H$_2$S and HS$^{-}$ donor NaHS inhibits intracellular chloride channels

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Abstract. We have characterized the effect of H$_2$S 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$_2$S inhibited the chloride channels by decreasing the channel open probability in a concentration-dependent manner. The inhibitory effect of H$_2$S 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$_2$S with the NO donor S-nitroso-N-acetyl-DL-penicillamine had smaller effect (IC$_{50}$ = 180 μmol/l) than H$_2$S alone. We assume that the inhibitory effect of H$_2$S on chloride channels may be responsible for some of its numerous biological effects.

Key words: Chloride channels — H$_2$S — 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$_2$S dissociates to H$_2$S $\leftrightarrow$ HS$^{-}$ + H$^+$ and a trace of S$^{2-}$ (Dombkowski et al. 2004). Since it is not known, which is the active form of H$_2$S in vivo, we used term H$_2$S for a total mixture. Endogenously produced H$_2$S 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, sepsis, 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$_2$S is not fully understood. Vasorelaxant effects of H$_2$S 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$_2$S 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$_2$S-induced effect on proliferation and apoptosis (Yang and Wang 2007). A decrease in calcium influx and increase in calcium release by H$_2$S 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$_2$S (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$_2$S, we tested whether H$_2$S can influence intracellular chloride channels. We have found that H$_2$S inhibited chloride channels.

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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-glycerol-phosphatidylcholine and dioleoyl-glycerol-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 CaCl₂, 0.3 EGTA, 1 MgCl₂, 10/5 Hepes/Tris, pH 7.4, except of cis/trans KCl, which was 250/50 mmol/l. The free Ca²⁺ 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 H₂S and HS⁻ donor, which dissociates to Na⁺ and HS⁻, which then reacts with H⁺ to yield H₂S. It is reasonable to neglect the third species, S₂⁻, due to the high pKₐ (11.96). Free H₂S 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 H₂S, HS⁻, and S₂⁻.

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 H₂S 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-
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...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_{2}S-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_{2}S 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_{2}S, 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...
inhibited chloride channels, were in the range of its endogenous physiological concentrations. H$_2$S 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′-disothiocyanostilbene-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$_2$S donor NaHS with NO donors was reported to either enhance (Zhang 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$_2$S 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$_2$S 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$_2$S 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$_2$S 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.

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