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RESEARCH PAPER

Chloride channels mediate sodium sulphide-induced relaxation in rat uteri

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BACKGROUND AND PURPOSE

Hydrogen sulphide reduces uterine contractility and is of potential interest as a treatment for uterine disorders. The aim of this study was to explore the mechanism of sodium sulphide (Na_2S)-induced relaxation of rat uterus, investigate the importance of redox effects and ion channel-mediated mechanisms, and any interactions between these two mechanisms.

EXPERIMENTAL APPROACH

Organ bath studies were employed to assess the pharmacological effects of Na_2S in uterine strips by exposing them to Na_2S with or without Cl^- channel blockers (DIDS, NFA, IAA-94, T16Ainh-A01, TA), raised KCl (15 and 75 mM), K^+ channel inhibitors (glibenclamide, TEA, 4-AP), L-type Ca^{2+} channel activator (S-Bay K 8644), propranolol and methylene blue. The activities of antioxidant enzymes were measured in homogenates of treated uteri. The expression of bestrophin channel 1 (BEST-1) was determined by Western blotting and RT-PCR.

KEY RESULTS

Na_2S caused concentration-dependent reversible relaxation of spontaneously active and calcium-treated uteri, affecting both amplitude and frequency of contractions. Uteri exposed to 75 mM KCl were less sensitive to Na_2S compared with uteri in 15 mM KCl. Na_2S-induced relaxations were abolished by DIDS, but unaffected by other modulators or by the absence of extracellular HCO_3^-, suggesting the involvement of chloride ion channels. Na_2S in combination with different modulators provoked specific changes in the anti-oxidant profiles of uteri. The expression of BEST-1, both mRNA and protein, was demonstrated in rat uteri.

CONCLUSIONS AND IMPLICATIONS

The relaxant effects of Na_2S in rat uteri are mediated mainly via a DIDS-sensitive Cl^--pathway. Components of the relaxation are redox- and Ca^{2+}-dependent.

Abbreviations

ANO (TMEM16), anoctamin chloride channels; BEST, bestrophin chloride channels; CaCCs, calcium-activated chloride channels; CAT, catalase; GR, glutathione reductase; GSH-Px, glutathione peroxidase; K_{ATP}, ATP-sensitive K^+ channel; NFA, niflumic acid; ROS, reactive oxygen species

Tables of Links

TARGETS	
GPCRs[a]	**Transporters**[c]
β-adrenoceptor	Cl^-/HCO_3^- exchanger
Ion channels[b]	**Enzymes**[d]
CaCC	Cystathionine β-synthase
K_{ATP} channels	Cystathionine γ-lyase
K_v channels	GR
TMEM16 channel	Guanylyl cyclase

LIGANDS	
4-AP	NaHS
Adrenaline	Niflumic acid (NFA)
CGRP	Nitric oxide (NO)
DIDS	Propranolol
Glibenclamide	S-Bay K 8644
H_2O_2	Tannic acid (TA)
IAA-94	TEA

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d]Alexander *et al.*, 2013a,b,c,d).

Introduction

The smooth muscle layer of the uterus, the myometrium, undergoes profound remodelling that allows rapid changes to occur in different physiological (and pathophysiological) conditions. In a non-pregnant state, myometrial activity is at its highest level during oestrus (Crane and Martin, 1991). Abnormal contractility can underlie disorders such as dysmenorrhea and endometriosis. Several potential inhibitors of myometrial activity have been identified: CGRP (Anouar *et al.*, 1998), NO (Buxton *et al.*, 2001) and carbon monoxide (CO; Acevedo and Ahmed, 1998). Endogenously produced hydrogen sulphide (H_2S), which, together with NO and CO, is a recognized gasotransmitter, also appears to be a signalling molecule in rat uterus. Both enzymes responsible for its endogenous production (cystathionine β-synthase and cystathionine γ-lyase) were identified in all rat intrauterine tissues (Patel *et al.*, 2009). Hydrogen sulphide is a potent inhibitor of spontaneous contractions of the pregnant myometrium (Sidhu *et al.*, 2001). It is thought that the mechanisms of H_2S-induced relaxations mainly involve effects on ATP-sensitive K^+ channels (K_{ATP}; Zhao *et al.*, 2001; Tang *et al.*, 2005; Robinson and Wray, 2012). However, contrary to this, it was found that K_{ATP} channels are not involved in the relaxant effects of H_2S (Dhaese and Lefebvre, 2009). Even though K_{ATP} channels are expressed in myometrium, they are only of limited functional importance (Aaronson *et al.*, 2006) suggesting that H_2S has other targets (Robinson and Wray, 2012).

It is not currently known whether the biological effects of H_2S are mediated directly by H_2S or by its derivatives, the most important being the thiolate anion HS^-. We recently showed that the relaxant effects of the molecular and anionic form H_2S/HS^-, which originate from exogenous sodium sulphide (Na_2S) at physiological pH, might be different (Mijušković *et al.*, 2014). It seems plausible that, because of its negative charge, HS^- affects Cl^- channels (Van der Sterren *et al.*, 2011). Chloride channels have been proposed to mediate the effects of hydrogen sulphide (Tang *et al.*, 2010). In myometrium, the major anionic conductance is mediated by currents evoked by Ca-activated Cl^- channels (CaCC channels; Song *et al.*, 2009; Bernstein *et al.*, 2014). However, the molecular identity of CaCCs is still not known. Several candidates have been proposed including bestrophins (BEST; Leblanc *et al.*, 2005; Greenwood, 2008), CLCA (Leblanc *et al.*, 2005) and the anoctamin (ANO or TMEM) family of proteins (Caputo *et al.*, 2008; Scudieri *et al.*, 2012; Pedemonte and Galietta, 2014). A study by Bernestein and co-workers showed that TMEM channels are important mediators of murine uterine smooth muscle contractility (Bernstein *et al.*, 2014). Recently, DIDS was identified as an inhibitor of BEST channels (Liu *et al.*, 2014). Also a few studies have shown that DIDS (Cl^- channel inhibitor) abolishes the vasorelaxant responses to hydrogen sulphide (Streeter *et al.*, 2012; White *et al.*, 2013). Cells with pacemaker activity that were reported in spontaneously active smooth muscles and interstitial cells of Cajal (ICC)-like cells have been found in rat and human uteri (Ciontea *et al.*, 2005; Duquette *et al.*, 2005). Furthermore, calcium-activated chloride channels have been shown to be involved in the generation of slow waves in ICC (Singh *et al.*, 2014).

In addition, HS^- has a higher affinity towards metalloproteins (such as anti-oxidant enzymes) compared with H_2S (Searcy *et al.*, 1995; Haouzi and Klingerman, 2013); HS^- acts as a very powerful nucleophile towards the oxidants (Li and Lancaster, 2013). Recent studies have shown that hydrogen sulphide and reactive oxygen species (ROS) signalling systems are integrated (Predmore *et al.*, 2012). This integration appears to be important in the regulation of the mechanism that underlies myometrial contractility, as ion channels, particularly the –SH groups exposed in the channel structure, are highly sensitive to ROS (Paulsen and Carroll, 2013). ROS has been shown to have a role in the regulation of uterine contractility (Appiah *et al.*, 2009). Taken together, H_2S/HS^-–ROS crosstalk might result in new ways of modulating ion channels. Therefore, determining the activity of enzymes that regulate the production or degradation of ROS is important when assessing the effects of hydrogen sulphide.

To the best of our knowledge, the mechanism of the Na_2S-induced relaxation in non-pregnant uteri has not been examined. Hence, we investigated the involvement of ion channels in the relaxation of rat uteri induced by derivatives of H_2S/HS^-. We measured the activity of antioxidant enzymes with the aim of elucidating whether different components of the relaxation are redox-sensitive and whether

the relaxation occurs as a consequence of disturbances in the redox homeostasis.

Methods

Experimental model

All protocols for handling rats were approved by the Local Animal Care Committee of the Institute for Biological Research (Belgrade, Serbia). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Virgin female Wistar rats (~225 g, 10–12 weeks of age) were used in these experiments. The animals were kept under standard laboratory conditions (12 h light, 12 h dark and 21 ± 2°C). All rats were housed in individual cages and given standard diet and tap water *ad libitum*. Female rats were examined for their stage in the oestrus cycle, determined by a daily vaginal lavage (Marcondes *et al.*, 2002), and then killed by decapitation. In brief, the uterine horns were rapidly excised and carefully cleaned of all fat and surrounding connective tissue, rinsed in De Jalon's solution and used immediately.

Isolated organ bath studies

Uteri were mounted separately in 10 mL organ chambers with one end tied to a tissue holder and the other to a wire connected to a force transducer (Experimetria, Budapest, Hungary). The chambers contained De Jalon's solution maintained at 37°C and aerated with a gas mixture of 95% oxygen and 5% carbon dioxide. Uteri were allowed to stabilize for 1 h, at 1 g tension by imposing a resting tension, until a stable resting tone was acquired. Changes in isometric force were recorded on a TSZ-04-E Tissue Bath System (Experimetria).

Three different types of experimental protocols were employed in this study. In the first protocol, spontaneously active uteri were used. In the second protocol, uteri were pre-contracted with 4.9 mM $CaCl_2$ (concentration was predetermined in preliminary experiments). Concentration-response curves to 20–200 μM Na_2S were obtained by adding the agent directly to the organ bath. All experiments involved obtaining a single concentration–response curve to Na_2S in a single uterine preparation. In each experiment, appropriate controls were run under the same experimental conditions using uterus horns obtained from the same rat (the total duration of each experiment did not exceed 2 h after uteri stabilization). These responses were used to normalize the tissue response from experiment to experiment.

In the third protocol, the effects of Na_2S were examined in the presence of raised KCl (15 and 75 mM). A depolarizing solution (KCl 75 mM) caused a rapid contraction, followed by a slight relaxation and prolonged contraction plateau. Additionally, uteri were pre-contracted with 15 mM KCl, which caused phasic contractions. Once a stable contraction was obtained, a concentration-response curve to Na_2S was generated by adding the agent cumulatively.

The relaxation response to cumulative concentrations of the H_2S donor, Na_2S (20–200 mM) was examined on both spontaneous and calcium-induced contractions in the presence of the following: 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS, 100 μM, an inhibitor of chloride channels and chloride-bicarbonate (Cl^-/HCO_3^-), 2-[(5-ethylt-1,6-tdihydro-4-methyl-6-oxo-2-pyrimidinyl)thio]-N-[4-(4-methoxyphenyl)-2-thiazolyl]-acetamide (T16Ainh-AO1, 5 μM, an inhibitor of TMEM16A chloride channels), tannic acid (TA, 50 μM, Ca^{2+}-activated chloride channel blocker), niflumic acid (NFA 10 μM, Ca^{2+}-activated chloride channel blocker), *R*(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5yl)-oxy]acetic acid 94 (IAA-94, 25 μM, CLC1 channel blocker), glibenclamide (2 μM, an inhibitor of K_{ATP} channel), tetraethylammonium (TEA, 10 mM, non-selective K^+ channel blocker), 4-aminopyridine (4-AP, 1 mM, voltage-dependent K^+ (K_v) channel blocker), (4*S*)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-trifluoromethyl) phenyl]-3-pyridinecarboxylic acid methyl ester (S-Bay K 8644, 0.1 μM, L-type Ca^{2+} channel activator), propranolol (1 μM, an inhibitor of β-adrenoceptors), methylene blue (1 μM, an inhibitor of guanylyl cyclase). Bicarbonate-free buffer was used to inhibit bicarbonate exchange aimed to discriminate between chloride channels and exchangers.

Tissue preparation for antioxidant enzyme activity assays

In order to prepare the samples for analytical procedures, thawed uteri (both treated with Na_2S and untreated – controls) were homogenized at 0–4°C in five volumes (w v^{-1}) of ice-cold 0.25 M sucrose, 1 mM EDTA and 0.05 M Tris–HCl buffer, pH 7.4. All procedures were performed on ice. The homogenates were centrifuged for 60 min at 105 000× *g*, 4°C and the resulting supernatants were used for determining total protein concentration and enzyme activities (using a Shimadzu UV-160 spectrophotometer, Shimadzu Scientific Instruments, Kyoto, Japan).

Enzyme assays

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined using the adrenaline method (Misra and Fridovich, 1972) based on the increase in absorbance at 480 nm. This method is based on the capacity of SOD to inhibit the autoxidation of adrenaline to an adrenochrome. The reaction was carried out in 50 mmol·L^{-1} sodium carbonate buffer, pH 10.2, and was initiated by the addition of 0.3 mmol·L^{-1} adrenaline. One unit of SOD was defined as the amount of protein required to halve the rate of substrate auto-oxidation.

Catalase (CAT; EC 1.11.1.6) activity was measured spectrophotometrically, as previously described (Claiborne, 1985). The reaction mixture contained 50 mmol·L^{-1} Tris pH 8, 10 mmol·L^{-1} H_2O_2 and appropriate amounts of samples. The decomposition of H_2O_2 was monitored continuously at 240 nm ($\varepsilon_{240\ nm}$ = 0.040 mmol·L^{-1}·cm^{-1}) with a Shimadzu spectrophotometer and only the initial linear rate was used to estimate the CAT activity. The blank did not contain H_2O_2 and the activity was calculated after subtraction of the blank value. The amount of enzyme activity that decomposed 1 mmol of H_2O_2 min^{-1} was defined as one unit of activity. Specific activities were expressed as IU·mg^{-1} protein.

Glutathione peroxidase (GSH-Px; EC 1.11.1.9) activity was measured following the spectrophotometric method of (Paglia and Valentine, 1967) based on the measurement of

NADPH consumption [i.e. NADPH oxidation by glutathione reductase (GR), 500 U·mg^{-1} protein, Sigma, St Louis, MO, USA] at 340 nm. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM GSH, 1 mM sodium azide, 1 IU·mL^{-1} GR from bakers' yeast, 0.2 mM NADPH and 3 mM t-butyl hydroperoxide as substrate and appropriate amounts of sample).The blank did not contain sample and the activity was calculated after subtraction of the blank value. The rate of NADPH oxidation was monitored at 37°C using a Shimadzu spectrophotometer on the basis of the decrease in absorbance at 340 nm ($\varepsilon_{340\ nm}$ = 6.22 mmol·L^{-1}·cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme required to transform 1 nmol of substrate min^{-1} under the above described assay conditions. Specific activities are expressed as IU·mg^{-1} protein.

GR (GSSG-Rx; EC 1.6.4.2) activity was assayed as described previously (Glatzle *et al.*, 1974). This assay is based on NADPH oxidation concomitant with GSSG reduction. The reaction mixture consisted of 0.5 M sodium phosphate buffer (pH 7.5), 0.1 mM EDTA, 0.1 mM NADPH, 0.1 mM GSSG and appropriate amounts of samples. The rate of NADPH oxidation was monitored at 37°C with a Shimadzu spectrophotometer, after the decrease in absorbance at 340 nm ($\varepsilon_{340\ nm}$ = mmol·L^{-1}·cm^{-1}). The blank did not contain GSSG and the activity was calculated after subtraction of the blank value. One unit of enzyme activity was defined as 1 nmol of NADPH oxidized min^{-1} at 25°C. Specific activities are expressed as IU·mg^{-1} protein.

Protein assay

The protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) using BSA as the standard.

Tissue preparation for Western blot analysis assays

Uteri were homogenized at 0–4°C in four volumes (w v^{-1}) of RIPA buffer: (25 mM Tris–HCl pH 7.4, 50 mM NaCl, 1 mM EDTA-Na_2, 1 mM EGTA-Na_2, 2 mM DTT, 1% NP-40, protease and phosphatase inhibitors). The homogenates were centrifuged for 20 min at 16 000× *g*, 4°C and the resulting supernatants were used for determining total protein concentration and Western blot analysis.

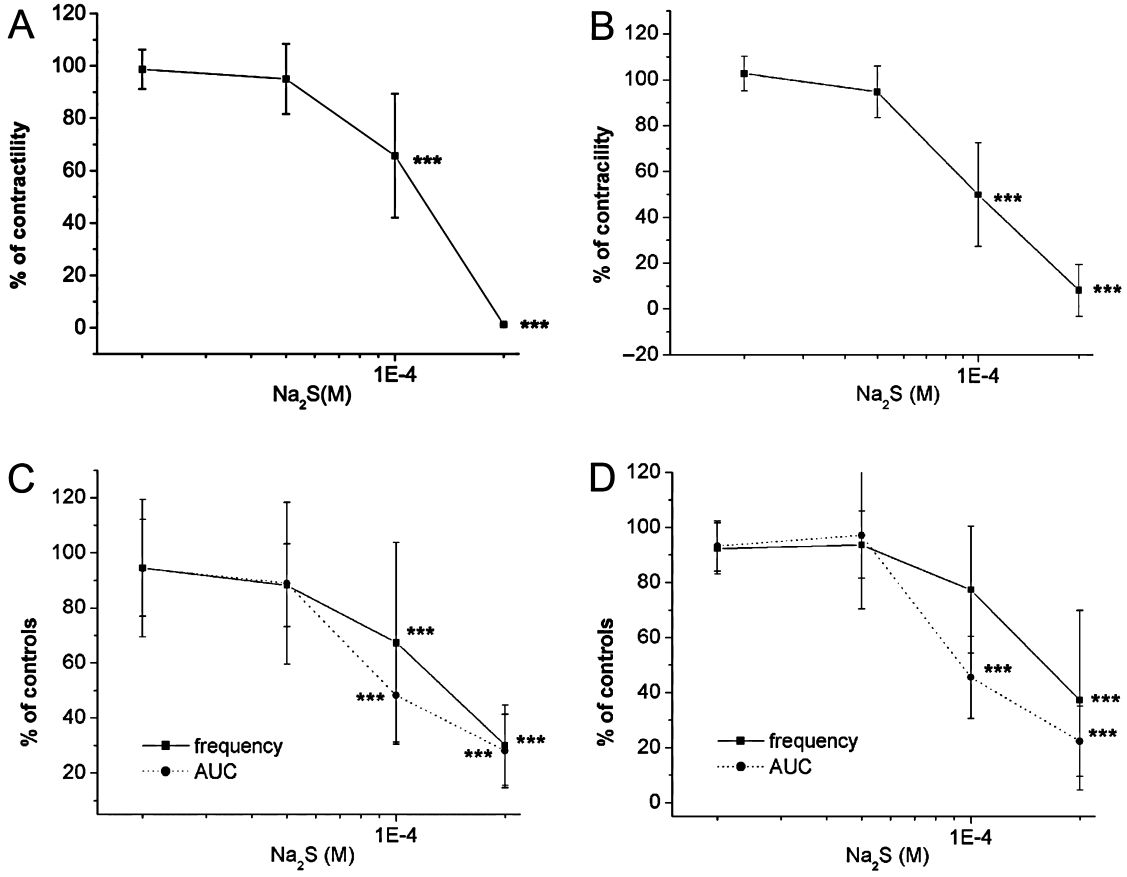

Figure 1

Na_2S-induced relaxation of spontaneously (A) and Ca^{2+}-stimulated (B) contracting rat uteri. The concentration-dependent decrease in frequency and AUC in response to Na_2S of spontaneously (C) and Ca^{2+}-stimulated (D) active rat uteri. Data are presented as mean ± SD (n = 7). Both curves were analysed separately by one-way ANOVA (factor: Na_2S concentration; $P < 0.05$ was considered as significant) and *post hoc* compared using Tukey's HSD test (***$P < 0.001$).

Western blot analysis

Proteins were solubilized in Laemmle's sample buffer, subjected to 7.5% SDS-PAGE along with molecular mass references (10–170 kDa, Fermentas, Hanover, MD, USA). After electrophoresis, proteins were transferred onto a PVDF membrane (Immobilon-FL, Millipore, Billerica, MA, USA) using a blot system (Transblot, BioRad, Hercules, CA, USA). Membranes were blocked for 1 h with 2% non-fat dry milk dissolved in PBS at room temperature and incubated overnight at 4°C with anti-BEST-1 primary antibody (CAT, 1:2000, Alomone Laboratories, Jerusalem, Israel). Anti-β-actin antibody (AC-15, 1:5000, Sigma-Aldrich, St Louis, MO, USA) was used as an equal loading control. After being washed, blots were incubated with HRP-linked secondary antibody (1:5000, Santa Cruz, Santa Cruz, CA, USA) for 1 h at room temperature. Blots were developed using a chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

RNA isolation and reverse transcription

Total RNA was extracted from the uterus of each animal using TRIzol (Life Technologies, Grand Island, NY, USA). The RNA was quantified spectrophotometrically by reading the optical density at 260 and 280 nm (OD 260/280 > 1.8 was considered satisfactory). Complementary DNA was synthesized (3 µg total RNA in a final volume of 10 µL) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's recommendations.

Real-time PCR

PCR amplification of cDNA was performed in a real-time PCR machine ABI Prism 7000 (Applied Biosystems) with SYBR-Green PCR master mix (Applied Biosystems) as indicated: 2 min at 50°C for dUTP activation, 10 min at 95°C for initial denaturation of cDNA, followed by 40 cycles each consisting

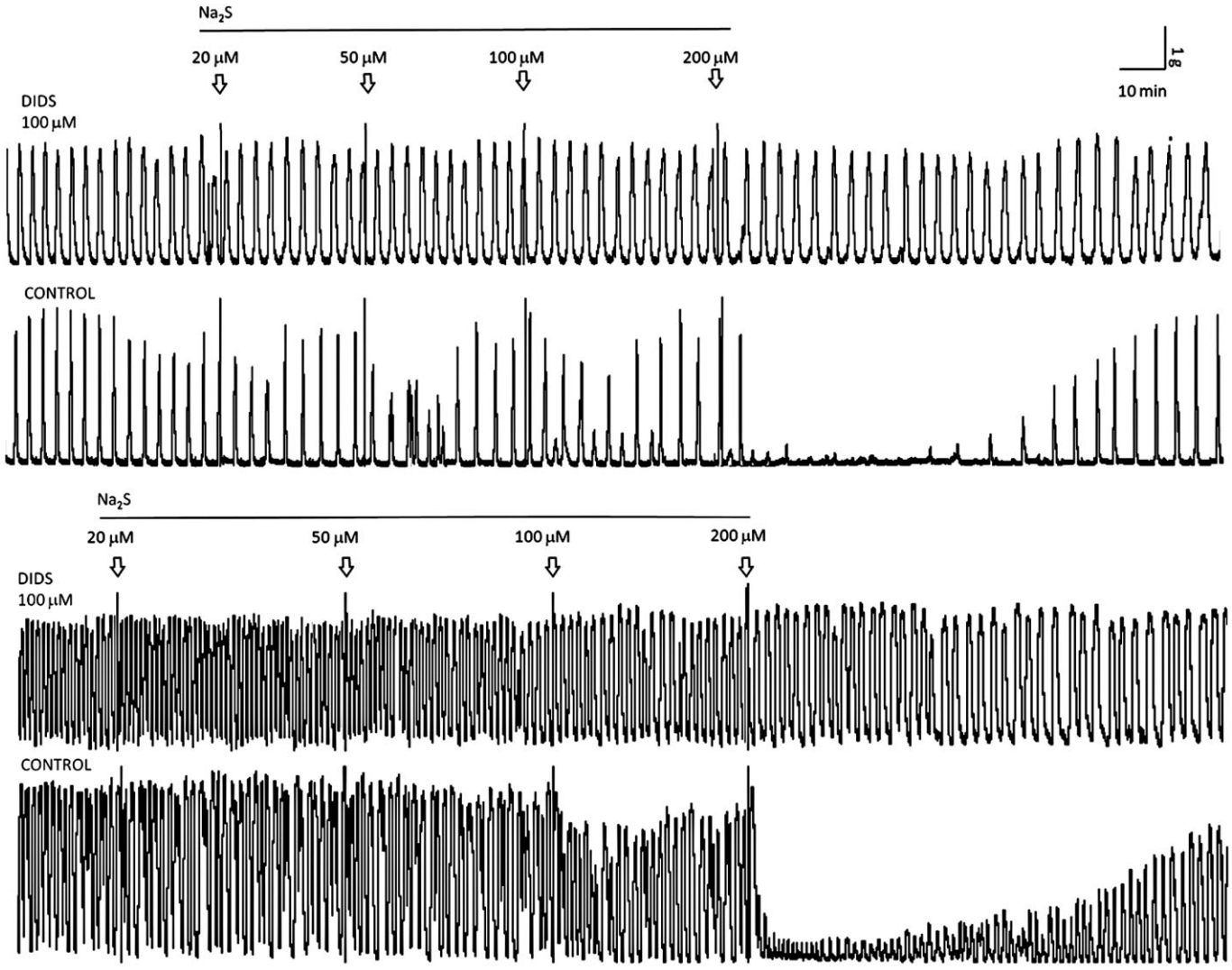

Figure 2

Representative isometric recordings of effects of Na_2S on spontaneously contracting rat uteri (upper) and after stimulation with Ca^{2+} (lower) with DIDS (100 µM) pretreatment and without (control; n = 6–10).

of denaturation at 95°C for 15 s, annealing and extension at 60°C for 60 s. The primer sequences used were purchased from Invitrogen (Invitrogen, Life Technologies): BEST-1 forward 5′-gcaccattggacgcttctta; BEST-1 reverse 5′- actgctgaggagggaagacc. PCR amplification was performed in triplicate. Relative expression was determined using the formula $2^{-(Cti-Cta)}$, where Cti is the cycle threshold value of the gene of interest and Cta was the cycle threshold value of endogenous control (GAPDH, actin, cyclophilin A and 18S). RNA data are presented as average relative levels versus endogenous control, A ± SD. The results were analysed by Sequence Detection Software version 1.2.3 for 7000 System SDS Software RQ Study Application (Applied Biosystems) with a confidence level of 95% ($P \leq 0.05$).

Materials

De Jalon's solution contained (in mM): NaCl 154, KCl 5.64, $NaHCO_3$ 5.95, $CaCl_2$ 0.41 and glucose 2.77. The composition of bicarbonate free De Jalon's buffer was the same as the De Jalon's solution, except $NaHCO_3$ was replaced with HEPES (10 mM) and NaCl increased to maintain osmolarity. H_2S/HS^- was produced in solution using Na_2S. All chemicals were obtained from Sigma-Aldrich unless otherwise stated. IAA-94 and NFA were obtained from Tocris (Abingdon, UK); S-Bay K from Alomone Laboratories, Jerusalem, Israel. All chemicals were dissolved in distilled water except for glibenclamide, which was dissolved in polyethylene glycol; T16Ainh-A01, IAA-94, NFA and S-Bay K 8644 were dissolved in DMSO.

Data analysis

Statistical analysis (descriptive statistics, ANOVA) was performed according to protocols described by Hinkle *et al.* (1994) and Manley (1986). The effects of treatments on uterine contractions were calculated as the percentage of control, untreated, contractions. All data are expressed as the mean ± SEM or SD (type of the error presented is indicated in each figure legend). Differences between groups were tested by factorial ANOVA on logarithmic-transformed data and considered statistically significant when $P < 0.05$. The activity of antioxidant enzymes was compared using one-way ANOVA followed by a Tukey's HSD *post hoc* test.

Results

Effects of Na_2S on isolated uteri

Na_2S caused a reversible concentration-dependent relaxation of spontaneous and Ca^{2+}-induced contractions of rat isolated uteri (Figure 1), which was measured based on a decrease in amplitude, frequency and AUC (Figure 1). The reduction in frequency and AUC caused by Na_2S was statistically significant at a concentration of 200 μM (significant ANOVA effect of concentration and *post hoc* comparison by Tukey's HSD test; Figure 1). Representative tracings are shown in Figure 2.

It was observed that the relaxant effects of Na_2S in the presence of a raised extracellular KCl (Figure 3) differed, being higher when strips were pre-contracted with a lower (15 mM) concentration of KCl compared with a higher (75 mM) concentration of KCl (two-way ANOVA, significant KCl concentration effect, $P < 0.001$). Total relaxation of contractions induced by the lower KCl concentration was obtained with 100 μM Na_2S. However, 100 and 200 μM Na_2S also induced a statistically significant relaxation of contractions induced by the higher KCl concentration.

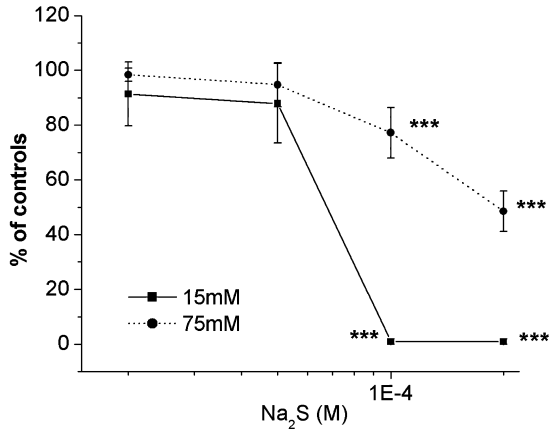

Figure 3

The relaxant effect of Na_2S on rat uteri precontracted with 15 or 75 mM KCl. Data are presented as mean ± SD ($n = 7$). Both curves were analysed separately by one-way ANOVA (factor: Na_2S concentration; $P < 0.05$ was considered significant) and *post hoc* compared using Tukey's HSD test (***$P < 0.001$).

Involvement of Cl^- channels in the relaxation of spontaneously active and Ca^{2+}-stimulated rat uteri

Pretreatment with DIDS completely abolished any relaxant effect of Na_2S on spontaneously active uteri (Figure 4A). DIDS also abolished the relaxation induced by Na_2S of Ca^{2+}-stimulated active uteri (Figure 5A). In order to investigate the role of the Cl^-/HCO_3^- exchange, an HCO_3^--free solution was used, but it had no effect on the Na_2S-induced relaxation (Figures 4B and 5B). Pretreatment of spontaneously active uteri with T16Ainh-AO1 and TA potentiated the relaxant effect of Na_2S (Figure 4C and D) but was without effect in the Ca^{2+}-stimulated uteri (Figure 5C and D). Pretreatment of spontaneously active uteri with NFA also potentiated the relaxant effect of Na_2S, lowering the concentration required for relaxation (ANOVA effect of treatment $P < 0.001$; Figure 4E). In contrast, in Ca^{2+}-stimulated active uteri, pretreatment with NFA had no effect (Figure 5E). Pretreatment with IAA-94 had no effect on Na_2S-induced relaxation in either spontaneous or Ca^{2+}-stimulated active uteri (Figures 4F and 5F).

Involvement of K^+ channels in relaxation of spontaneously active and Ca^{2+}-stimulated rat uteri

Pretreatment of spontaneously active uteri with glibenclamide or 4-AP had no effect (Figure 6A and C), whereas TEA increased the relaxant effect of Na_2S (significant ANOVA treatment effect, $P < 0.001$; *post hoc* Tukey's HSD test, $P < 0.001$) (Figure 6B). In contrast, pretreatment of Ca^{2+}-stimulated active uteri with glibenclamide attenuated the Na_2S (200 μM)-mediated relaxation (significant ANOVA treatment $P < 0.01$, *post hoc* Tukey's test, $P < 0.001$) (Figure 7A). Pretreatment of Ca^{2+}-stimulated active uteri with TEA and 4-AP had no effect on the Na_2S-mediated relaxation (Figure 7B and C).

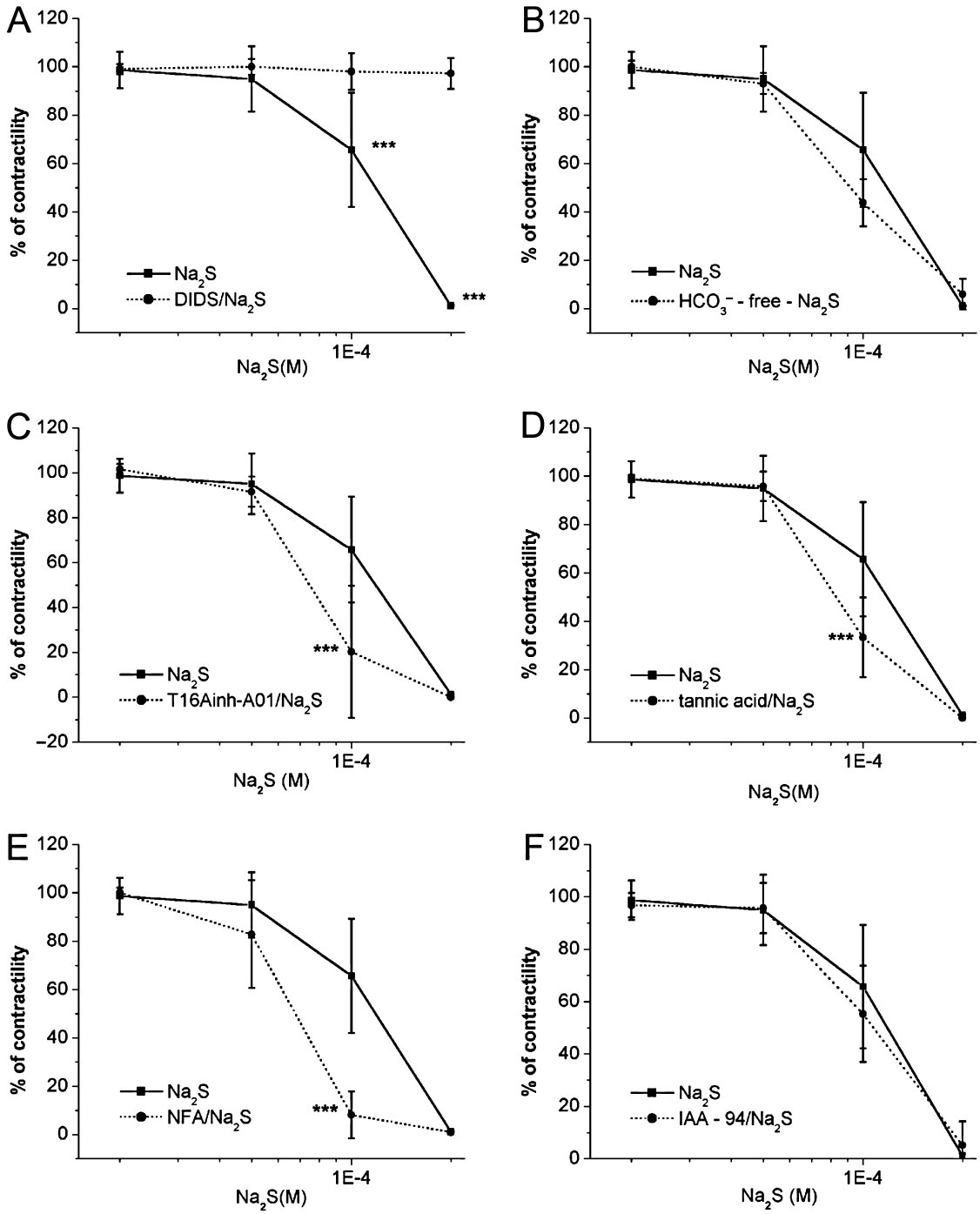

Figure 4

The effects of Na_2S on spontaneously active rat uteri in the absence and presence of (A) DIDS (100 μM), (B) HCO_3^-, (C) $T16A_{inh}$-A01 (5 μM), (D) TA (50 μM), (E) NFA (10 μM) and (F) IAA-94 (25 μM). Data are presented as mean ± SD ($n = 7–10$). Data were analysed by two-way ANOVA (factors: Na_2S concentration and pretreatment) and *post hoc* compared using Tukey's HSD test. Only statistically significant effects between pretreatment and Na_2S are shown; ***$P < 0.001$.

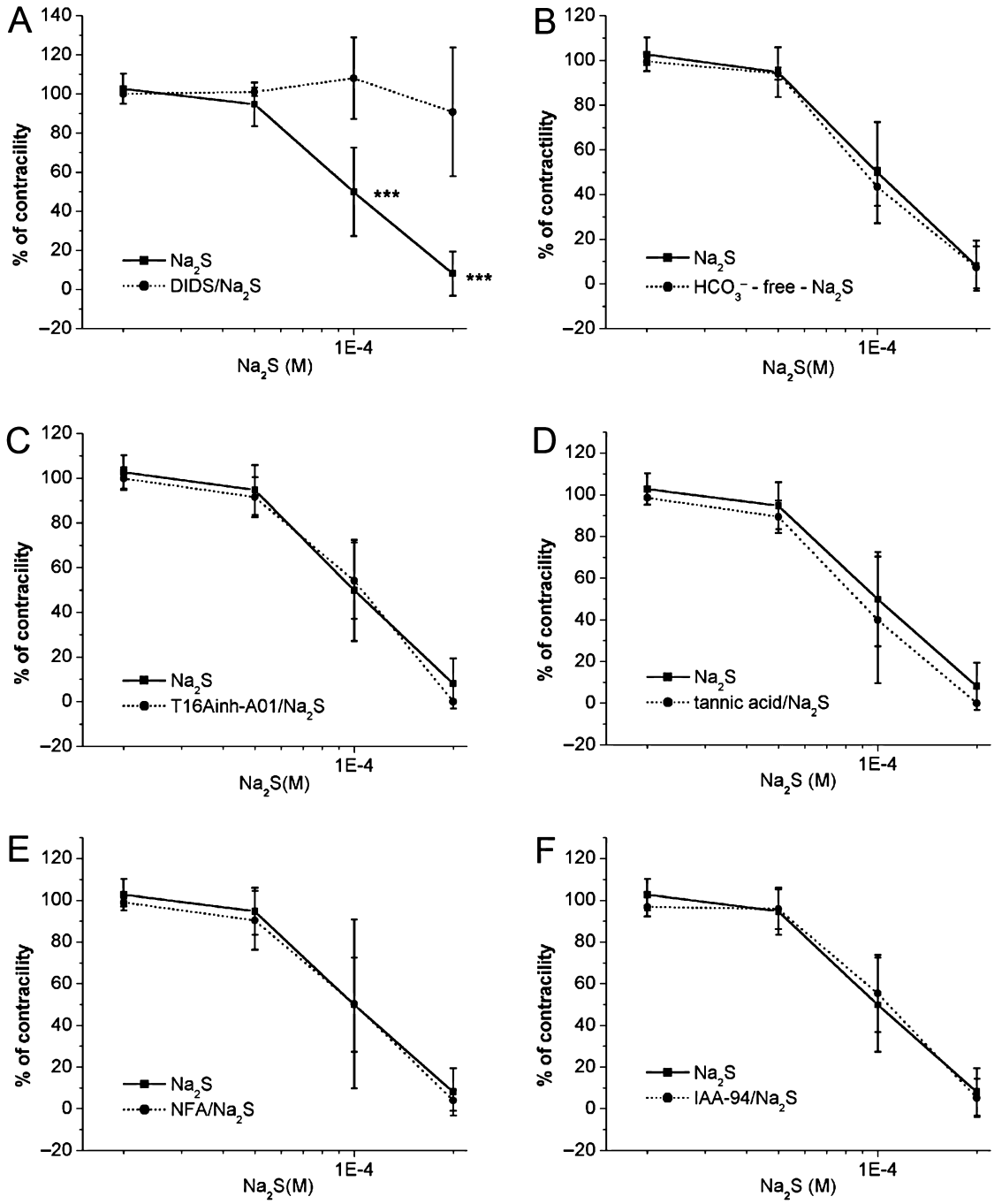

Figure 5

The effects of Na_2S on Ca^{2+}-stimulated rat uteri in the absence and presence of (A) DIDS (100 μM), (B) HCO_3^-, (C) $T16A_{inh}$-A01 (5 μM), (D) TA (50 μM), (E) NFA (10 μM), and (F) IAA-94 (25 μM). Data are presented as mean ± SD (n = 7–10). Data were analysed by two-way ANOVA (factors: Na_2S concentration and pretreatment) and *post hoc* compared using Tukey's HSD test. Only statistically significant effects between pretreatment and Na_2S are shown; *P < 0.05, **P < 0.01, ***P < 0.001.

Involvement of L-type Ca^{2+} channels in relaxation of spontaneously active and Ca^{2+}-stimulated rat uteri

S-Bay K 8644 pretreatment had no effect on the relaxant effect of Na_2S on both types of active uteri. (Figures 6D and 7D).

Effect of Na_2S on spontaneously active and Ca^{2+}-stimulated rat uteri in the presence of propranolol and methylene blue

Pretreatment with methylene blue increased the relaxation to Na_2S of spontaneously active uteri, but the concentration

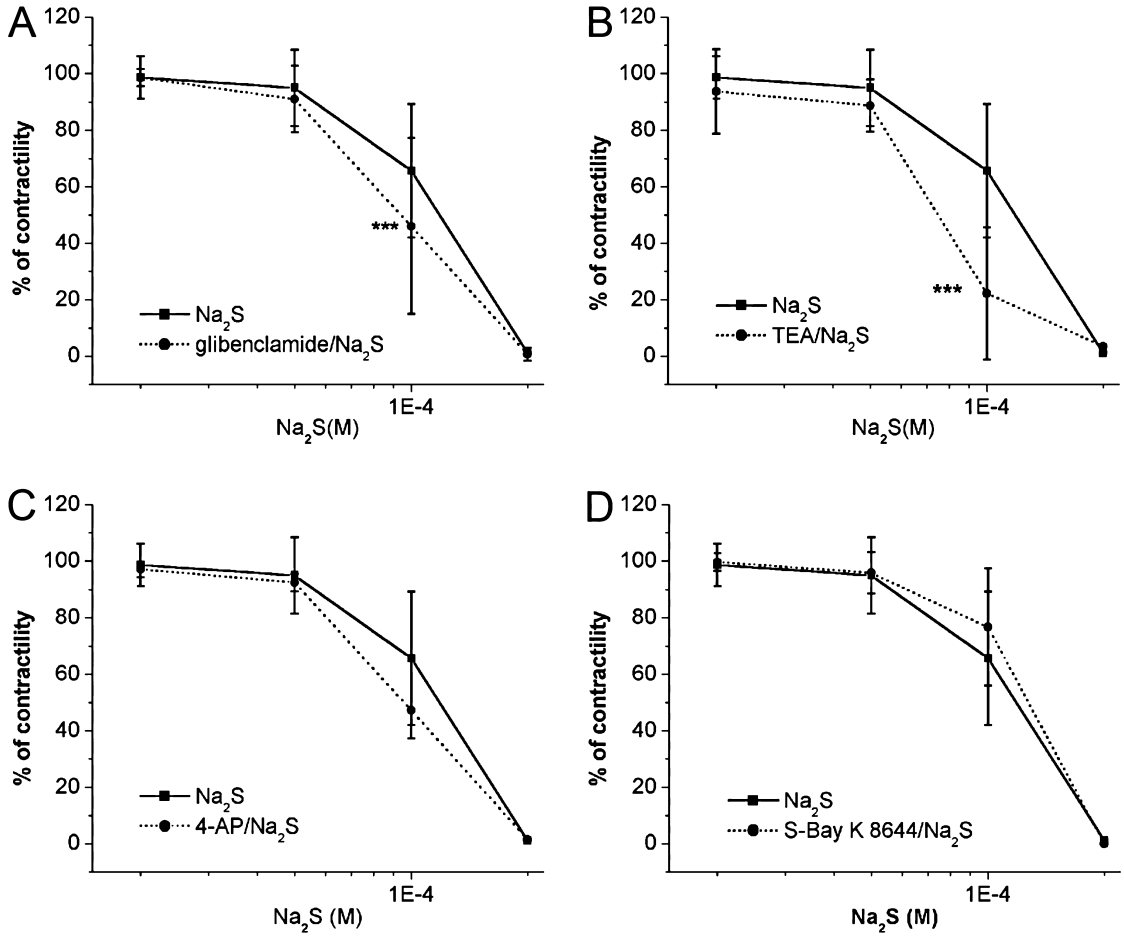

Figure 6

The effects of Na_2S on spontaneously active rat uteri in the absence and presence of (A) glibenclamide (2 μM), (B) TEA (10 mM), (C) 4-AP (1 mM) and (D) S-Bay K 8644 (0.1 μM). Data are presented as mean ± SD (n = 7–10). Data were analysed by two-way ANOVA (factors: Na_2S concentration and pretreatment) and *post hoc* compared using Tukey's HSD test. Only statistically significant effects between pretreatment and Na_2S are shown; *P < 0.05, **P < 0.01, ***P < 0.001.

required for complete relaxation was the same (ANOVA effect of treatment P < 0.001, *post hoc* Tukey's test, P < 0.001; Figure 8A). Pretreatment with propranolol had no effect on Na_2S-mediated relaxation of spontaneously active uteri (Figure 8B). In contrast, pretreatment of Ca^{2+}-stimulated active uteri with methylene blue reduced the concentration required for complete relaxation to Na_2S (significant ANOVApretreatment P < 0.05 effect, *post hoc* Tukey's test, P < 0.001; Figure 8C). Propranolol had no effect on the uterine relaxation of Ca^{2+}-stimulated active uteri induced by Na_2S (Figure 8D).

BEST-1 expression in rat uteri by RT-PCR and Western blot

The expression of BEST-1 channels was confirmed both at mRNA level (Figure 9A) and protein level (Figure 9B) in a non-pregnant rat uterus in oestrus.

Effect of Na_2S on the activity of antioxidant enzymes in spontaneously and Ca^{2+}-induced active rat uteri

Treatment of uteri with Na_2S led to changes in the activity of antioxidant enzymes, but the intensity and the quality of the changes depended on the type of activation (spontaneous or Ca^{2+}-mediated) and pretreatment. In spontaneously active uteri, pretreatment with MB led to a statistically significant decrease in SOD (P < 0.05), CAT, GSH-Px and GR activities after the addition of Na_2S compared with Na_2S controls. Uteri that were treated with glibenclamide before treatment with Na_2S had lower SOD and GSH-Px activities compared with those of Na_2S controls. When uteri were pretreated with propranolol, there was a decrease in CAT (P < 0.05) and GSH-Px activity after Na_2S application. Pretreatment with DIDS had no effect (Figure 10). In Ca^{2+}-stimulated uteri, pretreatment with MB led to a decrease in SOD, GSH-Px (P < 0.05) and GR

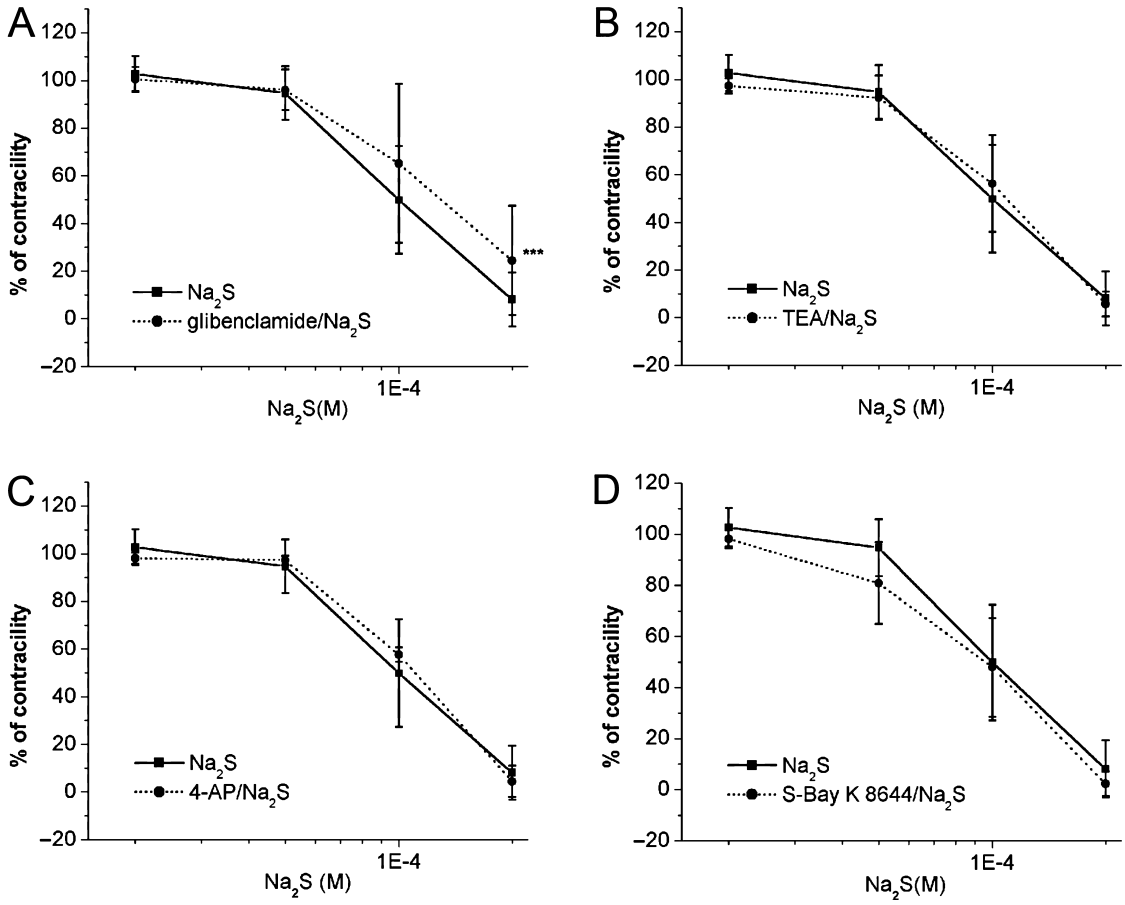

Figure 7

The effects of Na_2S on Ca^{2+}-stimulated rat uteri in the absence and presence of (A) glibenclamide (2 μM), (B) TEA (10 mM), (C) 4-AP (1 mM) and (D) S-Bay K 8644 (0.1 μM). Data are presented as mean ± SD (n = 7–10). Data were analysed by two-way ANOVA (factors: Na_2S concentration and pretreatment) and *post hoc* compared using Tukey's HSD test. Only statistically significant effects between pretreatment and Na_2S are shown; *P < 0.05, **P < 0.01, ***P < 0.001.

activities. Compared with the Na_2S control, pretreatment with propranolol slightly lowered only CAT (P < 0.05) activity. If uteri were pretreated with glibenclamide, lower SOD and CAT activities (P < 0.05) were recorded after the addition of Na_2S. Pretreatment with DIDS had no effect (Figure 11).

Discussion

Here, we demonstrated the relaxant effect of Na_2S (H_2S/HS^-) on spontaneous and Ca^{2+}-evoked contractions of non-pregnant rat uteri, giving insights into the mechanism of its action. We found that Na_2S provokes reversible concentration-dependent relaxation via a DIDS-sensitive Cl^- channel, which affects both the amplitude and the frequency of contractions. Its effect on the frequency of contractions appears to be mediated via pacemaker channels. CaCC channel inhibitors reduce the frequency of spontaneous contractions in myometrial strips (Jones *et al.*, 2004) and were proposed to be the main pacemaker channels in smooth muscles (Sanders *et al.*, 2012). CaCC channels are the main conducive pathway for Cl^- in myometrium, and have been proposed to contribute to membrane potential and the firing frequency of pacemakers cells (Wray *et al.*, 2015). A number of candidate proteins have been proposed to form CaCC, but only two families, BEST and TMEM proteins, replicate the properties of native CaCC in expression systems (Liu *et al.*, 2014). We showed that BEST-1 is expressed at the mRNA level in rat myometrium. Additionally, BEST-1 channels are expressed at the protein level in rat uterus during oestrus, suggesting a role for BEST-1 in the control of uterine contractility. A recent study found that DIDS is highly selective for BEST-1 compared with TMEM16A (ANO-1) channels (Liu *et al.*, 2014). Our data showed that CaCC channel inhibitors that target TMEM–T16Ainh-AO1, tannic acid and NFA fail to inhibit Na_2S-induced relaxation. It has been shown that T16Ainh-AO1 partially inhibits TMEM16A-mediated currents but has no

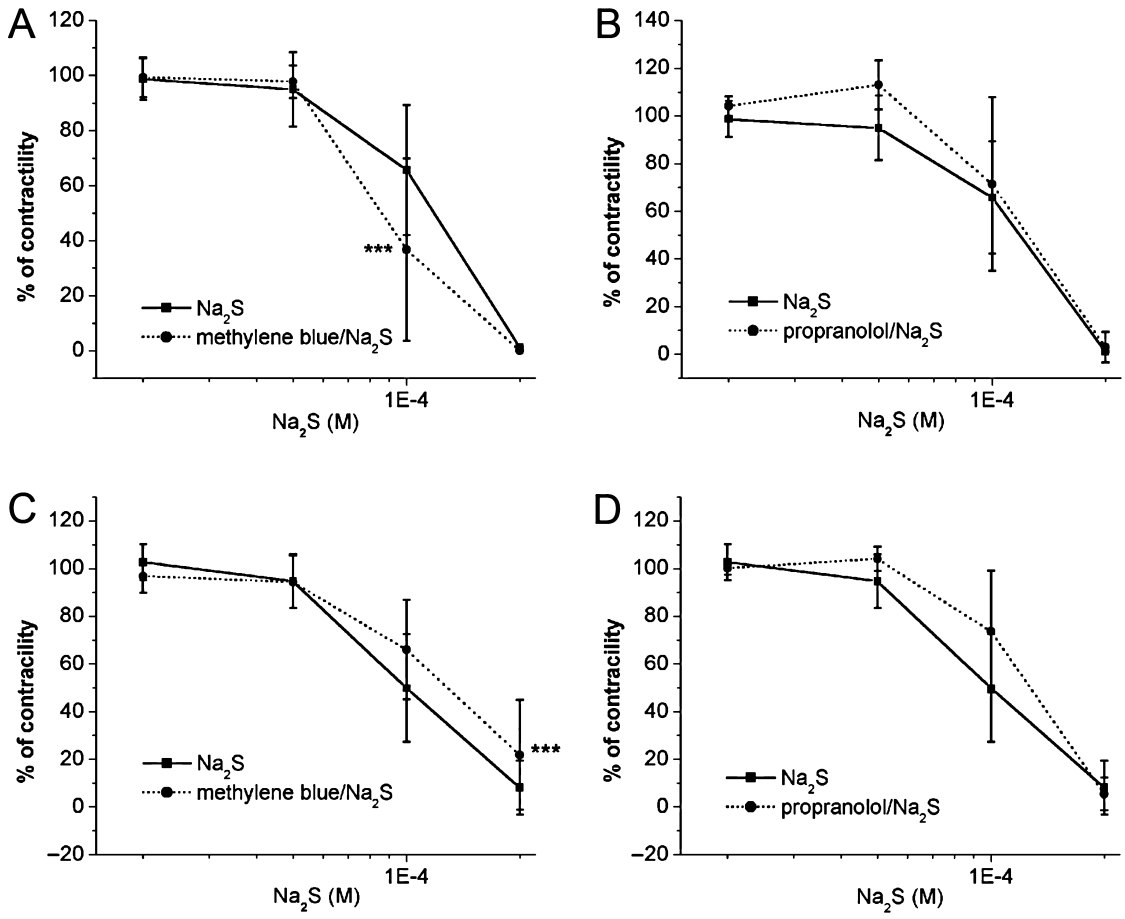

Figure 8

The effects of Na_2S on spontaneously active rat uteri in the absence and presence of (A) methylene blue (1 μM) and (B) propranolol (1 μM); and on Ca^{2+}-stimulated rat uteri in the absence and presence of (C) methylene blue (1 μM) and (D) propranolol (1 μM). Data are represented as mean ± SD (n = 7–10). Data were analysed by two-way ANOVA (factors: Na_2S concentration and pretreatment) and *post hoc* compared using Tukey's HSD test. Only statistically significant effects between pretreatment and Na_2S are shown; *P < 0.05, **P < 0.01, ***P < 0.001.

effect on those mediated by BEST-1 (Liu *et al.*, 2014). Furthermore, NFA is less potent at inhibiting BEST-1 currents as compared with its effect on TMEM16A (Liu *et al.*, 2014). It is worth mentioning that intracellular chloride channels have been recognized previously as mediators of hydrogen sulphide's effects (Malekova *et al.*, 2009). We found that the intracellular channel (CLIC1) blocker IAA-94 did not affect the relaxant response to Na_2S of calcium-induced contractions. In line with our results, it has been shown that DIDS completely abolishes responses to Na_2S in the small mesenteric rat artery (White *et al.*, 2013). It has been speculated that the vasorelaxant effects of H_2S occur via the DIDS-sensitive Cl^-/HCO_3^- exchanger (Lee *et al.*, 2007). In order to investigate the role of Cl^-/HCO_3^- exchange, an HCO_3^--free solution was applied but it had no effect on the Na_2S-induced relaxation, which is in agreement with the findings of a recent study (Streeter *et al.*, 2012). Interestingly, BEST-1 channels were shown to be highly permeable to HCO_3^- anions (Qu and Hartzell, 2008).

The hydrogen sulphide anion HS^- accounts for 80% of the total hydrogen sulphide in conditions of physiological pH. Dombkowski and co-authors have proposed that HS^- can interfere with a Cl^- channel or transporter, and have noted that the effect is diminished when transmembrane Cl^- gradients are changed by using high concentrations of KCl (Dombkowski *et al.*, 2006). In line with this, the relaxant effects observed here were more pronounced on phasic contractions induced by 15 mM KCl compared with tonic contractions in high KCl (75 mM). Different effects of the two KCl concentrations also imply that an interaction between Cl^- and HS^- might have taken place.

On the other hand, BEST-mediated currents appear to be essential for phasic but not for tonic contractile responses of rat mesenteric arteries (Broegger *et al.*, 2011).

The potential involvement of K^+ channels in the mechanisms of hydrogen sulphide-induced relaxation was examined using a set of three inhibitors. The failure of TEA and 4-AP to affect relaxation implies that K^+ channels in general

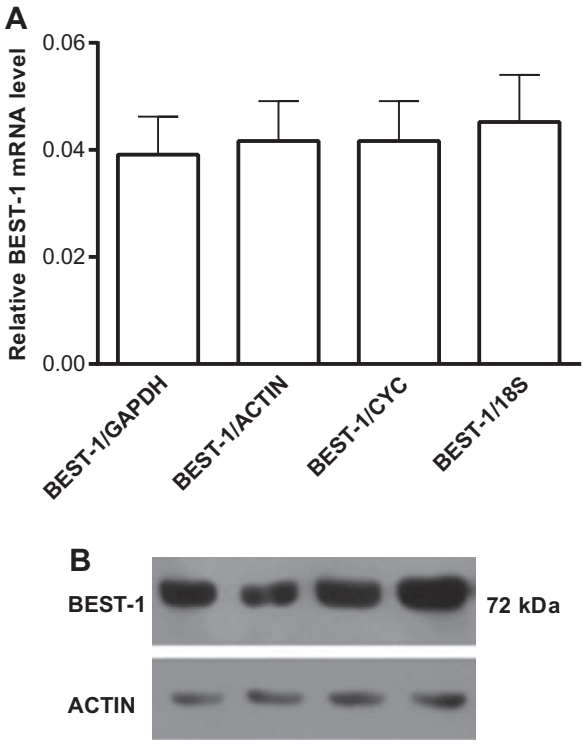

Figure 9

(A) RT-PCR data showing quantitative evidence of BEST-1 in rat uterus in oestrus compared with endogenous controls (GAPDH, actin, cyclin A and 18S). (B) Representative Western blot demonstrating bands (four different animals) of expected molecular mass (72 kDa) for the BEST-1 channel expressed in rat uterus in oestrus. Presence of ß-actin was used to illustrate comparable sample loading.

and more specifically, K_v channels were not involved. Uterine relaxation induced by GYY4137 (a slow-releasing, H_2S-generating compound) was shown to be mediated via K_{ATP} channels (Robinson and Wray, 2012). Our study showed that K_{ATP} channels were of limited importance since glibenclamide decreased Na_2S-induced relaxation only in Ca^{2+}-activated uteri at 200 μM, suggesting that the effects might be dependent on the modulation of Ca^{2+} mobilization and/or direct inhibition of Ca^{2+} entry, but this requires further clarification. It is important to note that L-type Ca^{2+} channels appear to be involved in myometrial relaxation induced by GYY4137 (slow-releasing, H_2S-generating compound) (Robinson and Wray, 2012). Our results showed that the L-type calcium channel activator S-Bay K failed to affect Na_2S-induced relaxation. The effects of H_2S appear to result from cysteine residue modification, S-sulfhydration of proteins such as K_{ATP} channels and L-type channels (Mustafa *et al.*, 2009; 2011; Zhang *et al.*, 2012). It is highly unlikely that S-sulfhydration occurs as a consequence of H_2S-mediated disulfide reduction since H_2S is expected to be a very weak reductant (Greiner *et al.*, 2013). Therefore, it was proposed that polysulphides as oxidizing species might be possible mediators of H_2S-induced S-sulfhydration (Greiner *et al.*, 2013). Diverse effects of slow and fast H_2S-releasing agents might be related to different rates of H_2S-derived species such as polysulphides. Interestingly, wild-type BEST-1 is inactivated by sulfhydryl-reactive agents, whereas BEST-1 mutant with point mutation on its regulatory cysteine residue is resistant to such inactivation (Sun *et al.*, 2002). BEST-1 appears to function as a regulator of voltage-dependent L-type Ca^{2+} channels (Reichhart *et al.*, 2010) possibly thorough a direct physical interaction with the β3- and β4-subunits of Ca^{2+} channels (Milenkovic *et al.*, 2011). It was proposed that BEST-1 functions as an intracellular Cl^- channel, which helps to accumulate and release Ca^{2+} from stores by conducting the counter ion for Ca^{2+} (Strauss *et al.*, 2014). Furthermore, its combined function as a Ca^{2+}-dependent anion channel and Ca^{2+} channel regulator would provide an efficient feedback loop to control Ca^{2+}-dependent Cl^- transport (Milenkovic *et al.*, 2011). This might explain why L-type channels were highlighted as targets for the effects of H_2S in smooth muscle (Streeter *et al.*, 2012). On the other hand, hydrogen sulphide modulates Ca^{2+} events originating from endoplasmic reticulum (ER: Kida *et al.*, 2013). Moreover, it was shown to reduce the intracellular Ca^{2+} transients that underlie spontaneous myometrium contractions (Robinson and Wray, 2012). Hydrogen sulphide's effects on intracellular Ca^{2+} were inhibited in the presence of the sGC inhibitor (Moustafa and Habara, 2014). Additionally, cAMP and cGMP were shown to be important in the regulation of uterine contractility (Aguilar and Mitchell, 2010). We found that the β-adrenoceptor signalling pathway was not operative in Na_2S-induced rat uterine relaxation, since propranolol did not antagonize the effect of Na_2S on either type of activation. We observed that methylene blue reduced Na_2S-induced relaxation, similar to glibenclamide, only in Ca^{2+}-activated uteri at 200 μM. These findings suggest that NO may modulate sulphide's action on uterus contraction. Recently, it was shown that powerful inhibitory effect of sulphide on rat uterus is unaffected by the presence of polysulphides but that it is diminished by interaction with NO donors such as S-nitrosoglutathione (GSNO; Berenyiova *et al.*, 2015). The NO donor SNP prevented H_2S-induced relaxation of rat uterus (Filipovic *et al.*, 2013). It is worth mentioning that DIDS-sensitive BEST channels are activated by NO, since DIDS completely inhibits GSNO-activated Cl^- conductance (Duta *et al.*, 2006).

Kunzelmann and co-workers have shown that endogenous BEST-1 primarily resides in the ER (Kunzelmann *et al.*, 2007). BEST channels appear to be regulated by ER stress and ROS-Ca^{2+} crosstalk (Lee *et al.*, 2012). Our data show that antioxidative enzyme activities from tissues pretreated with DIDS, followed by Na_2S exposure were not different from activities measured in uteri treated only with Na_2S. This implies that there is no feedback from DIDS-sensitive Cl^- channels to the activity of the antioxidant system. However, inhibition of the K_{ATP} channels did affect the antioxidant system. Namely, uteri pretreated with glibenclamide showed decreased SOD activity compared with uteri treated only with Na_2S. Decreased GSH-Px activity was found only in the case of spontaneously active uteri. Our results imply that the limited effects of glibenclamide observed only on calcium-induced contractions might be associated with its

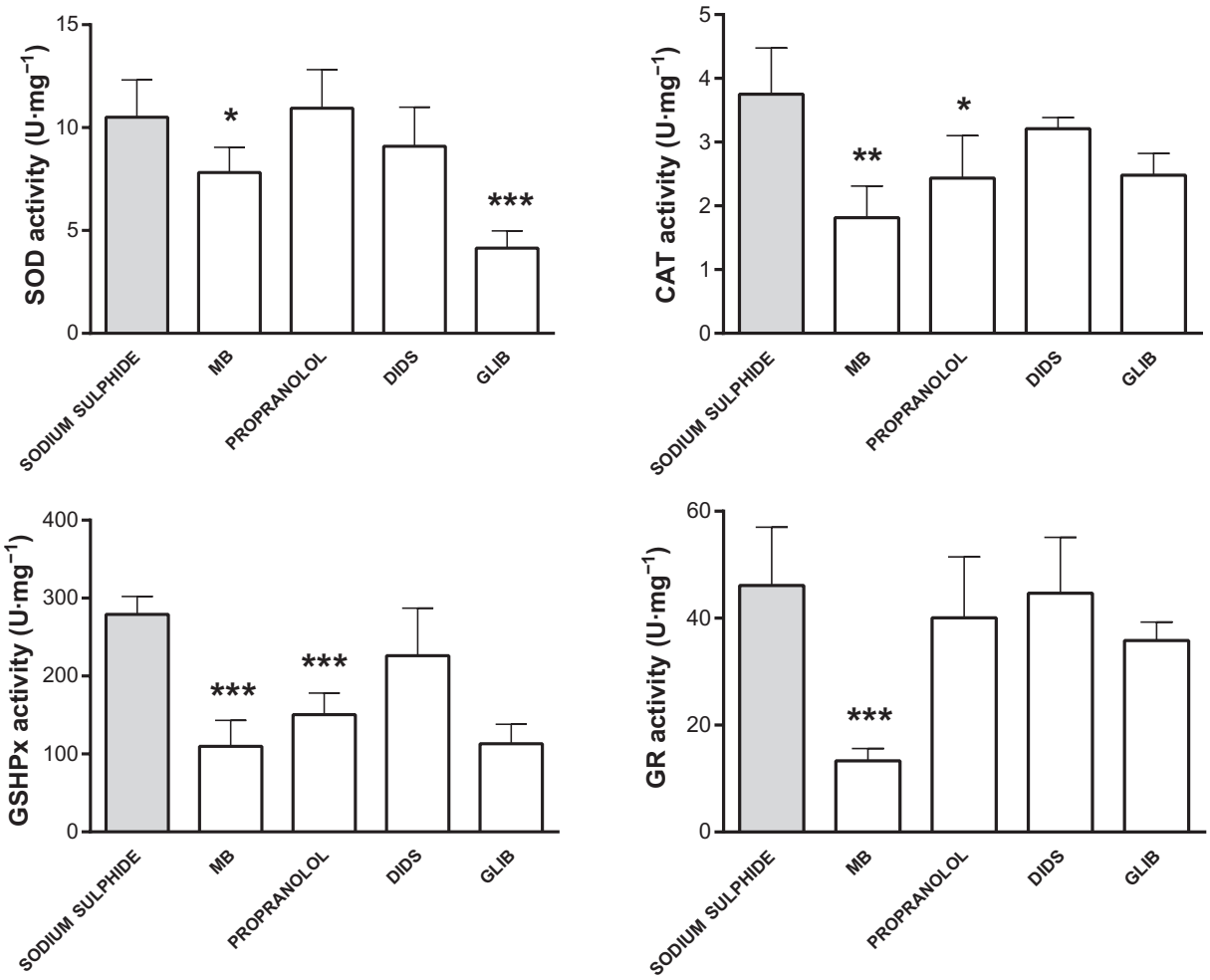

Figure 10

The activity of antioxidant enzymes: SOD, CAT, GSH-Px and GR in spontaneously active uteri treated with Na_2S and pretreated with methylene blue (MB), propranolol, DIDS and glibenclamide. Data are presented as mean ± SD ($n = 7$). Data were analysed by one-way ANOVA and *post hoc* compared using Tukey's HSD test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

ability to inhibit crosstalk between intracellular ROS and Ca^{2+}, probably via its effects on K_{ATP} channels on mitochondria (Li *et al.*, 2014).

In conclusion, DIDS-sensitive CaCC channels are responsible for the majority of the effects of sodium sulphide on non-pregnant rat uteri. The present findings suggest a novel mechanism that offers a better explanation for the effects of hydrogen sulphide, which may help in the design of a suitable therapy for the management of dysfunctional uteri.

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Author contributions

A. M., A. N. K., Z. O. D. and M. S. performed the research. A. M. and M. B. S. designed the study. D. B. analysed the data. A. M., M. B. S. and D. B. wrote the paper.

Conflict of interest

None.

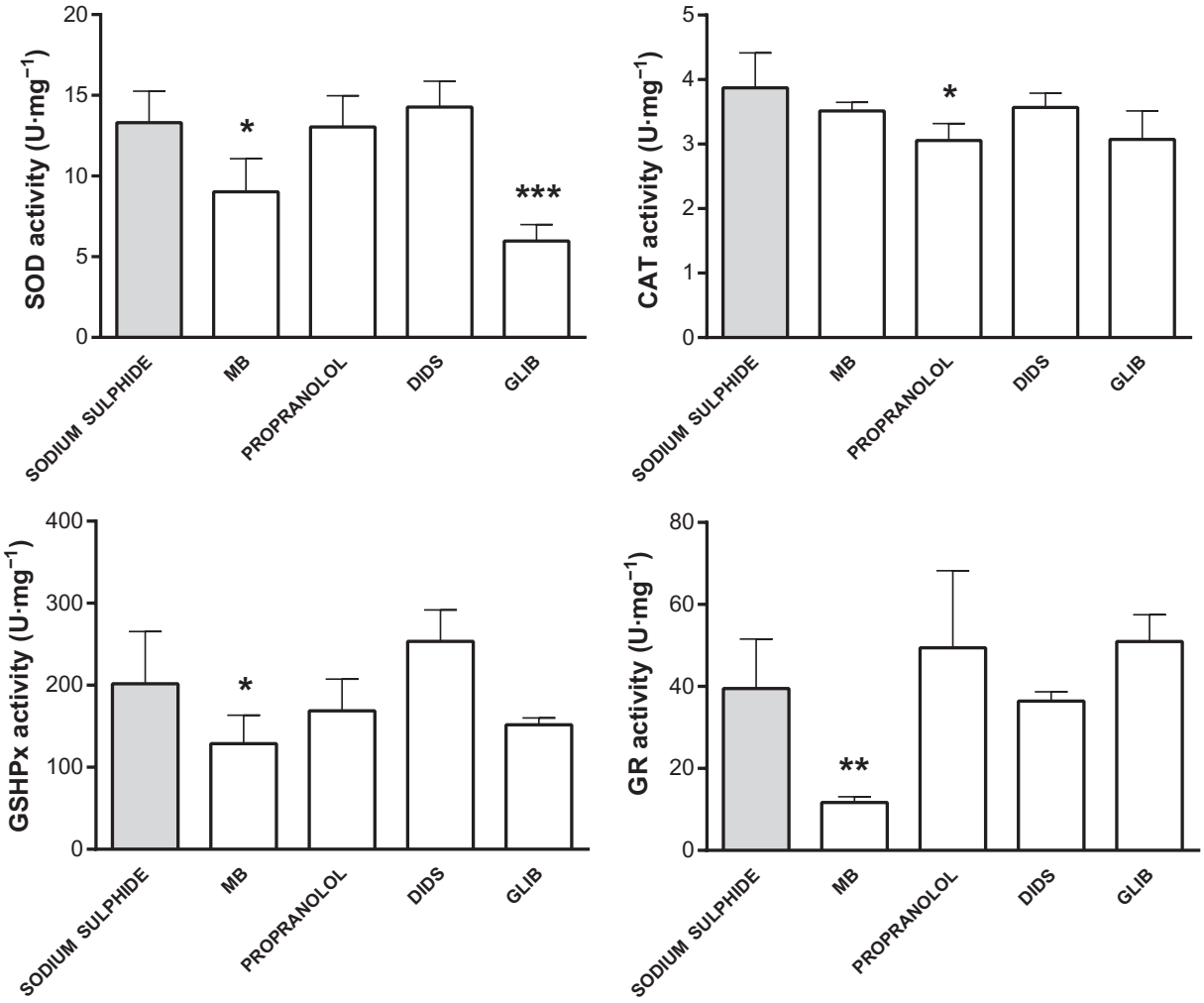

Figure 11

The activity of antioxidant enzymes: SOD, CAT, GSH-Px and GR in Ca^{2+}-stimulated active uteri treated with Na_2S and pretreated with MB, propranolol, DIDS and glibenclamide. Data are presented as mean ± SD (n = 7). Data were analysed by one-way ANOVA and *post hoc* compared using Tukey's HSD test. *P < 0.05, **P < 0.01, ***P < 0.001.

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