

Since mixing of H_2S donor NaHS with NO donors was reported to either enhance (Zhao and Wang 2002), attenuate (Hosoki et al 1997; Ali et al. 2006) or inhibit a vasorelaxant effect of NO, we studied effect of the mixture of NaHS with SNAP on the chloride channels. An inhibitory effect of the mixture was smaller compared to the effect of H_2S alone. We may assume that chemical reactions among products of NaHS and SNAP might occur. This proposal might be supported by observation of forming a novel nitrosothiol generated by reaction between H_2S and NO (Whiteman et al. 2006).

It is now appreciated that chloride channels play important functional roles in diverse processes, such as blood pressure regulation, cell cycle and apoptosis, muscle tone, volume regulation, synaptic transmission and also cellular excitability (Nilius and Droogmans 2003; Miller 2006; Puljak and Kilic 2006). Considering an involvement of chloride channels and H_2S in similar physiological and pathological processes (Hosoki et al. 1997; Zhao et al. 2001; Geng et al. 2004; Johansen et al 2006; Lee et al. 2006; Sivarajah et al. 2006; Chen et al. 2007; Lowicka and Beltowski 2007; Xu et al. 2007; Yang and Wang 2007) we may assume that inhibitory effect of H_2S and/or HS^- on chloride channels is responsible for some of its biological activities.

Acknowledgements. Financial support by Slovak Agency for Promotion Research and Development under the contract APVV-0397-07 and Science Grant Agency VEGA, project 2/6012/6 is gratefully acknowledged.

References

Ali M. Y., Ping C. Y., Mok Y. Y., Ling L., Whiteman M., Bhatia M., Moore P. K. (2006): Regulation of vascular nitric oxide in vitro and in vivo; a new role for endogenous hydrogen sulphide? Br. J. Pharmacol. **149,** 625–634; doi:10.1038/sj.bjp.0706906 PMid:17016507 PMCid:2014646

Chen C. Q., Xin H., Zhu Y. Z. (2007): Hydrogen sulfide: third gaseous transmitter, but with great pharmacological potential. Acta Pharmacol. Sin. **28,** 1709–1716; doi:10.1111/j.1745-7254.2007.00629.x PMid:17959020

Dombkowski R. A., Russell M. J., Olson K. R. (2004): Hydrogen sulfide as an endogenous regulator of vascular smooth muscle tone in trout. Am. J. Physiol., Regul. Integr. Comp. Physiol. **286,** R678–685; doi:10.1152/ajpregu.00419.2003

Geng B., Yang J. H., Qi Y. F., Zhao J., Pang Y. Z., Du J. B., Tang C. S. (2004): H2S generated by heart in rat and its effects on cardiac function. Biochem. Biophys. Res. Commun. **313,** 362–368; doi:10.1016/j.bbrc.2003.11.130 PMid:14684169

Hayman K. A., Spurway T. D., Ashley R. H. (1993): Single anion channels reconstituted from cardiac mitoplasts. J. Membr. Biol. **136,** 181–190; doi:10.1007/BF02505762 PMid:7508981

Hosoki R., Matsuki N., Kimura H. (1997): The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. Biochem. Biophys. Res. Commun. **237,** 527–531; doi:10.1006/bbrc.1997.6878 PMid:9299397

Johansen D., Ytrehus K., Baxter G. F. (2006): Exogenous hydrogen sulfide (H2S) protects against regional myocardial ischemia-reperfusion injury. Evidence for a role of KATP channels. Basic Res. Cardiol. **101,** 53–60; doi:10.1007/s00395-005-0569-9 PMid:16328106

Lee S. W., Hu Y. S., Hu L. F., Lu Q., Dawe G. S., Moore P. K., Wong P. T., Bian J. S. (2006): Hydrogen sulphide regulates calcium homeostasis in microglial cells. Glia **54,** 116–124; doi:10.1002/glia.20362 PMid:16718684

Lowicka E., Beltowski J. (2007): Hydrogen sulfide (H2S) – the third gas of interest for pharmacologists. Pharmacol. Rep. **59,** 4–24

Malekova L., Kominkova V., Ferko M., Stefanik P., Krizanova O., Ziegelhoffer A., Szewczyk A., Ondrias K. (2007a): Bongkrekic acid and atractyloside inhibits chloride channels from mitochondrial membranes of rat heart. Biochim. Biophys. Acta **1767,** 31–44; doi:10.1016/j.bbabio.2006.10.004

Malekova L., Tomaskova J., Novakova M., Stefanik P., Kopacek J., Lakatos B., Pastorekova S., Krizanova O., Breier A., Ondrias K. (2007b): Inhibitory effect of DIDS, NPPB, and phloretin on intracellular chloride channels. Pflügers Arch. **455,** 349–357; doi:10.1007/s00424-007-0300-9 PMid:17611769

Miller C. (2006): ClC chloride channels viewed through a transporter lens. Nature **440,** 484–489; doi:10.1038/nature04713 PMid:16554809

Nilius B., Droogmans G. (2003): Amazing chloride channels: an overview. Acta Physiol. Scand. **177,** 119–147; doi:10.1046/j.1365-201X.2003.01060.x PMid:12558550

Ondrias K., Stasko A., Cacanyiova S., Sulova Z., Krizanova O., Kristek F., Malekova L., Knezl V., Breier A. (2008): H2S and HS-donor NaHS releases nitric oxide from nitrosothiols, metal nitrosyl complex, brain homogenate and murine L1210 leukaemia cells. Pflügers Arch. **457,** 271–279; doi:10.1007/s00424-008-0519-0 PMid:18458940

Puljak L., Kilic G. (2006): Emerging roles of chloride channels in human diseases. Biochim. Biophys. Acta **1762,** 404–413

Sivarajah A., McDonald M. C., Thiemermann C. (2006): The production of hydrogen sulfide limits myocardial ischemia and reperfusion injury and contributes to the cardioprotective effects of preconditioning with endotoxin, but not ischemia in the rat. Shock **26,** 154–161; doi:10.1097/01.shk.0000225722.56681.64 PMid:16878023

Whiteman M., Li L., Kostetski I., Chu S. H., Siau J. L., Bhatia M., Moore P. K. (2006): Evidence for the formation of a novel nitrosothiol from the gaseous mediators nitric oxide and hydrogen sulphide. Biochem. Biophys. Res. Commun. **343,** 303–310; doi:10.1016/j.bbrc.2006.02.154 PMid:16540095

Whitfield N. L., Kreimier E. L., Verdial F. C., Skovgaard N., Olson K. R. (2008): Reappraisal of H2S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling. Am. J. Physiol., Regul. Integr. Comp. Physiol. **294,** R1930–1937; doi:10.1152/ajpregu.00025.2008

Xu M., Wu Y. M., Li Q., Wang F. W., He R. R. (2007): Electrophysiological effects of hydrogen sulfide on guinea pig papillary muscles in vitro. Acta Physiol. Sin. **59,** 215–220

Yang G.-D., Wang R. (2007): H2S and cellular proliferation and apoptosis. Acta Physiol. Sin. **59,** 133–140

Zhao W., Zhang J., Lu Y., Wang R. (2001): The vasorelaxant effect of H2S as a novel endogenous gaseous KATP channel opener. EMBO J. **20,** 6008–6016; doi:10.1093/emboj/20.21.6008 PMid:11689441 PMCid:125693

Zhao W., Wang R. (2002): H2S-induced vasorelaxation and underlying cellular and molecular mechanisms. Am. J. Physiol., Heart Circ. Physiol. **283,** H474–480

Received: July 31, 2008
Final version accepted: September 3, 2008