Title Page

Targeting mitochondrial large-conductance calcium-activated potassium channel by hydrogen sulfide via heme-binding site

Agnieszka Walewska, Adam Szewczyk, Milena Krajewska, Piotr Koprowski

Laboratory of Intracellular Ion Channels, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland (A.W., A.S., M.K., P.K.)

Running Title Page

H₂S activates heme-inhibited mitoBK_{Ca} channels

Address correspondence to:

Piotr Koprowski, Laboratory of Intracellular Ion Channels, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur Street, Warsaw 02-093, Phone (+48 22) 5892343, E-mail: p.koprowski@nencki.edu.pl

The number of text pages: 25

The number of tables: 0

The number of figures: 5

The number of references: 96

The number of words in the *Abstract*: 195

The number of words in the *Introduction*: 733

The number of words in the *Discussion*: 1884

A list of nonstandard abbreviations:

BK_{Ca} – large-conductance calcium-activated potassium channel; BK_{Ca}-DEC – DEC splice variant of BK_{Ca} channel; HBM – heme-binding motif; HBP – heme-binding peptide; mitoBK_{Ca} – mitochondrial large-conductance calcium-activated potassium channel; imd – imidazole.

A recommended section assignment:

Cellular and Molecular

Abstract

Reperfusion together with the preceding ischemic period results in serious damage to brain and heart tissues. Activation of potassium channels from the inner mitochondrial membrane leads to cytoprotection during such events. The mitochondrial large-conductance calcium-activated potassium channel (mitoBK_{Ca}) is one of these cytoprotective channels. It was previously shown that BK_{Ca} channels are blocked by hemin, which is present in excess during hemorrhage. In the experiments described in this work, we checked whether NaHS, known as a donor of gasotransmitter hydrogen sulfide (H₂S), which can play an important role in cytoprotection, interacts with mitoBK_{Ca} channels. Indeed, using the biotin-switch method, it was found that mitoBK_{Ca} channels undergo S-sulfhydrylation in the presence of NaHS. Although patch-clamp experiments showed that NaHS has negligible effects on the activity of mitoBK_{Ca} channels, NaHS has been shown to almost fully activate hemin-inhibited mitoBK_{Ca} channels. The effects of NaHS were mimicked by imidazole, suggesting a common mechanism of activation of mitoBK_{Ca} channels inhibited by heme/hemin by molecules able to coordinate the iron ion of porphyrin. A set of absorption spectroscopy experiments with the 23 amino acid model peptides containing the heme-binding motif CXXCH suggested previously unrecognized roles of cysteines in heme binding.

Significance Statement

The activity of mitochondrial channels including mitoBK_{Ca} seems to play a significant role in cytoprotection during ischemia/reperfusion. Hemin, which is present in excess during hemorrhage, can potentially bind to and inhibit mitoBK_{Ca} activity. We found that hydrogen sulfide does not affect mitoBK_{Ca} activity unless it is blocked by hemin. In this case, hydrogen sulfide activates hemin-inhibited mitoBK_{Ca} by binding to hemin iron. The hydrogen sulfide effect could be mimicked in patch-clamp experiments by imidazole probably acting by a similar mechanism.

Introduction

For a number of years, hydrogen sulfide (H₂S) was known only as a toxic molecule. However, the beneficial effects of H₂S action in living organisms were recently discovered, and the molecule was classified as a gasotransmitter, next to carbon monoxide (CO) (Untereiner *et al.*, 2012) and nitric oxide (NO) (Mir & Maurya, 2018). H₂S displays a broad spectrum of activities, e.g., attenuating myocardial ischemia-reperfusion injury (Calvert *et al.*, 2010, Elrod *et al.*, 2007, Wang *et al.*, 2011), protecting against apoptosis (Wu *et al.*, 2015), regulating Ca²⁺ homeostasis (Lee *et al.*, 2006), acting as an endogenous vasoactive factor (Zhao *et al.*, 2001) and as a preventive and therapeutic agent in age-associated diseases (Zhang *et al.*, 2013).

The chemical mechanisms underlying the action of H₂S are complex (Bełtowski, 2015, Dongó *et al.*, 2018). Due to its strong reducing nature, H₂S reacts with ROS (Xie *et al.*, 2016), the modified -SH groups of cysteines (Benchoam *et al.*, 2020), and the metallic centers of heme proteins (Boubeta *et al.*, 2020). Due to its high reactivity toward ubiquitous chemical groups, H₂S has a plethora of molecular targets (Kimura, 2015). Potassium channels, such as ATP-sensitive potassium channel (K_{ATP}) (Mustafa *et al.*, 2011, Tang *et al.*, 2005) and the large-conductance calcium-activated potassium (BK_{Ca}) channel (Sitdikova *et al.*, 2010, 2014, Telezhkin *et al.*, 2009), are also targets for H₂S. The BK_{Ca} channel is a tetrameric protein composed of four pore-forming α subunits. Each α subunit consists of a short N-terminus, seven transmembrane segments, and a large C-terminus containing two RCK (regulating conductance of K⁺) domains located in the cytoplasm (Lee & Cui, 2010, Yang *et al.*, 2015). Electrophysiological studies have revealed that the BK_{Ca} channel is modulated by various stimuli, including Ca²⁺ (Adams *et al.*, 1982, Barrett *et al.*, 1982, Latorre *et al.*, 2017, Pallotta *et al.*, 1981), voltage (Horrigan & Aldrich, 2002, Latorre *et al.*, 2017), membrane tension (Kirber *et al.*, 1992, Mallouk & Allard, 2000, Wawrzekiewicz-Jałowicka *et al.*, 2018), and

redox state (DiChiara & Reinhart, 1997, Gao & Fung, 2002, Gong *et al.*, 2000, Soh *et al.*, 2001). BK_{Ca} is also inhibited by the binding of heme (Fe(II) protoporphyrin IX) (Jaggar *et al.*, 2005, Tang *et al.*, 2003) and its oxidized form hemin (Fe(III) protoporphyrin IX) (Horrigan *et al.*, 2005, Tang *et al.*, 2003) to the heme-binding motif (HBM), which is located between RCK domains and composed of two cysteines and a histidine -CXXCH- (Tang *et al.*, 2003). Heme may inhibit BK_{Ca} channel opening by influencing the flexible linker between the RCK domains (Horrigan *et al.*, 2005, Tang *et al.*, 2003); however, many questions remain about the effects of axial ligand(s) or the importance of cysteines in heme binding to the BK_{Ca} channel.

The BK_{Ca} channel is present not only in the plasma membrane but also in the inner membrane of mitochondria (mitoBK_{Ca}) (Bednarczyk *et al.*, 2006, Kicinska *et al.*, 2016, Siemen *et al.*, 1999, Xu *et al.*, 2002). The mitoBK_{Ca} channel could be formed by a DEC splice variant (BK_{Ca}-DEC) encoded by the common *KCNMA1* gene (Singh *et al.*, 2013). Similar to plasmalemmal BK_{Ca} channels, mitoBK_{Ca} is also regulated by hemin (Augustynek *et al.*, 2014). Mitochondria are the main source of endogenous H₂S in the brain (Shibuya *et al.*, 2009), and H₂S regulates mitochondrial energy metabolism (Szabo, 2021) and ROS generation (Cao *et al.*, 2019, Murphy *et al.*, 2019). It has been suggested that the activation of mitoBK_{Ca} leads to the cytoprotection of cardiomyocytes insulted during ischemia/reperfusion (Xu *et al.*, 2002) due to the modulation of ROS signaling (Heinen *et al.*, 2007, Kulawiak *et al.*, 2008). Therefore, whether and how mitoBK_{Ca} activity is modulated by H₂S remains an intriguing issue. Previous research on the impact of H₂S on the activity of BK_{Ca} channels is ambiguous, thus showing either channel activation (Jackson-Weaver *et al.*, 2013, Li *et al.*, 2020) or inhibition (Fernandes *et al.*, 2015, Li *et al.*, 2010, Telezhkin *et al.*, 2010). Therefore, further studies are needed in this field.

In this article, we applied NaHS as an H₂S donor (Reiffenstein *et al.*, 1992, Sitdikova *et al.*, 2010) to investigate its impact on the activity of mitoBK_{Ca} channels. We found that

although the α -subunit of the mitoBK_{Ca} channel is S-sulfhydrated, sulfhydration does not significantly change the activity of this channel. However, we found that H₂S is an activator of mitoBK_{Ca} channels inhibited by hemin. In addition, we investigated in more detail the mechanism of this activation.

Materials and Methods

Chemicals

Stock solutions were prepared as mentioned below, and then diluted to the desired concentrations. Hemin (Sigma-Aldrich, St. Louis, MO, USA) was diluted in DMSO to 10 mM and stored at -20°C. NaHS (Fluorochem Ltd., Hadfield, United Kingdom) and imidazole (Sigma-Aldrich, St. Louis, MO, USA) were diluted in water to 100 mM and 1 M concentrations, respectively. NaHS stock solutions were prepared immediately before use. The pH of the imidazole stock solution was adjusted with HCl to 7.2-7.4, and the solution was stored in a tightly closed vessel at room temperature. Model peptides (Life Tein LLC, New Jersey, USA) containing heme-binding motifs were dissolved in water to prepare stock solutions (40 µmol/L), which were stored at -80°C.

Cell culture

Astrocytoma U-87MG cells (U-87MG, see details Bednarczyk *et al.*, 2013) and the HEK 293 cell line (original HEK 293 cell line from ATCC, Manassas, Virginia, USA) stably expressing the DEC isoform of the human BK_{Ca} channel (HEK 293-BK_{Ca}-DEC) were cultured in DMEM (Laboratory of General Chemistry, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland) supplemented with 2 mM L-glutamine (Gibco, Carlsbad, CA, USA), 10% FBS (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). DMEM for the HEK 293-BK_{Ca}-DEC cell line was additionally supplemented with 500 µg/mL geneticin (Gibco, Carlsbad, CA, USA). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. The cells were fed and reseeded when they reached approximately 70-90% confluence, which usually occurred every fourth day.

Mitochondria isolation

Mitochondria from the U-87MG and HEK 293-BK_{Ca}-DEC cell lines were isolated as previously described (Bednarczyk *et al.*, 2013) with modifications. Briefly, the cells were washed with PBS (Laboratory of General Chemistry, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland), harvested by scraping in PBS, collected in a 50 mL Falcon tube, and centrifuged at 800 x g for 10 minutes. The cell pellet was resuspended and homogenized in SEH buffer (250 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH=7.2). The homogenate was transferred into an Eppendorf tube and centrifuged at 9200 x g for 10 min. Next, the pellet was resuspended in SEH buffer and centrifuged at 780 x g for 10 min. The supernatant containing mitochondria was moved to a new Eppendorf tube and centrifuged at 9200 x g for 10 min. The mitochondrial pellet obtained after the last centrifugation was resuspended in a small volume of SEH buffer (20-300 μ L). All centrifugations were performed at 4°C, and the other manipulations were performed on ice.

Detection of S-sulphydration of the α subunit of the mitoBK_{Ca} channel by the biotin-switch method

In all experiments, NaHS (Fluorochem Ltd. Hadfield, United Kingdom) was used as an H₂S donor (Reiffenstein *et al.*, 1992, Sitdikova *et al.*, 2010). To detect S-sulphydration, a published biotin-switch protocol was used with slight modifications (Park *et al.*, 2015). Mitochondria isolated from U-87MG cells were diluted, resuspended in SEH buffer, and incubated with 1 mM NaHS (experimental sample) for 30 min on ice. The control sample was not treated with NaHS. Next, the experimental sample was diluted to 1,5 mL with SEH buffer and centrifuged at 9200 x g for 10 min to remove NaHS and pellet mitochondria. Subsequently, both the control sample and experimental sample have been subjected to the same procedures. Mitochondrial pellets were resuspended in HEN buffer (250 mM HEPES,

50 mM NaCl, 1 mM EDTA, 1% NP-40, 0,1 mM neocuproine) with protease inhibitors (protease inhibitor cocktail, cOmplete, EDTA-free; Roche, Basel, Switzerland) with the addition of 50 mM water-soluble methylsulfonyl benzothiazole (MSBT-A; BePharm, Ltd, Shanghai, China) and incubated at 37°C for 1 hour. MSBT-A was removed on Micro Bio-Spin™ P-6 Gel Columns (Bio-Rad, Hercules, CA, USA) by centrifugation at 1000 x g for 4 min. Next, the samples were incubated with 20 mM biotin-linked cyanoacetate (CN-biotin; Enamine, New Jersey, USA) at 37°C for 1 hour followed by the removal of unreacted CN-biotin on Micro Bio-Spin™ P-6 Gel Columns. Flow-through from the Micro Bio-Spin™ P-6 Gel Columns contained both S-sulphydrated and non-S-sulphydrated proteins, which were separated using CaptAvidin Agarose (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) as described below. CaptAvidin Agarose was applied to the column (Mobicol “Classic” with 1 closed screw cap; MoBiTec Molecular Biotechnology, Goettingen, Germany) with a 35 µm pore size filter (MoBiTec Molecular Biotechnology, Goettingen, Germany) and centrifuged two times at 1000 x rpm for 2 min. Next, the column with CaptAvidin agarose was washed with PBS (three volumes of CaptAvidin Agarose) and centrifuged at 1500 x rpm for 75 s followed by a wash with 50 mM Tris · HCl, pH=8.0 (two volumes of CaptAvidin Agarose) and centrifugation at 1500 x rpm for 75 s. After applying S-sulphydration and the biotin-switch protocol, the proteins were applied on the column with CaptAvidin Agarose and the column was centrifuged at 1500 x rpm for 1 min. Flow-through from the column contained non-S-sulphydrated (nonbiotinylated proteins), while on the agarose S-sulphydrated proteins were captured. CaptAvidin agarose-capture proteins were analyzed by SDS-PAGE and Western blotting (*Supplemental data, Fig.1*)

SDS-Page and Western-blot

Samples were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% skimmed milk in TBST

buffer (20 mM Tris, 125 mM NaCl, 0.1% Tween-20; pH 7.5). Next, the blocked membranes were incubated with the anti- α subunit of mitoBK_{Ca} channel primary antibody (1:200 in 5% milk in TBST; NeuroMab Anti-Slo1/BK alpha potassium channel, clone L6/60) followed by incubation with anti-mouse secondary antibody (1:5000 in 5% milk in TBST; Amersham ECLTM Anti-mouse IgG, HRP-linked whole Ab (from sheep), GE Healthcare) coupled to horseradish peroxidase. The blots were developed using enhanced chemiluminescence solution (ECL, GE Healthcare) and X-ray films (GE Healthcare).

Mitoplast preparation

Mitoplasts for the patch-clamp experiments were prepared by swelling the mitochondria isolated from U-87MG or HEK 293-BK_{Ca}-DEC cells as previously described (Walewska *et al.*, 2018). Briefly, mitochondria (1-2 μ L) were incubated in 40 μ L of hypotonic solution (5 mM HEPES, 100 μ M CaCl₂, pH=7.2) for approximately 2 min. Then, 10 μ L of high sucrose buffer (1.5 M sucrose, 30 mM HEPES, 100 μ M CaCl₂, pH=7.2) was added to restore the isotonicity of the medium.

Patch-clamp experiments

Patch-clamp experiments were performed according to a published protocol (Bednarczyk *et al.*, 2021). Briefly, the experiments were carried out in patch-clamp inside-out mode, where modulators were applied to the matrix side of the channel. Mitoplasts (0.5-1 μ L) and modulators were added by mixing directly to the recording chamber filled with a high calcium solution containing 150 mM KCl, 10 mM HEPES, and 100 μ M CaCl₂ at pH=7.2. The patch-clamp pipettes made of borosilicate glass (Harvard Apparatus GC150-10, Holliston, MA, USA) with a resistance of 10-20 M Ω were filled with a high-calcium solution. After establishing the gigaohm seal, the membrane patch was excised by tapping the pipette holder. The current was recorded using a patch-clamp amplifier (Axopatch 200B, Molecular Devices

Corporation, San Jose, CA, USA), low-pass filtered at 1 kHz, and sampled at a frequency of 2.5 kHz. Clampfit 10.7 software in the single-channel search mode was used to calculate the probability of channel opening ($P(o)$). Calculations of $P(o)$ were performed for ten-second recordings of the mitoBK_{Ca} channel at different voltages (from -60 mV to 60 mV, 20 mV interval) and continuous recordings at 40 mV with a different length depending on the type of experiment. The Fe(III) protoporphyrin IX (hemin) used in the patch-clamp experiments was dissolved in DMSO to obtain stock solutions and stored at -20°C, while imidazole was dissolved in water (pH 7.2-7.4, HCl) and stored in a tightly closed vessel at room temperature. NaHS stock was prepared in water immediately before use.

Absorption spectroscopy

Electronic absorbance spectra were measured with an Agilent Cary 60 UV-Vis Spectrophotometer in the 250-650 nm range. Hemin, NaHS, imidazole, and model peptides (Life Tein LLC, New Jersey, USA) were added sequentially from the stock solutions to high-calcium buffer (150 mM KCl, 10 mM HEPES, and 100 μ M CaCl₂ at pH=7.2) purged with nitrogen gas for 30-60 minutes before the start of the experiment. The order of substance addition and their concentrations are shown in figure captions. Model peptides containing the heme-binding motif -DAKEVKRAFFYCKACHDDITDPK- and peptides containing single, double, and triple substitutions of two cysteines and histidine (underlined in the sequence) with serines and arginine, respectively, were dissolved in water to prepare the stock solution and stored at -80°C.

Raw absorbance spectra were corrected for the absorbance of the added substances. These corrected absorbance spectra were used to plot the dependence of the wavelength for maximum absorbance (λ_{max}) on the imidazole concentration (*Supplemental data, Fig. 3*) The difference spectra were calculated (*Supplemental data, Fig. 4*) to plot the dependence of the difference between the maximum and minimum absorbance as a function of the imidazole

concentration and fitted by the Hill model $A = A_{max} \frac{I^N}{K^N + I^N}$ (Fig. 5G and *Supplemental data, Fig. 5*). All calculations were performed with Origin2019 (OriginLab Corporation) and Excel (MS Office).

Statistical analysis

Student's t-test or one-way ANOVA with means comparisons test was used in statistical analysis of the obtained results depending on the type of experiment. The following ranges of p values were adopted: 0.05–0.01 (*), 0.01–0.001 (**), and <0.001 (***). All experiments were done in an exploratory manner.

Results

S-sulfhydration of cysteines of the α subunit of the mitoBK_{Ca} channel by NaHS does not significantly affect channel activity

In all our experiments, we used NaHS as an H₂S donor. First, we checked whether cysteines in the α subunit of the mitoBK_{Ca} channel, present endogenously in U-87MG cells undergo S-sulfhydration and, if so, what is the effect of sulfhydration on mitoBK_{Ca} channel activity (Fig. 1). The “biotin-switch” assay was used to biochemically detect the S-sulfhydration of cysteines in the α subunit of the mitoBK_{Ca} channel (Fig. 1A; for a detailed description, see “*Materials and methods*” and *Supplemental data, Fig. 1*). In this method, biotinylation of a protein indicates that the -SH groups underwent S-sulfhydration (Park *et al.*, 2015). We found that among proteins that were immobilized on avidin-agarose was the α subunit of the mitoBK_{Ca} channel (Fig. 1B). However, we were able to detect this protein only if the sample was treated with NaHS. This result suggested that some cysteines in the pore-forming subunit of mitoBK_{Ca} could indeed be S-sulfhydrated.

To check whether this S-sulfhydration affects channel activity, patch-clamping of mitoplasts isolated from the U-87MG cell line was carried out (Fig. 1C; for a detailed description, see “*Materials and methods*”). Fig. 1D shows representative current-time recordings of mitoBK_{Ca} channel activity in the control conditions and the presence of 500 μ M NaHS at different voltages. No changes in the activity of the channel were found (Fig. 1D). The open probability (P(o)) values under the control conditions and in the presence of 500 μ M NaHS at different voltages were similar (Fig. 1E). Fig. 1F shows changes in the percent channel activity recorded at 40 mV after treatment of the mitoBK_{Ca} channel with NaHS. The mean percent of channel activity after treatment with 100 μ M, 500 μ M and 1 mM NaHS was 98.05% ($\pm$ 10.88, n=4), 95.05% ($\pm$ 9.36, n=4) and 96.93% ($\pm$ 24.37, n=4), respectively. There

was no decrease in channel activity at 40 mV after NaHS treatment relative to the control conditions.

NaHS is an activator of hemin-inhibited mitoBK_{Ca} channels

Fig. 2A shows the changes in the P(o) of the mitoBK_{Ca} channel during the course of the representative experiment. Hemin gradually inhibited the activity of the mitoBK_{Ca} channel from P(o)=0.71 (in the last minute of control) to P(o)=0.26 (in the 5th minute after the application of 3 μ M hemin). Subsequent application of NaHS in the presence of hemin resulted in a rapid increase in P(o) to 0.60 (in the 1st minute after NaHS addition). Surprisingly, simultaneous washout of both hemin and NaHS with the control solution resulted in transient inhibition of the mitoBK_{Ca} channel. Immediately after washout, we observed a decrease in the P(o) to 0.29 relative to the P(o) of the mitoBK_{Ca} channel in the presence of hemin and NaHS. However, after 2 minutes, the mitoBK_{Ca} channel became more active and the P(o) increased to 0.76 (Fig. 2A). Representative single mitoBK_{Ca} channel recordings showed that hemin inhibition of the activity of the mitoBK_{Ca} channel was characterized by the presence of hallmark blocking events with flickery channel characteristics (Fig. 2B - rectangles in the second upper trace). After the addition of NaHS, hemin hallmark blocking events are no longer present. However, immediately after washout with the control solution, these blocking events for hemin were visible again (Fig. 2B - rectangles in the second lower trace). Fig. 2C shows changes in the percent of channel activity recorded at 40 mV after treatment of the mitoBK_{Ca} channel with hemin and NaHS. Dose-dependent inhibition of the mitoBK_{Ca} channel with 100 nM and 500 nM hemin was observed, with corresponding mean percent channel activity values of 50.57% ($\pm$ 38.07, n=4; p<0.05 vs. control, *) and 10.99% ($\pm$ 17.03, n=6; p<0.001 vs. control, ***), respectively. The addition of NaHS to the hemin-inhibited channel resulted in an increase in mean channel activity from

this low value to 85.43% (± 32.80 , $n=5$; $p<0.001$ vs. 500 nM hemin, ***) and 111.22% (± 16.07 , $n=6$; $p<0.001$ vs. 500 nM hemin, ***) after the application of 500 μ M and 1 mM NaHS, respectively. Washout did not change the channel activity significantly (immediately after washout 86.47% ± 29.50 , $n=5$; 2 minutes after washout 98.13% ± 5.05 , $n=4$; ≥ 3 minutes after washout 106.27% ± 9.84 , $n=5$) (Fig. 2C). Statistical analysis of the activity of mitoBK_{Ca} channels recorded at 40 mV treated with hemin and NaHS shows large variability (Fig. 2C). However, general trends are noticeable, with inhibition by hemin, reactivation of hemin-inhibited channels by NaHS, and transient decline of the activity after washout.

Modulation of the activity of the DEC isoform of the BK_{Ca} channel by hemin and NaHS.

Previously, we were unable to detect the transcript for the BK_{Ca}-DEC channel splice variant in the U-87MG cell line (Walewska *et al.*, 2018). It was therefore unclear what other isoforms of the BK_{Ca} channel could be present in the mitochondria of these cells. We and others (Ahring *et al.*, 1997) found that HEK 293 cells lack BK_{Ca} activity. Therefore, to investigate the effects of NaHS in a more defined molecular environment, we chose the HEK 293 cell line, which stably expresses the BK_{Ca}-DEC channel (Gałęcka *et al.*, 2021).

Fig. 3 shows the modulation of BK_{Ca}-DEC channel activity from the inner mitochondrial membrane (mitoBK_{Ca}-DEC) by hemin and NaHS. After the addition of 300 nM hemin, the P(o) decreased gradually from 0.96 to 0.60 and finally to 0.24 in 500 nM hemin. The application of 100 μ M NaHS reactivated the mitoBK_{Ca}-DEC channel to a P(o) of 0.91 in the 1st minute after NaHS addition. However, the activity of the mitoBK_{Ca}-DEC channel after washout remained at a high level, and no transient channel inhibition (Fig. 3A) was observed as in the case of the U-87MG cell line (Fig. 2). In the representative single mitoBK_{Ca}-DEC channel recording, channel inhibition with hallmark hemin blocking events was observed (Fig. 3B, top trace). NaHS application resulted in an increase in the P(o) of the channel and a

lack of blocking events. However, washout of both hemin and NaHS did not significantly change the P(o) of the channel. No characteristic hemin blocking events were visible after washout (Fig. 3B). To substantiate this observation, statistical analysis of multiple (n=10) experiments was carried out (Fig. 3C). Dose-dependent inhibition of the mitoBK_{Ca}-DEC channel with 100 nM, 300 nM and 500 nM hemin was observed, with corresponding mean percent channel activity values of 84.36% (± 3.18 , n=9), 41.33% (± 28.93 , n=9; $p < 0.05$,*) and 12.95% (± 14.27 , n=10; $p < 0.001$,***), respectively. The addition of NaHS to the hemin-inhibited channel resulted in an increase in the mean channel activity to 54.98% (± 49.15 , n=9) and 82.84% (± 20.20 , n=6; $p < 0.01$, **) after the application of 500 μ M and 1 mM NaHS, respectively. Washout did not change the channel activity significantly (immediately after washout 98.02% ± 17.19 , n=9; 2 minutes after washout 95.88% ± 17.95 , n=10) relative to the channel activity in the presence of 500 nM hemin and 1 mM NaHS (82.84% ± 20.20 , n=6) (Fig. 3C).

The above results indicate that NaHS activates hemin-inhibited mitoBK_{Ca}-DEC channels like mitoBK_{Ca} channels from the U-87MG cell line.

Imidazole mimics the effects of NaHS on hemin-inhibited mitoBK_{Ca} channels

H₂S is a reducing agent that could potentially reduce Fe(III) ions in porphyrins under specific conditions (Bianco *et al.*, 2017, Giuffrè & Vicente, 2018). To examine whether we could mimic the effects of NaHS with a redox nonreactive molecule, we chose imidazole, which is a known coordination ligand of iron porphyrins (Collman *et al.*, 1975, Perry *et al.*, 2002).

Fig. 4 shows a set of patch-clamp experiments carried out on mitoplasts obtained from mitochondria of the U-87MG cell line (Fig. 4A) and HEK 293-BK_{Ca}-DEC cell line (Fig. 4B). In these experiments, hemin was added up to 1 μ M, and at this concentration, the channels

were almost blocked (Fig. 4A i). In the U-87MG cell line, the $P(o)$ of the mitoBK_{Ca} channel activity decreased from 0.74 under the control conditions to 0.37 in the 5th minute after 500 nM hemin application to a final value of 0.04 in the 2nd minute after 1 μ M hemin administration (Fig. 4A i, left panel). In the HEK 293-BK_{Ca}-DEC cells, multiple channels were observed and the NP(o) decreased from 3.80 in control conditions to 2.30 in the 3rd minute after 500 nM hemin application to a final value of 0.20 in the 3rd minute after 1 μ M hemin administration (Fig. 4B i, right panel).

The addition of 50 mM imidazole resulted in immediate channel reactivation: the $P(o)$ increased to 0.78 for mitoBK_{Ca} from the mitoplasts of U-87MG cells (Fig. 4A i, left panel) and the NP(o) increased to 2.97 for mitoBK_{Ca}-DEC from mitoplasts of HEK 293-BK_{Ca}-DEC cells (Fig. 4B i, right panel). This effect was not voltage-dependent (*Supplemental data, Fig. 2*). Simultaneous washout of both hemin and imidazole resulted in evident transient inhibition of the activity of mitoBK_{Ca} channels. For channels from U-87MG cells immediately after washout, the $P(o)$ decreased to 0.003 and then gradually increased to 0.76 in the 10th minute after washout (Fig. 4A i, left panel). For the channels from the HEK 293-BK_{Ca}-DEC cells, the NP(o) decreased to 1.44 and then gradually increased to 2.89 in the 9th minute after washout (Fig. 4B i, right panel).

Representative traces of channel activities provide more insight into channel behavior (Figs. 4A ii and 4B ii). After the addition of imidazole, at positive (pipette negative) potentials a decrease in single-channel conductance relative to the control solution could be observed (for the mitoBK_{Ca} channel from the U-87 cell line from 294.3 pS to 212.0 pS and for the mitoBK_{Ca}-DEC channel from 279.3 pS to 204.3 pS). The single-channel conductance returned to the control values after the washout step. In Figs. 4A ii and 4B ii, characteristic hemin blocking events are not visible after 1 μ M hemin application because of complete channel inhibition. However, they were visible at lower hemin concentrations (not shown). These

blocking events were not observed in the presence of imidazole. Strikingly, after washout blocking events reappeared (Fig. 4A ii and 4B ii, black dashed rectangles) along with a transient inhibitory effect, suggesting that hemin was still bound within the channel.

We observed large patch-to-patch variability; however, an investigation of single-channel behavior and statistical analyses of multiple patches supported the findings described above (Figs. 4A iii and 4B iii). For mitoBK_{Ca} channels from the U-87MG cell line, the addition of 500 nM and 1 μ M hemin resulted in a statistically significant ($p < 0.01$, **) decrease in channel activity to 32.88% (± 41.12 ; $n = 6$) and 16.50% (± 30.56 ; $n = 4$), respectively. Subsequent administration of 50 mM imidazole in the presence of hemin resulted in an increase in channel activity to 91.01% (± 25.52 ; $n = 7$; $p < 0.01$ vs. 1 μ M hemin, **). Finally, simultaneous washout of both compounds resulted in transient channel inhibition with a decrease in channel activity to 35.73% (± 37.75 ; $n = 6$; $p < 0.05$ vs. 50 mM imidazole, **) and a gradual increase in channel activity to 59.82% (± 33.66 ; $n = 5$) 4 minutes after washout and to 77.34% (± 31.63 ; $n = 5$) 9 minutes after washout (Fig. 4A iii).

Similar observations were perceived for the mitoBK_{Ca}-DEC channel (Fig. 4B iii). A statistically significant decrease in channel activity of 47.63% was observed for 500 nM hemin (± 29.54 ; $n = 7$; $p < 0.01$ vs. control, **) and of 10.42% was observed for 1 μ M hemin (± 19.31 ; $n = 5$; $p < 0.001$ vs. control, ***). After treatment of hemin-inhibited channels with 50 mM imidazole, the percent channel activity increased to 94.62% (± 14.69 ; $n = 7$; $p < 0.001$ vs. 1 μ M hemin, ***). Simultaneous washout of hemin and imidazole caused transient channel inhibition, with a decrease in channel activity to 54.89% (± 34.13 ; $n = 5$) and a gradual increase in channel activity to 69.12% (± 24.13 ; $n = 4$) 4 minutes after washout and 75.24% (± 24.14 ; $n = 5$) 8 or more minutes after washout (Fig. 4B iii).

Altogether, the phenomena associated with modulation of channel activity by hemin and imidazole are in accordance with the data obtained for hemin and NaHS. Both imidazole and

NaHS reactivated channels that were inhibited by hemin. In addition, washouts resulted in transient inhibition of channel activity with a gradual recovery of activity over time. These observations indicate a similar if not equivalent mechanism of regulation of the activity of the mitoBK_{Ca} channel by NaHS and imidazole, which possibly occurs by binding the iron ion of hemin. However, observable differences occurred between the mitoBK_{Ca} and mitoBK_{Ca}-DEC channels with respect to modulation by NaHS (see “*Discussion*”).

Spectroscopic investigation of the interactions of hemin, H₂S, and imidazole with a model peptide containing the heme-binding motif

To gain more insights into the interplay between the ligands hemin, H₂S, and imidazole and the channel, we took advantage of heme-binding peptide (HBP), which is a simple model system that was previously introduced to demonstrate the binding of hemin to the fragment of the BK_{Ca} channel containing the -CKACH- motif (Tang *et al.*, 2003).

UV-Vis absorption spectroscopy has been used in numerous studies to investigate the interaction of metal porphyrins with ligands (Collier *et al.*, 1979). Hemin is poorly soluble in water but soluble in DMSO. In DMSO, the hemin spectrum is characterized by a narrow Soret band at approximately 408 nm (Collier *et al.*, 1979). The UV-Vis absorption spectrum of hemin in aqueous solutions exhibits a broad Soret absorption maximum of approximately 384 nm (Karnaukhova *et al.*, 2014) due to the extensive formation of stacked dimers (De Villiers *et al.*, 2007). It is well known that imidazole forms complexes with iron porphyrins that exhibit distinctive spectra; however, most of the studies were carried out in organic solvents, such as chloroform (Patra *et al.*, 2010, Yoshimura & Ozaki, 1984). We carried out our measurements in a more biologically relevant buffer identical to that in which patch-clamp experiments were performed. Titration of hemin (20 μM) with increasing concentrations of imidazole (up to 50 mM) resulted in a shift of wavelength in the maximal absorbance (λ_{max}) to

436 nm (Fig. 5A). At lower concentrations, intermediate λ_{max} (*Supplemental data, Fig. 3*) or a shoulder of approximately 413 nm was visible. No isosbestic point could be identified, indicating multiple absorbing species. In addition to the Soret peak, after imidazole application, two additional peaks (Q bands) were present at 542 nm and 565 nm (Fig. 5A). The difference absorption spectrum for this type of reaction has minimum and maximum values near 371 and 446 nm, respectively (*Supplemental data, Fig 4Q*) A plot of the difference in absorbance at 446 and 371 nm as a function of the imidazole concentration is shown in Fig. 5G (black squares). The relation could be fit by the Hill model (see “*Materials & Methods*”) with a K_d of 6.10 mM and $N=5.17$ (*Supplemental data, Table 1*), indicating a cooperative process. Subsequent addition of NaHS to up to 1 mM resulted in lower values of A_{max} . This experiment suggested that imidazole is coordinated by hemin dimers, ultimately leading to monomerization of hemin and the formation of mono- and bis-imidazole hemin complexes with λ_{max} 413 and 436 nm, respectively. Due to the high imidazole concentration, the addition of relatively low concentrations of NaHS was not effective. Therefore, in the next experiment, we titrated hemin (20 μM) first with increasing concentrations of NaHS (up to 1 mM), and then performed titration with imidazole (up to 100 mM) (Fig. 5B). Here, the addition of NaHS resulted in a small 10 nm bathochromic shift of λ_{max} to 397 nm. However, in the presence of NaHS, higher concentrations of imidazole were required to achieve the maximal shift to 436 nm (Fig. 5B). Fitting of the plot of the difference in absorbance at 442 and 368 nm (maximum and minimum of difference spectra in *Supplemental data, Fig. 4R*) as a function of the imidazole concentration (Fig. 5G, green squares) produced values of $K_d=15.95$ and $N=3.34$. The increased K_d and lower cooperativity indicated that imidazole competes with NaHS for binding to hemin.

In consecutive experiments, the HBP model peptide was introduced. Fig. 5C shows that the application of 40 $\mu\text{mol/L}$ HBP resulted in a hypsochromic shift of λ_{max} from 384 nm for

20 μ M hemin to 376 nm for hemin with HBP. Subsequent application of imidazole caused a gradual bathochromic shift with increasing concentrations of imidazole to a final λ_{max} of 428 nm. At lower concentrations, intermediate λ_{max} (*Supplemental data, Fig. 3A*) or a shoulder of approximately 413 nm was visible. Subsequent addition of NaHS up to 1 mM resulted in only marginal changes in the spectra. The difference absorption spectrum of hemin with HBP after imidazole binding has minimum and maximum values of approximately at 368 nm and 433 nm, respectively (*Supplemental data, Fig. 4A*). A plot of the difference in absorbance at 433 and 368 nm as a function of the imidazole concentration is shown in Fig. 5G (black circles). The relation could be fit by the Hill model with a K_d of 32.41 mM and $N=3.24$ (*Supplemental data, Table 1*). When NaHS was applied in this system as the first compound (Fig. 5D), we noticed few differences compared to that for the conditions without this H_2S donor. First, a slightly lower λ_{max} (422 nm) was achieved with the maximal imidazole content (*Supplemental data, Fig. 3A*). Second, the analysis of the difference spectra (Fig. 5G and *Supplemental data, Fig. 4B*) showed that the N coefficient was smaller (2.46 vs. 3.20; *Supplemental data, Table 1*). Moreover, changes in K_d relative to the conditions without NaHS were not observed (32.86 mM vs. 32.41 mM; *Supplemental data, Table 1*). From the above experiments, we concluded that i) higher concentrations of imidazole (increased K_d) were required to achieve the λ_{max} value in the presence of HBP, suggesting competition between the binding of imidazole and HBP to hemin; ii) the λ_{max} of hemin/imidazole complexes in the presence and absence of HBP was different (428 nm vs. 436 nm), which suggested that the binding of imidazole to the iron ion of hemin did not result in HBP dissociation but rather the formation of a tertiary complex; and iii) NaHS interacts with hemin both in the presence and absence of HBP.

To determine whether the impact of HBP on the titration characteristics of hemin by imidazole is specific for the -CXXCH- motif, the analysis described above was performed for

a series of single, double, and triple substitutions of two cysteines and histidine with serines and arginine, respectively (Tang *et al.*, 2003) (Fig. 5E-F and *Supplemental data, Figs. 3-5; Supplemental data, Table 1*). We found various degrees of the contribution of these amino acids to the competitive behavior of HBP in the imidazole titration assay. Unexpectedly, we found that the substitution of histidine to arginine had no effect while the substitution of serine to cysteine was additive (*Supplemental data, Table 1*). The K_d for imidazole titration in the presence of HBP single cysteine mutants was 0.76 (for the first cysteine, C1) and 0.9 (for the second cysteine, C2) of that of the wild-type HBP, while for the double cysteine mutant HBP_C1, C2 K_d was only 0.45 (*Supplemental data, Table 1*). Curiously, the presence of NaHS affected the K_d of imidazole binding to hemin only if one or two cysteines were mutated, thus indicating the interplay between these residues and hydrogen sulfide in hemin binding (*Supplemental data, Table 1*).

Discussion

H₂S has become a popular research subject because increasing evidence has been obtained on the beneficial effects it exerts on living organisms (Cicccone *et al.*, 2021, Li *et al.*, 2021, Scammahorn *et al.*, 2021). Especially exciting is the role of H₂S as a mediator of cytoprotection during ischemia/reperfusion, which is also observed for CO and NO (Zhang *et al.*, 2021). Several lines of evidence suggest that mitoBK_{Ca} channels are involved in cytoprotection during ischemia/reperfusion (Frankenreiter *et al.*, 2017, Goswami *et al.*, 2019, Xu *et al.*, 2002). Previous data (see below) have shown that H₂S could affect the activity of BK_{Ca} channels from the plasma membrane; however, research on the effects of H₂S on the activity of mitoBK_{Ca} channels is lacking. Therefore, we sought to determine whether H₂S impacts the activity of the mitoBK_{Ca} channel and identify the possible mechanisms of H₂S action.

H₂S converts oxidatively modified -SH groups of cysteines to -SSH adducts, which may regulate protein functions (Mustafa *et al.*, 2009). Published studies showed that ion channels undergo direct regulation by H₂S. Differing effects of H₂S were shown for BK_{Ca} channels. H₂S potentiated BK_{Ca} currents in human uterine artery smooth muscle cells (Li *et al.*, 2020). H₂S also increased the activity of BK_{Ca} channels in rat pituitary tumor cells, and this effect was linked to its action on sulfhydryl groups of the channel protein (Sitdikova *et al.*, 2010).

In contrast, BK_{Ca} channels were inhibited by H₂S in colonic smooth muscle cells (SMCs) (Quan *et al.*, 2015) and human-induced pluripotent stem cell-derived mesenchymal stromal cells (Zhao *et al.*, 2013). BK_{Ca} was also found to be inhibited by H₂S in carotid body glomus cells, where channel inhibition by H₂S was rapid, reversible, and voltage-independent; however, the exact mechanism of H₂S action was not shown (Telezhkin *et al.*, 2010). The abovementioned data on the regulation of the activity of the BK_{Ca} channel from the plasma

membrane are not consistent and did not identify the mechanisms by which H₂S targets these BK_{Ca} channels.

In our research, we show for the first time that H₂S sulfhydrates the -SH groups of cysteines of the α -subunits of mitoBK_{Ca} channels in the U-87MG cell line treated with NaHS. Sulfhydration was not observed in the control, suggesting that the level of endogenous sulfhydration was low or that this modification of the α subunit of BK_{Ca} was chemically unstable. In addition, our patch-clamp experiments indicated that sulfhydration did not significantly change the channel activity in either the U-87MG cell line or the HEK 293 cell line that stably expresses the DEC isoform of the BK_{Ca} channel.

H₂S has broad reactivity with intracellular targets, and in addition to thiols, it also reacts with ferrous (Fe²⁺) or ferric (Fe³⁺) heme, i.e., hemin (Mishanina *et al.*, 2015). It is well established that BK_{Ca} channel activity is inhibited by hemin, which binds within a -CXXCH-motif located in the C-terminus of the channel (Horrigan *et al.*, 2005, Tang *et al.*, 2003). This motif is also present in cytochrome c, where heme c is covalently bound to the protein *via* thioether bonds formed by two cysteines of HBM (Mavridou *et al.*, 2013). In contrast, heme b is only transiently bound to BK_{Ca} channels (Tang *et al.*, 2003). Hemin inhibits the activity of the mitoBK_{Ca} channel from the U-87MG cell line (Augustynek *et al.*, 2014) and the DEC isoform of the BK_{Ca} channel expressed in the HEK 293T cell line (Gałecka *et al.*, 2021), thus confirming the presence of functional HBM in mitoBK_{Ca} channels. We observed that the hemin application not only inhibited channel activity (decrease in P(o)) but also resulted in characteristic burst-like blocking events. Heme is a receptor of H₂S and other gasotransmitters, and CO activates heme-inhibited BK channels (Jaggar *et al.*, 2005, Rotko *et al.*, 2020, Yi *et al.*, 2010). We found that the addition of NaHS caused reactivation (an increase in P(o)) of mitoBK_{Ca} channels that were inhibited by hemin. Moreover, the disappearance of hemin-blocking events was observed in the presence of NaHS, indicating

direct interference with hemin inhibition. Strikingly, after simultaneous washout of hemin and NaHS, we observed a transient decrease in $P(o)$ of the mitoBK_{Ca} channel from U-87MG cells with a reoccurrence of characteristic hemin blocking events. This finding suggests that i) hemin remained bound within the channel after coordination of H₂S with the channel, and ii) H₂S dissociates from hemin with fast off-rate, which is followed by slow dissociation of hemin from HBM. In comparison, after simultaneous washout of hemin and NaHS, BK_{Ca}-DEC channel hemin blocking events were rarely visible while the decrease in $P(o)$ was not significant. These differences may result from the distinctive architecture of HBD and differences in the dissociation rates of H₂S from hemin and hemin from HBM. Such differences could be due to the presence of particular isoforms of the BK_{Ca} channel. C615 and H616 within the CKACH motif (HBM) are responsible for inhibition by hemin (Tang *et al.*, 2003), and H616 serves as the axial ligand for iron ions (Yi *et al.*, 2010). Indeed the presence of iron ion is critical for inhibition since protoporphyrin IX (PPIX) and Mg(II)PPIX do not inhibit the activity of mitoBK_{Ca} (see *Supplemental data, Fig. 6*). However, the sequence similarity to cytochrome c indicated that distant M691 might be the second axial ligand for heme binding (Yusifov *et al.*, 2014). This methionine is separated from HBM by a variable stretch of amino acids, which can include the STREX sequence responsible for the mechanosensitivity of the BK_{Ca} channel (Naruse *et al.*, 2009). We previously found that the STREX variant is expressed in U87-MG cells and a subset of mitoBK_{Ca} channels in this cell line is mechanosensitive (Walewska *et al.*, 2018), thus supporting the possible molecular variability surrounding HBM.

C615S substitution results in a lack of inhibition by hemin; however, no data could point to a role in heme binding by the cysteines of HBM. Instead, these cysteines could form a disulfide bridge under oxidative conditions, thereby modulating HBM affinity toward heme

(Yi *et al.*, 2010). These oxidized cysteines could potentially be S-sulhydrated, which would affect channel inhibition by hemin.

H₂S coordinates preferentially to the free sixth position of ferric iron (Fe^{III}). However, hydrogen sulfide is a redox-active molecule, and upon coordination, it can form not only stable Fe^{III}-SH₂ complexes but also reduce ferric iron, thereby producing unstable Fe^{II}-SH₂ intermediates (Pietri *et al.*, 2011). To determine whether the effects of NaHS could be mimicked by a redox inert molecule, we examined imidazole, the side chain of histidine known to be coordinated by iron porphyrins (Erman *et al.*, 2015, Yoshimura & Ozaki, 1979).

We found that imidazole activated hemin-inhibited mitoBK_{Ca} channels from U-87MG and BK_{Ca}-DEC channels from HEK 293 cells with the disappearance of hemin blocking events. After washout of both hemin and imidazole, transient inhibition of channel activity was observed with the reappearance of hemin blocking events. The effects after washout were similar for NaHS and imidazole in experiments with the mitoBK_{Ca} channels from U-87MG cells, although they slightly differed for BK_{Ca}-DEC from HEK 293 cells. In the latter case, after washout of hemin and imidazole, transient inhibition was observed, while after washout of hemin and NaHS, no transient inhibition was observed, as mentioned above. These differences underscore the possible sequence variability around the hemin binding site, as discussed above.

To further investigate the binding of ligands to the channel, we used HBP as a model system. The addition of HBP to hemin did not change the spectra significantly. Specifically, we did not observe the appearance of a sharper peak at approximately 406 nm, which was observed when the heme-binding domain (HBD), a 134-residue linker between RCK1 and RCK2, was combined with hemin (Yi *et al.*, 2010). However, our results were compatible with an earlier study by Jaggar, who also used a short 23 aa long HBP (Jaggar *et al.*, 2005). The spectra suggest that no histidine was involved in the binding, which was further

confirmed by imidazole titration of mutant peptides with H to R substitutions (see *Supplemental data, Table 1*). Interestingly, we observed that cysteines play a role in HBP binding to hemin based on imidazole titration (see *Supplemental data, Table 1*). Altogether, HBP seems to be an inadequate model for the interaction of hemin with the BK_{Ca} channel, and further experiments are needed with the use of whole protein domains, such as HBD or HBD containing STREX exons, to investigate the interplay of ligands such as H₂S or imidazole with hemin-bound proteins.

One limitation of this study is the use of relatively high NaHS concentrations of 500-1000 μ M. These concentrations were selected according to literature data that described NaHS effects in electrophysiological recordings (Tang *et al.*, 2005; Sun *et al.*, 2008; Sitdikova *et al.*, 2010; Sekiguchi *et al.*, 2014; Kuksis and Ferguson, 2015). For instance, Sitdikova (Sitdikova *et al.*, 2010) used NaHS in the range from 10 μ M to 10 mM and found that the activity of BK_{Ca} in the plasma membrane of GH3 pituitary cells was modulated with EC₅₀ of 167 and 2000 μ M. The physiological concentrations of H₂S are still under debate but they are probably much lower in between the nanomolar (total tissue levels) to micromolar (locally) range (Furne *et al.*, 2008; Wintner *et al.*, 2010; Peng *et al.*, 2011). However, the use of higher concentrations of H₂S donor NaHS in patch-clamp experiments has justifications. NaHS dissociates in water to Na⁺ and HS⁻, and HS⁻ associates with H⁺ to produce H₂S. In neutral solutions, one-third of NaHS exists as H₂S and the remaining two-thirds are present as HS⁻ (Reiffenstein *et al.*, 1992). H₂S is volatile and it was shown that during the 10 minutes of the experiment approximately 40-50% of H₂S could be lost (Sitdikova *et al.*, 2014). Moreover, patch-clamp recordings are carried out at ambient oxygen, and significant oxidation of hydrogen sulfide to elemental sulfur S⁰ could be expected (Rathore *et al.*, 2021). Indeed, almost immediately after NaHS application, we observe the cloudy appearance of the bath solution due to precipitation of S⁰. Taking together all the above considerations, we can

estimate that the effective concentration of H₂S is less than 20% of that of NaHS i.e. around 100 – 200 μM. These concentrations are still higher than accepted average intracellular H₂S concentrations but close to pharmacologically relevant concentrations used in *in vitro* studies reported in the literature. In addition, local H₂S concentrations *in vivo* could be significantly higher than measured average values. For instance, it was shown that plasmalemmal BK_{Ca} bind hemoxygenase-2, an enzyme that degrades heme and produces another gasotransmitter carbon monoxide a known activator of heme-inhibited BK_{Ca} channels suggesting high CO concentrations in the channel vicinity (Williams *et al.*, 2004; Jaggar *et al.*, 2005).

There is also an experimental reason to use higher concentrations in our studies. There is large inherent variability in patch-clamp recordings due to their single-molecule nature and variability within BK_{Ca} channels. By using high concentrations of blocker (hemin) and activator (NaHS) we can shift the response of the system to all or none regime effectively minimizing result variability. Altogether, we acknowledge that the concentrations of H₂S used in our studies are higher than the average concentrations of H₂S measured in tissues. For that reason, our results should be considered as mechanistic studies on a molecular mechanism of H₂S action on mitoBK_{Ca} but not on the physiological significance of this phenomenon.

Acknowledgements

We are grateful to Bogusz Kulawiak for sharing the HEK 293-BK_{Ca}-DEC cell line.

Authorship Contributions

Participated in research design: Walewska, Szewczyk, Koprowski

Conducted experiments: Walewska, Krajewska

Contributed new reagents or analytic tools: Walewska, Koprowski

Performed data analysis: Walewska, Koprowski

Wrote or contributed to the writing of the manuscript: Walewska, Szewczyk, Krajewska,
Koprowski

References

- Adams, P. R., Constanti, A., Brown, D. A. & Clark, R. B. (1982) Intracellular Ca^{2+} activates a fast voltage-sensitive K^+ current in vertebrate sympathetic neurones. *Nature* **296**(5859):746-749. doi:10.1038/296746a0
- Ahring, P. K., Strøbaek, D., Christophersen, P., Olesen, S.-P. & Johansen, T. E. (1997) Stable expression of the human large-conductance Ca^{2+} -activated K^+ channel α - and β -subunits in HEK293 cells. *FEBS Lett.* **415**(1):67-70. John Wiley & Sons, Ltd. doi:10.1016/S0014-5793(97)01096-X
- Augustynek, B., Kudin, A. P., Bednarczyk, P., Szewczyk, A. & Kunz, W. S. (2014) Hemin inhibits the large conductance potassium channel in brain mitochondria: a putative novel mechanism of neurodegeneration. *Exp. Neurol.* **257**:70-75. doi:10.1016/j.expneurol.2014.04.022.
- Barrett, J. N., Magleby, K. L. & Pallotta, B. S. (1982) Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol.* **331**(1):211-230. doi:10.1113/jphysiol.1982.sp014370
- Bednarczyk, P., Skalska, J., Glab, M., Kulawiak, B., Dolowy, K. & Szewczyk, A. (2006) Large conductance potassium ion channel from rat brain mitochondria. *Biochim. Biophys. Acta-Bioenergetics* **461**:1–12.
- Bednarczyk, P., Kampa, R. P., Gałęcka, S., Sek, A., Walewska, A. & Koprowski, P. (2021) Patch-Clamp Recording of the Activity of Ion Channels. *Methods Mol Biol.* **2276**:235-248. doi: 10.1007/978-1-0716-1266-8_18.
- Bednarczyk, P., Wieckowski, M. R., Broszkiewicz, M., Skowronek, K., Siemen, D. & Szewczyk, A. (2013) Putative Structural and Functional Coupling of the Mitochondrial BKCa Channel to the Respiratory Chain. *PLoS One* **8**(6):e68125. doi:10.1371/journal.pone.0068125

- Bełtowski, J. (2015) Hydrogen sulfide in pharmacology and medicine - An update. *Pharmacol. Reports* 67(3):647-658. doi:10.1016/j.pharep.2015.01.005
- Benchoam, D., Cuevasanta, E., Möller, M. N. & Alvarez, B. (2020) Persulfides, at the crossroads between hydrogen sulfide and thiols. *Essays Biochem.* **64**(1):155-168. Portland Press Ltd. doi:10.1042/ebc20190049
- Bianco, C. L., Toscano, J. P. & Fukuto, J. M. (2017) An Integrated View of the Chemical Biology of NO, CO, H₂S, and O₂. In: *Nitric Oxide: Biology and Pathobiology: Third Edition*, 9-21. Elsevier Inc. doi:10.1016/B978-0-12-804273-1.00002-8
- Boubeta, F. M., Bieza, S. A., Bringas, M., Palermo, J. C., Boechi, L., Estrin, D. A. & Bari, S. E. (2020, February) Hemeproteins as Targets for Sulfide Species. *Antioxidants Redox Signal.* 32(4):247-257. Mary Ann Liebert Inc. doi:10.1089/ars.2019.7878
- Calvert, J. W., Elston, M., Nicholson, C. K., Gundewar, S., Jha, S., Elrod, J. W., Ramachandran, A., et al. (2010) Genetic and pharmacologic hydrogen sulfide therapy attenuates ischemia-induced heart failure in mice. *Circulation* **122**(1):11-19. Circulation. doi:10.1161/CIRCULATIONAHA.109.920991
- Cao, X., Ding, L., Xie, Z. Z., Yang, Y., Whiteman, M., Moore, P. K. & Bian, J. S. (2019, July) A Review of Hydrogen Sulfide Synthesis, Metabolism, and Measurement: Is Modulation of Hydrogen Sulfide a Novel Therapeutic for Cancer? *Antioxidants Redox Signal.* 31(1):1-38. Mary Ann Liebert Inc. doi:10.1089/ars.2017.7058
- Ciccone, V., Genah, S. & Morbidelli, L. (2021, March) Endothelium as a source and target of H₂S to improve its trophism and function. *Antioxidants.* 10(3):486 MDPI AG. doi:10.3390/antiox10030486
- Collier, G. S., Pratt, J. M., Wet, C. R. De & Tshabalala, C. F. (1979) Studies on haemin in dimethyl sulphoxide/water mixtures. *Biochem. J.* **179**(2):281-289. doi:10.1042/bj1790281

- Collman, J. P., Gagne, R. R., Reed, C. A., Halbert, T. R., Lang, G. & Robinson, W. T. (1975) 'Picket fence porphyrins', synthetic models for oxygen binding hemoproteins. *J Am Chem Soc.* **97**(6):1427-39. doi: 10.1021/ja00839a026. doi:10.1002/chin.197522413
- DiChiara, T. J. & Reinhart, P. H. (1997) Redox modulation of hsl^o Ca²⁺-activated K⁺ channels. *J. Neurosci.* **17**(13):4942-4955. doi:10.1523/jneurosci.17-13-04942.1997
- Dongó, E., Beliczai-Marosi, G., Dybvig, A. S. & Kiss, L. (2018) The mechanism of action and role of hydrogen sulfide in the control of vascular tone. *Nitric Oxide* **81**:75-87. doi:10.1016/j.niox.2017.10.010
- Elrod, J. W., Calvert, J. W., Morrison, J., Doeller, J. E., Kraus, D. W., Tao, L., Jiao, X., et al. (2007) Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. *Proc. Natl. Acad. Sci. U. S. A.* **104**(39):15560-15565. doi:10.1073/pnas.0705891104
- Erman, J. E., Chinchilla, D., Studer, J. & Vitello, L. B. (2015) Binding of imidazole, 1-methylimidazole and 4-nitroimidazole to yeast cytochrome c peroxidase (CcP) and the distal histidine mutant, CcP(H52L). *Biochim. Biophys. Acta - Proteins Proteomics* **1854**(8):869–881. Elsevier. doi:10.1016/j.bbapap.2015.04.013
- Fernandes, V. S., Xin, W. & Petkov, G. V. (2015) Novel mechanism of hydrogen sulfide-induced guinea pig urinary bladder smooth muscle contraction: role of BK channels and cholinergic neurotransmission. *Am. J. Physiol. Physiol.* **309**(2):C107-C116. American Physiological Society. doi:10.1152/ajpcell.00021.2015
- Frankenreiter, S., Bednarczyk, P., Kniess, A., Bork, N. I., Straubinger, J., Koprowski, P., Wrzosek, A., et al. (2017) cGMP-Elevating Compounds and Ischemic Conditioning Provide Cardioprotection Against Ischemia and Reperfusion Injury via Cardiomyocyte-Specific BK Channels. *Circulation* **136**(24):2337–2355. doi:10.1161/circulationaha.117.028723

- Furne J., Saeed A., and Levitt M. D. (2008) Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. *Am J Physiol - Regul Integr Comp Physiol* **295**(5):R1479-85. doi: 10.1152/ajpregu.90566.2008.
- Gałecka, S., Kulawiak, B., Bednarczyk, P., Singh, H. & Szewczyk, A. (2021) Single channel properties of mitochondrial large conductance potassium channel formed by BK-VEDEC splice variant. *Sci. Rep.* **11**(1):1-12. Nature Publishing Group UK. doi:10.1038/s41598-021-90465-3
- Gao, T. M. & Fung, M. L. (2002) Decreased large conductance Ca^{2+} -activated K^{+} channel activity in dissociated CA1 hippocampal neurons in rats exposed to perinatal and postnatal hypoxia. *Neurosci. Lett.* **332**(3):163-166.. doi:10.1016/S0304-3940(02)00946-1
- Giuffrè, A. & Vicente, J. B. (2018) Hydrogen sulfide biochemistry and interplay with other gaseous mediators in mammalian physiology. *Oxid. Med. Cell. Longev.* **2018**:6290931. Hindawi Limited. doi:10.1155/2018/6290931
- Gong, L. W., Gao, T. M., Huang, H. & Tong, Z. (2000) Redox modulation of large conductance calcium-activated potassium channels in CA1 pyramidal neurons from adult rat hippocampus. *Neurosci. Lett.* **286**(3):191-194. doi:10.1016/S0304-3940(00)01121-6
- Goswami, S. K., Ponnalagu, D., Hussain, A. T., Shah, K., Karekar, P., Gururaja Rao, S., Meredith, A. L., Khan M. & Singh H. (2019) Expression and Activation of BK_{Ca} Channels in Mice Protects Against Ischemia-Reperfusion Injury of Isolated Hearts by Modulating Mitochondrial Function. *Front. Cardiovasc. Med.* **5**:194. Frontiers. doi:10.3389/fcvm.2018.00194
- Heinen, A., Aldakkak, M., Stowe, D. F., Rhodes, S. S., Riess, M. L., Varadarajan, S. G. & Camara, A. K. S. (2007) Reverse electron flow-induced ROS production is attenuated by activation of mitochondrial Ca^{2+} -sensitive K^{+} channels. *Am. J. Physiol. - Hear. Circ. Physiol.* **293**(3) H1400-H1407. doi:10.1152/ajpheart.00198.2007

- Horrigan, F. T. & Aldrich, R. W. (2002) Coupling between voltage sensor activation, Ca^{2+} binding and channel opening in large conductance (BK) potassium channels. *J. Gen. Physiol.* **120**(3):267-305. doi:10.1085/jgp.20028605
- Horrigan, F. T., Heinemann, S. H. & Hoshi, T. (2005) Heme regulates allosteric activation of the Slo1 BK channel. *J. Gen. Physiol.* **126**(1):7–21. doi:10.1085/jgp.200509262
- Jackson-Weaver, O., Osmond, J. M., Riddle, M. A., Naik, J. S., Gonzalez Bosc, L. V., Walker, B. R. & Kanagy, N. L. (2013) Hydrogen sulfide dilates rat mesenteric arteries by activating endothelial large-conductance Ca^{2+} -activated K^{+} channels and smooth muscle Ca^{2+} sparks. *Am. J. Physiol. - Hear. Circ. Physiol.* **304**(11):H1446-H1454. doi:10.1152/ajpheart.00506.2012
- Jaggar, J. H., Li, A., Parfenova, H., Liu, J., Umstot, E. S., Dopico, A. M. & Leffler, C. W. (2005) Heme is a carbon monoxide receptor for large-conductance Ca^{2+} -activated K^{+} channels. *Circ. Res.* **97**(8):805-812. doi:10.1161/01.RES.0000186180.47148.7b
- Karnaukhova, E., Rutardottir, S., Rajabi M., M., Rosenlöf, L. W., Alayash, A. I. & Åkerström, B. (2014) Characterization of heme binding to recombinant $\alpha 1$ -microglobulin. *Front. Physiol.* **5**:465. Frontiers Media S.A. doi:10.3389/fphys.2014.00465
- Kicinska, A., Augustynek, B., Kulawiak, B., Jarmuszkiewicz, W., Szewczyk, A. & Bednarczyk, P. (2016) A large-conductance calcium-regulated K^{+} channel in human dermal fibroblast mitochondria. *Biochem. J.* **473**(23):4457–4471. doi:10.1042/BCJ20160732
- Kimura, H. (2015) Hydrogen sulfide and polysulfides as signaling molecules. *Proc. Japan Acad. Ser. B Phys. Biol. Sci.* **91**(4):131-59. doi:10.2183/pjab.91.131
- Kirber, M. T., Ordway, R. W., Clapp, L. H., Walsh, J. V. & Singer, J. J. (1992) Both membrane stretch and fatty acids directly activate large conductance Ca^{2+} -activated K^{+}

- channels in vascular smooth muscle cells. *FEBS Lett.* **297**(1–2):24-28. doi:10.1016/0014-5793(92)80319-C
- Kuksis M., and Ferguson A. V. (2015) Actions of a hydrogen sulfide donor (NaHS) on transient sodium, persistent sodium, and voltage-gated calcium currents in neurons of the subfornical organ. *J Neurophysiol.* **114**(3):1641-51. doi: 10.1152/jn.00252.2015
- Kulawiak, B., Kudin, A. P., Szewczyk, A. & Kunz, W. S. (2008) BK channel openers inhibit ROS production of isolated rat brain mitochondria. *Exp. Neurol.* **212**(2):543-547. doi:10.1016/j.expneurol.2008.05.004
- Latorre, R., Castillo, K., Carrasquel-Ursulaez, W., Sepulveda, R. V., Gonzalez-Nilo, F., Gonzalez, C. & Alvarez, O. (2017) Molecular determinants of BK channel functional diversity and functioning. *Physiol. Rev.* **97**(1):39-87. doi:10.1152/physrev.00001.2016
- Lee, S. W., Hu, Y. S., Hu, L. F., Lu, Q., Dawe, G. S., Moore, P. K., Wong, P. T. H. & Bian J.S. (2006) Hydrogen sulphide regulates calcium homeostasis in microglial cells. *Glia* **54**(2):116-124. doi:10.1002/glia.20362
- Lee, U. S. & Cui, J. (2010) BK channel activation: Structural and functional insights. *Trends Neurosci.* **33**(9):415-23. doi:10.1016/j.tins.2010.06.004
- Li, M., Mao, J. & Zhu, Y. (2021) New therapeutic approaches using hydrogen sulfide donors in inflammation and immune-response. *Antioxid. Redox Signal.* **35**(5):341-356. doi:10.1089/ars.2020.8249
- Li, Q., Sun, B., Wang, X., Jin, Z., Zhou, Y., Dong, L., Jiang, L. H., et al. (2010) A crucial role for hydrogen sulfide in oxygen sensing via modulating large conductance calcium-activated potassium channels. *Antioxidants Redox Signal.* **12**(10):1179-1189. doi:10.1089/ars.2009.2926
- Li, Y., Bai, J., Yang, Y. H., Hoshi, N. & Chen, D. B. (2020) Hydrogen sulfide relaxes human uterine artery via activating smooth muscle bkca channels. *Antioxidants* **9**(11):1-18.

MDPI AG. doi:10.3390/antiox9111127

- Mallouk, N. & Allard, B. (2000) Stretch-induced activation of Ca^{2+} -activated K^{+} channels in mouse skeletal muscle fibers. *Am. J. Physiol. - Cell Physiol.* **278**(3):C473-C479. doi:10.1152/ajpcell.2000.278.3.c473
- Mavridou, D. A. I., Ferguson, S. J. & Stevens, J. M. (2013) Cytochrome c assembly. *IUBMB Life*. **65**(3):209-216. doi:10.1002/iub.1123
- Mir, J. M. & Maurya, R. C. (2018) A gentle introduction to gasotransmitters with special reference to nitric oxide: Biological and chemical implications. *Rev. Inorg. Chem.* **38**(4). doi:10.1515/revic-2018-0011
- Mishanina, T. V., Libiad, M. & Banerjee, R. (2015) Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways. *Nat. Chem. Biol.* **11**(7):457-464. doi:10.1038/nchembio.1834
- Murphy, B., Bhattacharya, R. & Mukherjee, P. (2019) Hydrogen sulfide signaling in mitochondria and disease. *FASEB J.* **33**(12):13098-13125 John Wiley and Sons Inc. doi:10.1096/fj.201901304R
- Mustafa, A. K., Gadalla, M. M., Sen, N., Kim, S., Mu, W., Gazi, S. K., Barrow, R. K., Yang, G., Wang, R. & Snyder, S.H.(2009) H_2S signals through protein S-Sulfhydration. *Sci. Signal.* **2**(96):ra72. doi:10.1126/scisignal.2000464
- Mustafa, A. K., Sikka, G., Gazi, S. K., Steppan, J., Jung, S. M., Bhunia, A. K., Barodka, V. M., Gazi, F. K., Barrow, R. K., Wang, R., Amzel, L. M., Berkowitz, D. E. & Snyder, S.H. (2011) Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulfhydrates potassium channels. *Circ. Res.* **109**(11):1259-1268. doi:10.1161/CIRCRESAHA.111.240242
- Naruse, K., Tang, Q. Y. & Sokabe, M. (2009) Stress-Axis Regulated Exon (STREX) in the C terminus of BK_{Ca} channels is responsible for the stretch sensitivity. *Biochem. Biophys.*

- Res. Commun.* **385**(4):634–639. doi:10.1016/j.bbrc.2009.05.105
- Pallotta, B. S., Magleby, K. L. & Barrett, J. N. (1981) Single channel recordings of Ca^{2+} -activated K^{+} currents in rat muscle cell culture. *Nature* **293**(5832):471-474. doi:10.1038/293471a0
- Park, C. M., Macinkovic, I., Filipovic, M. R. & Xian, M. (2015) Use of the ‘Tag-Switch’ Method for the Detection of Protein S-Sulfhydration. In: *Methods in Enzymology* **555**:39-56. doi:10.1016/bs.mie.2014.11.033
- Patra, R., Chaudhary, A., Ghosh, S. K. & Rath, S. P. (2010) Axial ligand orientations in a distorted porphyrin macrocycle: Synthesis, structure, and properties of low-spin bis(imidazole)iron(III) and iron(II) porphyrinates. *Inorg. Chem.* **49**(5):2057-2067. doi:10.1021/ic9016504
- Peng H., Cheng Y., Dai C., King A. L., Predmore B. L., Lefer D. J., and Wang B. (2011) A fluorescent probe for fast and quantitative detection of hydrogen sulfide in blood. *Angew Chem Int Ed Engl.* **50**(41):9672-5. doi: 10.1002/anie.201104236
- Perry, C. B., Chick, T., Ntlokwana, A., Davies, G. & Marques, H. M. (2002) The co-ordination of ligands by iron porphyrins: A comparison of ligand binding by myoglobin from sperm whale and the haem undecapeptide from cytochrome c. *J. Chem. Soc. Dalton Trans.* (3):449-457. doi:10.1039/b107333h
- Pietri, R., Roman-Morales, E. & Lopez-Garriga, J. (2011) Hydrogen Sulfide and Hemeproteins: Knowledge and Mysteries. *Antioxid. Redox Signal.* **15**(2):393–404. doi:10.1089/ars.2010.3698
- Quan, X., Luo, H., Liu, Y., Xia, H., Chen, W. & Tang, Q. (2015) Hydrogen sulfide regulates the colonic motility by inhibiting both L-type calcium channels and BK_{Ca} channels in smooth muscle cells of rat colon. *PLoS One* **10**(3):e0121331. Public Library of Science. doi:10.1371/journal.pone.0121331

- Rathore D. S., Meena V. K. , Chandel C. P. S., and Gupta K. S. (2021) Kinetics of the Oxidation of Hydrogen Sulfide by Atmospheric Oxygen in an Aqueous Medium. *J. Atmos. Sci. Res.* **4**(3). doi: 10.30564/jasr.v4i3.3465
- Reiffenstein, R. J., Hulbert, W. C. & Roth, S. H. (1992) Toxicology of hydrogen sulfide. *Annu. Rev. Pharmacol. Toxicol.* **32**:109-34. doi:10.1146/annurev.pa.32.040192.000545
- Rotko, D., Bednarczyk, P., Koprowski, P., Kunz, W. S., Szewczyk, A. & Kulawiak, B. (2020) Heme is required for carbon monoxide activation of mitochondrial BK_{Ca} channel. *Eur. J. Pharmacol.* **881**:173191. Elsevier B.V. doi:10.1016/j.ejphar.2020.173191
- Scammahorn, J. J., Nguyen, I. T. N., Bos, E. M., Goor, H. van & Joles, J. A. (2021, March) Fighting oxidative stress with sulfur: Hydrogen sulfide in the renal and cardiovascular systems. *Antioxidants*. **10**(3):373. MDPI AG. doi:10.3390/antiox10030373
- Sekiguchi F., Miyamoto Y., Kanaoka D., Ide H., Yoshida S., Ohkubo T., and Kawabata A. (2014) Endogenous and exogenous hydrogen sulfide facilitates T-type calcium channel currents in Cav3.2-expressing HEK293 cells. *Biochem. Biophys. Res. Commun.* **445**(1):225-229. doi: 10.1016/j.bbrc.2014.01.185
- Shibuya, N., Tanaka, M., Yoshida, M., Ogasawara, Y., Togawa, T., Ishii, K. & Kimura, H. (2009) 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. *Antioxidants Redox Signal.* **11**(4) :703-714. doi:10.1089/ars.2008.2253
- Siemen, D., Loupatatzis, C., Borecky, J., Gulbins, E. & Lang, F. (1999) Ca²⁺-activated K channel of the BK-type in the inner mitochondrial membrane of a human glioma cell line. *Biochem. Biophys. Res. Commun.* **257**(2) 549-554. doi:10.1006/bbrc.1999.0496
- Singh, H., Lu, R., Bopassa, J. C., Meredith, A. L., Stefani, E. & Toro, L. (2013) MitoBK_{Ca} is encoded by the Kcnma1 gene, and a splicing sequence defines its mitochondrial location. *Proc. Natl. Acad. Sci. U. S. A.* **110**(26):10836-10841. National Academy of Sciences.

doi:10.1073/pnas.1302028110

- Sitdikova, G. F., Fuchs, R., Kainz, V., Weiger, T. M. & Hermann, A. (2014) Phosphorylation of BK channels modulates the sensitivity to hydrogen sulfide (H₂S). *Front. Physiol.* **5**:431. doi:10.3389/fphys.2014.00431
- Sitdikova, G. F., Weiger, T. M. & Hermann, A. (2010) Hydrogen sulfide increases calcium-activated potassium (BK) channel activity of rat pituitary tumor cells. *Pflugers Arch. Eur. J. Physiol.* **459**(3):389-397. doi:10.1007/s00424-009-0737-0
- Soh, H., Jung, W., Uhm, D. Y. & Chung, S. (2001) Modulation of large conductance calcium-activated potassium channels from rat hippocampal neurons by glutathione. *Neurosci. Lett.* **298**(2):115-118. doi:10.1016/S0304-3940(00)01737-7
- Sun Y. G., Cao Y. X., Wang W. W., Ma S. F., Yao T. & Zhu Y. C. (2008) Hydrogen sulphide is an inhibitor of L-type calcium channels and mechanical contraction in rat cardiomyocytes. *Cardiovasc. Res.* **79**(4):632-41. doi: 10.1093/cvr/cvn140
- Szabo, C. (2021, January) Hydrogen Sulfide, an Endogenous Stimulator of Mitochondrial Function in Cancer Cells. *Cells.* **10**(2):220. NLM (Medline). doi:10.3390/cells10020220
- Tang, G., Wu, L., Liang, W. & Wang, R. (2005) Direct stimulation of K_{ATP} channels by exogenous and endogenous hydrogen sulfide in vascular smooth muscle cells. *Mol. Pharmacol.* **68**(6):1757-1764. doi:10.1124/mol.105.017467
- Tang, X. D., Xu, R., Reynolds, M. F., Garcia, M. L., Heinemann, S. H. & Hoshi, T. (2003) Haem can bind to and inhibit mammalian calcium-dependent Slo1 BK channels. *Nature* **425**(6957):531-535. doi:10.1038/nature02003
- Telezhkin, V., Brazier, S. P., Cayzac, S., Müller, C. T., Riccardi, D. & Kemp, P. J. (2009) Hydrogen sulfide inhibits human BK_{Ca} channels. *Adv. Exp. Med. Biol.* **648**:65-72. doi:10.1007/978-90-481-2259-2_7
- Telezhkin, V., Brazier, S. P., Cayzac, S. H., Wilkinson, W. J., Riccardi, D. & Kemp, P. J.

- (2010) Mechanism of inhibition by hydrogen sulfide of native and recombinant BK_{Ca} channels. *Respir. Physiol. Neurobiol.* **172**(3):169-178. doi:10.1016/j.resp.2010.05.016
- Untereiner, A. A., Wu, L. & Wang, R. (2012) The role of carbon monoxide as a gasotransmitter in cardiovascular and metabolic regulation. In: *Gasotransmitters: Physiology and Pathophysiology* **9783642303388**:37-70. doi:10.1007/978-3-642-30338-8_2
- Villiers, K. A. De, Kaschula, C. H., Egan, T. J. & Marques, H. M. (2007) Speciation and structure of ferriprotoporphyrin IX in aqueous solution: Spectroscopic and diffusion measurements demonstrate dimerization, but not μ -oxo dimer formation. *J. Biol. Inorg. Chem.* **12**(1):101-117. doi:10.1007/s00775-006-0170-1
- Walewska, A., Kulawiak, B., Szewczyk, A. & Koprowski, P. (2018) Mechanosensitivity of mitochondrial large-conductance calcium-activated potassium channels. *Biochim. Biophys. Acta - Bioenerg.* **1859**(9):797-805. doi:10.1016/j.bbabo.2018.05.006
- Wang, X., Wang, Q., Guo, W. & Zhu, Y. Z. (2011) Hydrogen sulfide attenuates cardiac dysfunction in a rat model of heart failure: A mechanism through cardiac mitochondrial protection. *Biosci. Rep.* **31**(2):87-98. doi:10.1042/BSR20100003
- Wawrzkieicz-Jałowicka, A., Trybek, P., Machura, Ł., Dworakowska, B. & Grzywna, Z. J. (2018) Mechanosensitivity of the BK Channels in Human Glioblastoma Cells: Kinetics and Dynamical Complexity. *J. Membr. Biol.* **251**(5–6):667-679. doi:10.1007/s00232-018-0044-9
- Williams S. E. J., Wootton P., Mason H. S., Bould J., Iles D. E., Riccardi D., Peers C. & Kemp P. J. (2004) Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. *Science* **306**(5704):2093-7. doi: 10.1126/science.1105010
- Wintner E. A., Deckwerth T. L., Langston W., Bengtsson A., Leviten D., Hill P., Insko M. A., Dumpit R., Vandenekart E., Toombs C. F. & Szabo C. (2010) A monobromobimane-

- based assay to measure the pharmacokinetic profile of reactive sulphide species in blood. *Br. J. Pharmacol.* **160**(4): 941–957. doi: 10.1111/j.1476-5381.2010.00704.x
- Wu, D., Hu, Q., Liu, X., Pan, L., Xiong, Q. & Zhu, Y. Z. (2015) Hydrogen sulfide protects against apoptosis under oxidative stress through SIRT1 pathway in H9c2 cardiomyocytes. *Nitric Oxide - Biol. Chem.* **46**:204-12. doi:10.1016/j.niox.2014.11.006
- Xie, Z. Z., Liu, Y. & Bian, J. S. (2016) Hydrogen Sulfide and Cellular Redox Homeostasis. *Oxid. Med. Cell. Longev.* **2016**: ID 6043038. doi:10.1155/2016/6043038
- Xu, W., Liu, Y., Wang, S., McDonald, T., Eyk, J. E. Van, Sidor, A. & O'Rourke, B. (2002) Cytoprotective role of Ca^{2+} -activated K^+ channels in the cardiac inner mitochondrial membrane. *Science*. **298**(5595):1029-1033. doi:10.1126/science.1074360
- Yang, H., Zhang, G. & Cui, J. (2015) BK channels: Multiple sensors, one activation gate. *Front. Physiol.* **6**:29. doi:10.3389/fphys.2015.00029
- Yi, L., Morgan, J. T. & Ragsdale, S. W. (2010) Identification of a Thiol/Disulfide Redox Switch in the Human BK Channel That Controls Its Affinity for Heme and CO. *J. Biol. Chem.* **285**(26):20117-20127. doi:10.1074/jbc.M110.116483
- Yoshimura, T. & Ozaki, T. (1979) Equilibria of Imidazole Derivatives with (Protoporphyrin IX dimethyl ester)iron(III) Chloride. *Bull. Chem. Soc. Jpn.* **52**(8):2268-2275. The Chemical Society of Japan. doi:10.1246/bcsj.52.2268
- Yoshimura, T. & Ozaki, T. (1984) Imidazole, imidazolate, and hydroxide complexes of (protoporphyrin IX)iron(III) and its dimethyl ester as model systems for ferric hemoproteins: Electron paramagnetic resonance and electronic spectral study. *Arch. Biochem. Biophys.* **230**(2):466-482. Academic Press. doi:10.1016/0003-9861(84)90427-2
- Yusifov, T., Madhvani, R. V. & Olcese, R. (2014) The Cytochrome C-Like Domain of the Human BK Channel. *Biophys. J.* **106**(2):535a. Elsevier BV.

doi:10.1016/j.bpj.2013.11.2985

- Zhang, M.-L., Peng, W., Ni, J.-Q. & Chen, G. (2021) Recent advances in the protective role of hydrogen sulfide in myocardial ischemia/reperfusion injury: a narrative review. *Med. Gas Res.* **11**(2):83-87. Medknow. doi:10.4103/2045-9912.311499
- Zhang, Y., Tang, Z.-H., Ren, Z., Qu, S.-L., Liu, M.-H., Liu, L.-S. & Jiang, Z.-S. (2013) Hydrogen Sulfide, the Next Potent Preventive and Therapeutic Agent in Aging and Age-Associated Diseases. *Mol. Cell. Biol.* **33**(6):1104-1113. doi:10.1128/mcb.01215-12
- Zhao, W., Zhang, J., Lu, Y. & Wang, R. (2001) The vasorelaxant effect of H₂S as a novel endogenous gaseous K_{ATP} channel opener. *EMBO J.* **20**(21):6008-6016. doi:10.1093/emboj/20.21.6008
- Zhao, Y., Wei, H., Kong, G., Shim, W. & Zhang, G. (2013) Hydrogen sulfide augments the proliferation and survival of human induced pluripotent stem cell-derived mesenchymal stromal cells through inhibition of BK_{Ca}. *Cytotherapy* **15**(11):1395–1405. doi:10.1016/j.jcyt.2013.06.004

Footnotes

This work was supported by the Nencki Institute of Experimental Biology and, by the Polish National Science Centre grants nos. 2015/17/B/NZ1/02496 to A.S. and 2018/31/N/NZ1/00928 to A.W.

The authors declare there are no actual or perceived conflicts of interest with the contents of this article.

Figure legends

Fig. 1. S-sulfhydrylation of the α subunit of the mitoBK_{Ca} channel by NaHS and its effect

on channel activity **A.** Schematic representation of the “biotin-switch” assay used to detect protein S-sulfhydrylation. NaHS was used as a sulfhydryl donor. S-sulfhydrylation was followed by “the tagging step”, where both the -SH (non-S-sulfhydrylated) and -SSH (S-sulfhydrylated) groups were blocked by MSBT-A. In the next “switching step”, CN-biotin was introduced to specifically convert the -SSH groups to biotin-labeled adducts that could be captured or detected with the use of avidin. **B.** Western blot analysis of two fractions obtained after separation of proteins on CaptAvidin agarose: flow-through fraction with nonbiotinylated proteins that did not undergo S-sulfhydrylation and CaptAvidin agarose fraction with biotinylated proteins that were S-sulfhydrylated. The control sample was not treated with NaHS. An antibody against the α subunit of the mitoBK_{Ca} channel (anti-BK_{Ca} α) was used. **C.** Schematic representation of the patch-clamp protocol on the mitochondrial inner membrane: isolation of mitochondria from the U-87MG cell line, preparation of mitoplasts by hypoosmotic swelling of mitochondria, and patch-clamp recording from the inner membrane of mitochondria in inside-out configuration. **D.** Representative recordings of single mitoBK_{Ca} channel activity from the U-87MG cell line at different voltages under the control conditions and in the presence of 500 μ M NaHS; “c” with an arrow and grey solid line denotes the closed state of the channel; “o” with an arrow and grey dotted line denotes the open state of the channel. **E.** Open probability of mitoBK_{Ca} channels at different voltages in the control conditions (black line) and in the presence of 500 μ M NaHS (blue line). The data are presented as the mean $\pm$ SD (control conditions n=4; 500 μ M NaHS n=4) and were fitted using a Boltzmann function. A paired sample Student’s t-test was used to identify any significant differences. **F.** Statistical analysis of channel activity at 40 mV after the application of different NaHS concentrations. The data are presented as the percentage of the

channel activity with respect to the control conditions (mean $\pm$ SD with points representing a given repetition). A one-way ANOVA with Tukey means comparisons test was used to identify any significant differences. The $P(o)$ for the statistical analysis of channel activity was calculated for a one-minute-long recording starting one minute after NaHS application (unless otherwise stated).

Fig. 2. Effect of NaHS on the activity of mitoBK_{Ca} channels from the U87-MG cell line inhibited by hemin

A. Changes in the open probability of mitoBK_{Ca} channels during continuous experiments recorded at 40 mV. The gradual decrease in channel open probability after the application of hemin (3 μ M) followed by a sharp increase in channel activity after the addition of NaHS (1 mM). After washout with the control solution, a transient inhibitory effect was observed. The data were binned by one-minute intervals. A time interval of $\sim$ 1 min in which the compounds were added or washed out was excluded from the analysis. **B.** Representative recordings of single-mitoBK_{Ca} channel activity at 40 mV in the control conditions after the application of hemin followed by NaHS addition and washout step. Hallmark hemin blocking events are marked by dashed black rectangles; “c” with an arrow and grey solid line denotes the closed state of the channel; “o” with an arrow and grey dotted line denotes the open state of the channel. Open probabilities are shown on the right side of each trace. **C.** Statistical analysis of channel activity at 40 mV after the application of hemin (100 nM and 500 nM) and NaHS (500 μ M and 1 mM) and after washout. The data are presented as the mean percent of channel activity $\pm$ SD with marked points representing given repetitions. A one-way ANOVA with Tukey means comparisons test was used to identify any significant differences. The points of a given color come from the same experiment. The $P(o)$ for the statistical analysis of percent of channel activity was calculated for one-minute-long

recordings. The analysis start time is indicated on the graph in minutes from substance application or washout.

Fig. 3. Regulation of the activity of the mitoBK_{Ca}-DEC isoform by hemin and NaHS.

NaHS activates mitoBK_{Ca}-DEC channels inhibited by hemin; no transient inhibitory effect after washout is observed. **A.** Changes in the open probability of the mitoBK_{Ca} channel during continuous experiments recorded at 40 mV. The gradual decrease in channel open probability after the application of hemin (300 nM and 500 nM) followed by a sharp increase in channel activity after the addition of NaHS (500 μ M and 1 mM). No transient channel inhibition after simultaneous washout of either hemin or NaHS was observed. **B.** Representative recordings of single-mitoBK_{Ca}-DEC channel activity at 40 mV in control conditions after the application of hemin followed by NaHS addition and washout step; “c” with an arrow and grey solid line denotes the closed state of the channel; “o” with an arrow and grey dotted line denotes the open state of the channel. **C.** Statistical analysis of channel activity at 40 mV after the application of hemin and NaHS and after the washout step. The data are presented as the means $\pm$ SD of percent of channel activity, with marked points representing given repetitions. A one-way ANOVA with Games-Howell means comparisons test was used to identify any significant differences. The points of a given color come from the same experiment. The P(o) for the statistical analysis of percent of channel activity was calculated for one minute long recording. The analysis start time is indicated on the graph in minutes from substance application or washout. The P(o) of the mitoBK_{Ca}-DEC channel in control conditions is marked as 100%, while the P(o) of the mitoBK_{Ca}-DEC channel after treatment with hemin, NaHS, and after washout step is calculated as a percent of channel activity in control conditions.

Fig. 4. Regulation of the activity of the mitoBK_{Ca} channel (A) and mitoBK_{Ca}-DEC channel (B) by hemin and imidazole i. Changes in the open probability of the mitoBK_{Ca} channel and mitoBK_{Ca}-DEC channel during continuous experiments recorded at 40 mV. The gradual decrease in channel open probability after the application of hemin (500 nM and 1 μ M) was followed by a sharp increase in channel activity after the addition of imidazole (50 mM). Washout with the control solution results in transient channel inhibition. **ii.** Representative recording of single-mitoBK_{Ca} channel activity (A) or multiple mitoBK_{Ca}-DEC channels (B) at 40 mV in control conditions after the application of hemin followed by imidazole addition and washout step; “c” with an arrow and grey solid line denotes the closed state of the channel; “o₁”, “o₂”, “o₃”, “o₄” with an arrow and grey dotted line denotes the open states of the channel. Open probability values are shown. **iii.** Statistical analysis of mitoBK_{Ca} channel (A.) and mitoBK_{Ca}-DEC channel (B.) activity at 40 mV after the application of hemin and imidazole and after the washout step. The data are presented as the mean percent of channel activity $\pm$ SD, with marked points representing a given repetition. The points of a given color come from the same experiment. The P(o) and NP(o) for the statistical analysis of percent of channel activity were calculated for a one-minute-long recording. A one-way ANOVA with Tukey means comparisons test was used to identify any significant differences. The analysis start time is indicated on the graph in minutes from substance application or washout. Open probabilities of channels in control conditions are normalized, while after treatment with hemin and imidazole and after the washout step, the probabilities are calculated as a percent of channel activity in control conditions.

Fig. 5. Spectroscopic investigation of the interactions of hemin with H₂S, imidazole, and model peptides containing heme-binding motif (HBP) A.-F. Absorbance spectra of hemin after the administration of substances indicated in the legend. In all experiments, constant

concentrations of hemin (20 μ M) and HBP (40 μ mol/L) were used. Concentrations of NaHS and imidazole (Imd) were varied and are marked in the graph legends, such as is the order in which the substances are added. Numbers above the graph lines with corresponding colors specify wavelengths of maximal absorbance - λ_{max} . **G.** Plot of the difference in absorbance (at a wavelength where the maximum and minimum of absorbance is observed at different spectra) as a function of the imidazole concentration with Hill fitting. The data are presented as the mean $\pm$ SD and were fitted using a Hill function.

Figure 2

Figure 3

Figure 4

Figure 5