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Hydrogen sulfide mediates hypoxia-induced relaxation of trout urinary bladder smooth muscle

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Summary

Hydrogen sulfide (H_2S) is a recently identified gasotransmitter that may mediate hypoxic responses in vascular smooth muscle. H_2S also appears to be a signaling molecule in mammalian non-vascular smooth muscle, but its existence and function in non-mammalian non-vascular smooth muscle have not been examined. In the present study we examined H_2S production and its physiological effects in urinary bladder from steelhead and rainbow trout (*Oncorhynchus mykiss*) and evaluated the relationship between H_2S and hypoxia. H_2S was produced by trout bladders, and its production was sensitive to inhibitors of cystathionine β-synthase and cystathionine γ-lyase. H_2S produced a dose-dependent relaxation in unstimulated and carbachol pre-contracted bladders and inhibited spontaneous contractions. Bladders pre-contracted with 80 mmol l^{-1} KCl were less sensitive to H_2S than bladders contracted with either 80 mmol l^{-1} $KC_2H_3O_2$ (KAc) or carbachol, suggesting that some of the H_2S effects are mediated through an ion channel. However, H_2S relaxation of bladders was not affected by the potassium channel inhibitors, apamin, charybdotoxin, 4-aminopyridine, and glybenclamide, or by chloride channel/exchange inhibitors 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt, tamoxifen and glybenclamide, or by the presence or absence of extracellular HCO_3^-. Inhibitors of neuronal mechanisms, tetrodotoxin, strychnine and *N*-vanillylnonanamide were likewise ineffective. Hypoxia (aeration with N_2) also relaxed bladders, was competitive with H_2S for relaxation, and it was equally sensitive to KCl, and unaffected by neuronal blockade or the presence of extracellular HCO_3^-. Inhibitors of H_2S synthesis also inhibited hypoxic relaxation. These experiments suggest that H_2S is a phylogenetically ancient gasotransmitter in non-mammalian non-vascular smooth muscle and that it serves as an oxygen sensor/transducer, mediating the effects of hypoxia.

Key words: H_2S, hypoxia, smooth muscle, urinary bladder, trout.

Introduction

Hydrogen sulfide (H_2S) is a recently described endogenous gasotransmitter with considerable regulatory activity. H_2S is synthesized by, and has physiological actions in, a variety of mammalian tissues including brain (Abe and Kimura, 1996; Kimura, 2000; Kimura, 2002), vascular (Hosoki et al., 1997; Zhao et al., 2001; Zhao and Wang, 2002; Cheng et al., 2004), gastrointestinal (Hosoki et al., 1997; Teague et al., 2002), genitourinary (Patacchini et al., 2004; Patacchini et al., 2005) and reproductive (Hayden et al., 1989; Sidhu et al., 2001; Teague et al., 2002) tracts, pulmonary system (Shi et al., 2003; Zhang et al., 2003; Cheng et al., 2004), and heart (Zhang et al., 2003; Geng et al., 2004). H_2S relaxes most mammalian smooth muscles including systemic vessels (Hosoki et al., 1997; Zhao et al., 2001; Zhao and Wang, 2002; Cheng et al., 2004), intestine (Hosoki et al., 1997; Teague et al., 2002), uterus (Sidhu et al., 2001) and vas deferens (Teague et al., 2002). Rat urinary bladder detrusor muscle is the only tissue reported to date where H_2S produces a contraction (Patacchini et al., 2004; Patacchini et al., 2005). ATP-sensitive K^+ channels (K_{ATP}) have been proposed to mediate H_2S-mediated relaxation of vascular smooth muscle (Zhao et al., 2001; Zhao and Wang, 2002), but they do not appear to be involved in either gastrointestinal or reproductive smooth muscle relaxation (Teague et al., 2002). Contraction of the rat urinary bladder appears to be indirect *via* stimulation of capsaicin-sensitive afferent neurons (Patacchini et al., 2004; Patacchini et al., 2005).

We have shown that H_2S may relax or contract vascular smooth muscle in non-mammalian vertebrates and many vessels relax at lower H_2S concentrations ([H_2S]) and then contract at higher [H_2S] (Dombkowski et al., 2004;

Dombkowski et al., 2005; Olson, 2005). We (Olson et al., 2006) recently observed that H_2S also constricts, or has multi-phasic dilation/constriction effects on some mammalian vessels such as bovine and rat pulmonary arteries. To our knowledge, there is no evidence for a direct H_2S-mediated contraction of non-vascular smooth muscle, nor is there any information on the effects of H_2S on non-mammalian, non-vascular smooth muscle.

Recently, we (Olson et al., 2006) proposed that H_2S serves as a vascular oxygen sensor and that it plays an integral role in both hypoxic vasoconstriction of pulmonary vessels and cyclostome aortas and it is also involved in hypoxic dilation of mammalian systemic vessels. This model is based on continual oxidative inactivation of constitutively generated H_2S during normoxia and the development of vasoactive levels of H_2S when available oxygen falls. It is not known if this model is only applicable to vascular smooth muscle or if it is a feature of smooth muscle in general.

Spontaneous and agonist-induced contraction of rat urinary bladder, like that of systemic vessels, decreases when exposed to hypoxia (Leven et al., 1999; Whitbeck et al., 1999; Waring and Wendt, 2000), which is opposite to the contractile effect of H_2S, albeit indirect, in this same tissue (Patacchini et al., 2004; Patacchini et al., 2005). To our knowledge, the effects of hypoxia on non-mammalian non-vascular smooth muscle are not known.

The purpose of the experiments reported here were threefold: (1) to determine if H_2S affects contractile properties of non-mammalian, non-vascular smooth muscle and examine the mechanism(s) involved, (2) to evaluate the effects of hypoxia on the same tissue, and (3) to determine if H_2S mediates the hypoxic response. To this end we used urinary bladders from steelhead and rainbow trout and measured H_2S production, H_2S effects on spontaneous and agonist-induced contractions and possible mechanisms of action, effects of hypoxia on bladder contractions, and the effects of inhibiting H_2S synthesis on the hypoxic responses.

Materials and methods

Animals

Steelhead trout (*Oncorhynchus mykiss* Walbaum, skamania strain, 3–7 kg) of either sex were used for tissue production and most myography studies as the urinary bladders are large. Fish were captured by the Indiana Department of Natural Resources (DNR) during the fall migration of 2003 and 2004 and kept at the Richard Clay Bodine State Fish Hatchery until the spawning season (January–March). The fish were anesthetized in ethyl m-aminobenzoate methanesulfonate (MS-222), and after the spawn was collected by the DNR, urinary bladders were removed, placed in 4°C Hepes-buffered saline with glucose, and transported back to the laboratory where they were thoroughly cleaned of loose connective tissue and blood.

Because the steelhead trout were no longer available after March, we used urinary bladders from rainbow trout (*O. mykiss*, Walbaum, kamloops strain, 0.3–0.8 kg) for several experiments. Rainbow trout of either sex were purchased from a local hatchery (Homestead Trout Farm, Harrietta, MI, USA), and maintained throughout the year in 2000 liter tanks containing circulating well-water at 12–15°C, aerated with filtered room air, with a 12 h:12 h light:dark cycle. The fish were fed a maintenance diet of commercial trout pellets (Purina, St Louis, MO, USA). The rainbow trout were stunned by a blow to the head and the bladders prepared for myography, as described above. Although the bladders from the rainbow trout were considerably smaller than those from the steelhead trout, their sensitivity to NaHS, Na_2S, and N_2 was identical to steelhead bladders, therefore, 'trout' is used hereafter to refer to experiments on either strain. All procedures followed NIH guidelines and were approved by the local IACUC Committee.

Myography

Circular smooth muscle rings, approximately one-half centimeter long, and immediately distal to the trigone were mounted on 280 μm-diameter stainless steel wire hooks and suspended in 5 ml water-jacketed smooth muscle baths filled with 14°C Hepes buffer and aerated with room air. The bottom hooks were stationary; the upper ones were connected to Grass model FT03C force-displacement transducers (Grass Instruments, West Warwick, RI, USA). Tension was measured on a Grass Model 7E or 7F polygraph (Grass Instruments, West Warwick, RI, USA). Polygraph sensitivity was set to detect changes as small as 5 mg. Data was archived on a PC computer at 1 Hz using SoftWire (Measurement Computing, Middleboro, MA, USA). The chart recorders and software were calibrated prior to each experiment.

Baseline (resting) tension of approximately 200 mg was applied and continuously adjusted for at least 1 h prior to experimentation as the bladders exhibited substantial stress relaxation. The bladders were then contracted with 80 mmol l^{-1} KCl, washed twice, and resting tension re-established for a minimum for 30 min before further experimentation. A second KCl contraction was given in initial studies, but as this contraction was not different from the first it was omitted in later experiments.

H_2S responses

The cumulative dose–response characteristics of NaHS, which forms HS- and H_2S in solution similar to that produced by gassing with H_2S gas (Zhao et al., 2001), were examined in otherwise unstimulated bladders. Because H_2S inhibited spontaneous contractions and relaxed unstimulated bladders, the cumulative dose–response characteristics of NaHS were also examined in 10 μmol l^{-1} carbamylcholine chloride (carbachol, CARB)-prestimulated bladders. In later experiments, Na_2S, which also forms HS- and H_2S in solution, was used, because of its availability with a reduced amount of elemental sulfur impurities (Doeller et al., 2005). As the effects of Na_2S and NaHS were similar, H_2S is used in the context of either NaHS or Na_2S unless otherwise specified.

Potential mechanisms of H_2S-induced relaxation were evaluated in carbachol pre-stimulated bladders. The rings were

treated with the inhibitor, followed 15 min later by pre-stimulation with carbachol (10 μmol l^{-1}), and at the plateau contraction they were exposed to 1 mmol l^{-1} H_2S. Control rings were treated similarly omitting the inhibitor, and only one experiment was performed per ring. Potassium channels were inhibited with apamin (APA, 100 nmol l^{-1}), a small conductance K_{Ca} channel (SK_{Ca}) inhibitor, charybdotoxin (CTX, 50 nmol l^{-1}), a large conductance K_{Ca} channel (BK_{Ca}) inhibitor, 4-aminopyridine (4-AP, 100 μmol l^{-1}), a voltage sensitive K^+ channel inhibitor, and by using APA and CTX in combination. Chloride channel/exchangers were inhibited with glibenclamide (GLY, 10 μmol l^{-1}), a cystic fibrosis transmembrane conductance regulator (CFTR) and K_{ATP} channel inhibitor, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS, 400 μmol l^{-1}), an inhibitor of Cl^-/HCO_3^- exchange, and tamoxifen (TAM, 100 μmol l^{-1}), a volume-sensitive Cl^- channel inhibitor. The effects of the primary afferent nerve irritant *N*-vanillylnonanamide (10 μmol l^{-1}; a synthetic capsaicin with similar biological activity) were also examined.

In order to determine if the H_2S relaxation was affected by the type of pre-contraction stimulus, the effects of 1 mmol l^{-1} H_2S were also examined on bladders contracted with 80 mmol l^{-1} KCl or 80 mmol l^{-1} $KC_2H_3O_2$ (KAc). The effects of strychnine (10 μmol l^{-1}), a glycine/NMDA receptor antagonist, tetrodotoxin (TTX, 10 μmol l^{-1}), a fast Na^+ channel inhibitor, and a bicarbonate-based buffer (Cortland) on H_2S-induced relaxation were also examined in the KCl and KAc prestimulated bladders.

H_2S production

Pieces from ten different bladders were pooled for each experiment, blotted dry, weighed, and homogenized on ice in 100 mmol l^{-1} potassium phosphate buffer (pH 7.4). H_2S production was measured as described previously (Zhao et al., 2003), with minor modifications. Briefly, the homogenates were brought to a final volume of 1:10 tissue mass:nutrient buffer volume. Nutrient phosphate buffer contained 10 mmol l^{-1} cysteine and 2 mmol l^{-1} pyridoxal-5′-phosphate. In other experiments, the nutrient buffer also contained the cystathionine γ-lyase (CSE) inhibitor, D,L-propargylglycine (PPG; 20 mmol l^{-1}) or the cystathionine β-synthase (CBS) inhibitor, amino-oxyacetic acid (AOA; 1 mmol l^{-1}). The final mixture was then drawn into 10 ml polyethylene syringes, air bubbles were expelled, and the syringes sealed with three-way stopcocks and gently rotated for 24 h at room temperature. A glass bead in the syringe assisted mixing. At the end of the incubation, 1 ml samples of the homogenate solution were placed in 1.5 ml centrifuge tubes and immediately centrifuged. The supernatant was removed and mixed 1:1 with an antioxidant buffer made according to the manufacturer's specifications. This buffer converted all H_2S and HS^- to S^{2-}, which was then measured with a sulfide electrode (Lazar Research Laboratories, Los Angeles, CA, USA) on a Fisher Accumet AR50 pH meter (Fisher Scientific, Pittsburgh, PA, USA) following the manufacturer's directions. Standards were prepared from Na_2S, all measurements were done in triplicate.

Hypoxic responses

The effects of hypoxia, produced by gassing with N_2 rather than air, were examined in otherwise un-stimulated and 10 μmol l^{-1} carbachol, 80 mmol l^{-1} KCl, or 80 mmol l^{-1} KAc pre-contracted bladders. Similar to the mechanistic examination of H_2S, N_2 was also tested on prestimulated bladders. The effects of strychnine, tetrodotoxin and Cortland saline on the hypoxic response were also examined in KCl and KAc pre-contracted bladders.

Relationship between H_2S and hypoxia

Two experiments were employed to examine the relationship between H_2S and hypoxic relaxation. The first experiments were designed to determine if the relaxation produced by H_2S and hypoxia was additive or competitive. Bladders were pre-contracted with carbachol, KCl or KAc and then exposed to either 1 mmol l^{-1} H_2S or hypoxia. When tension stabilized, the other stimulus (hypoxia or H_2S, respectively) was applied. In the second experiments, the effects of inhibiting H_2S synthesis on hypoxic relaxation of un-stimulated and carbachol, KCl, or KAc pre-contracted bladders was examined. The CSE and CBS inhibitors (PPG, 10 mmol l^{-1}, and AOA, 1 mmol l^{-1}, respectively) were added at least 15 min prior to hypoxia.

Data analysis

Dose–response curves were fit for each vessel using Table Curve® (Jandel Corp., Chicago, IL, USA). Student's *t*-tests were used for comparisons between groups with SigmaStat® (Jandel Corp.). Results are provided as mean ± s.e.m. Significance was assumed at $P\leq 0.05$.

Chemicals

Unless otherwise stated all chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Na_2S and NaHS were purchased from Fisher Scientific (Pittsburgh, PA, USA). Concentrations for the Hepes-buffered trout saline (pH 7.8) were as follows (in mmol l^{-1}): 145 NaCl, 3 KCl, 0.57 $MgSO_4$, 2 $CaCl_2$, 5 glucose, 3 Hepes acid, and 7 Hepes Na^+ salt. Concentrations of the Cortland-buffered trout saline (pH 7.8) were as follows (in mmol l^{-1}): 124 NaCl, 3 KCl, 1.1 $MgSO_4$, 2 $CaCl_2$, 5.55 glucose, 12 $NaHCO_3$, 0.09 NaH_2PO_4, and 1.8 Na_2HPO_4. Hepes and Cortland buffers were used within 72 h of preparation. NaHS and Na_2S stock solutions for electrode calibration and bath application were used within 8 h of preparation.

Results

Effects of H_2S

H_2S concentrations at or above 100 μmol l^{-1} relaxed otherwise un-stimulated bladders and partially inhibited spontaneous contractions. Spontaneous contractions were completely inhibited when H_2S concentration reached

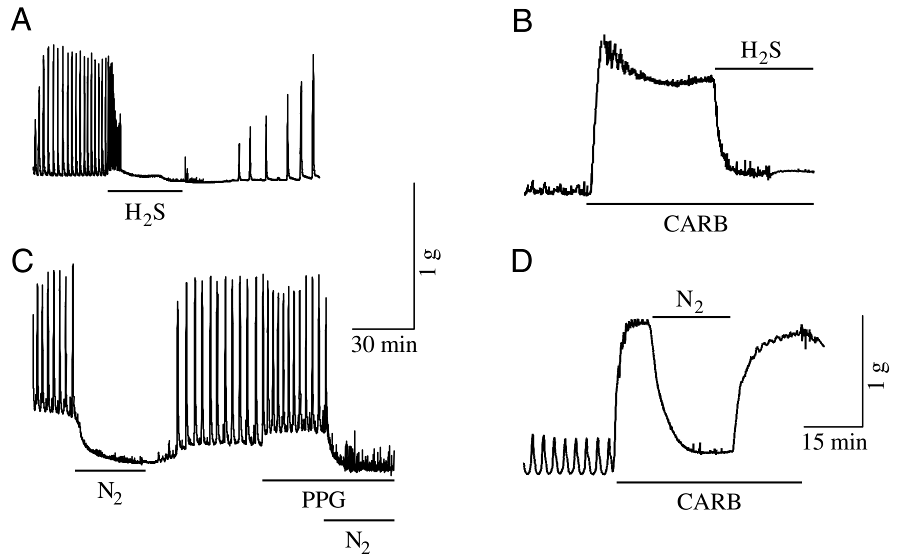

Fig. 1. Typical tracings of the effects of 1 mmol l^{-1} H_2S (as NaHS) on un-stimulated (A) and 10 μmol l^{-1} carbachol (CARB)-pre-contracted (B) trout bladders and the effects of hypoxia (N_2) on un-stimulated (C) and CARB-pre-contracted (D) bladders. C also shows the effect of 10 mmol l^{-1} of cystathionine γ-lyase inhibitor, D,L-propargylglycine (PPG; N=4) on un-stimulated bladders before and after exposure to N_2. Scale bars, force–time scale for A and C (left), B and D (right).

1 mmol l^{-1} (Fig. 1A). H_2S relaxed 10 μmol l^{-1} carbachol-pre-contracted bladders in a dose-dependent manner with an EC_{50} (concentration producing half-maximal response) of 129±50 μmol l^{-1} H_2S (N=8). At 1 mmol l^{-1} H_2S, 10 μmol l^{-1} carbachol-contracted bladders were nearly completely relaxed (Fig. 1B). As the efficacy of 1 mmol l^{-1} Na_2S was not significantly greater than that of 1 mmol l^{-1} NaHS in relaxing a carbachol contraction (98.8±7.4%; N=8 *versus* 82.2±6.7%; N=8, respectively; Fig. 2), 'H_2S' is used to denote either NaHS or Na_2S treatment. H_2S was significantly less efficacious in relaxing KCl-contracted bladders (27.1±6.2% relaxation) than it was in bladders contracted with either carbachol or KAc (52.6±14.8% relaxation; Fig. 2). There was no significant difference in the force of bladder contraction produced by carbachol (1.83±0.26 g; N=28), KCl (1.68±0.33 g; N=16), or KAc (1.66±0.24 g; N=16).

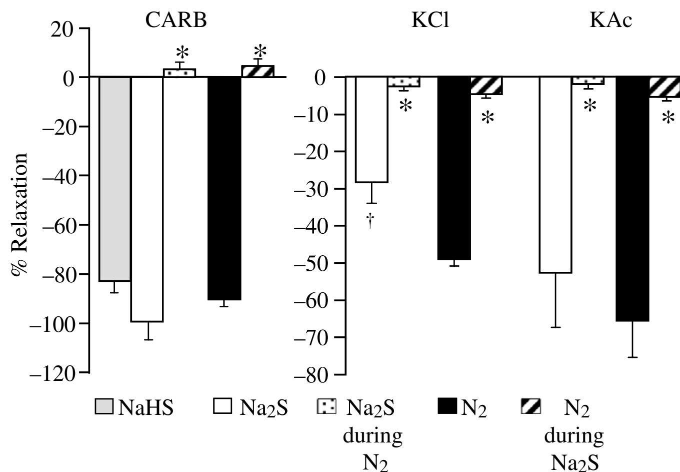

Fig. 2. Comparison of the relaxation efficacy of 1 mmol l^{-1} H_2S (as Na_2S) and hypoxia (N_2) on 10 μmol l^{-1} carbachol (CARB)-, 80 mmol l^{-1} KCl- and 80 mmol l^{-1} potassium acetate (KAc)-contracted trout urinary bladders (all N=8) and the effects of H_2S application on a pre-existing N_2 exposure and of N_2 application on a pre-existing H_2S exposure in pre-contracted bladders (all N=4). H_2S (as NaHS) relaxation of CARB-contracted vessels is also shown. Values are means ± s.e.m. H_2S was significantly less efficacious on KCl-contracted bladders than bladders pre-contracted with either CARB or KAc (†). Bladders relaxed with either H_2S or N_2 did not respond to the other (N_2 or H_2S, respectively), irrespective of the pre-contractile agonist (*). There was no difference in the relaxation produced by Na_2S and NaHS.

Potassium channel inhibitors: apamin (APA, 100 nmol l^{-1}; N=4), charybdotoxin (CTX, 50 nmol l^{-1}; N=4), 4-aminopyridine (4-AP, 100 μmol l^{-1}; N=4), and APA and CTX in combination (N=4) did not significantly affect on the magnitude of either a carbachol contraction or the ability of H_2S to relax the carbachol contraction (Fig. 3). Antagonists of chloride channels and/or exchangers, glibenclamide (GLY, 10 μmol l^{-1}; N=4) and tamoxifen (TAM, 100 μmol l^{-1}; N=4)

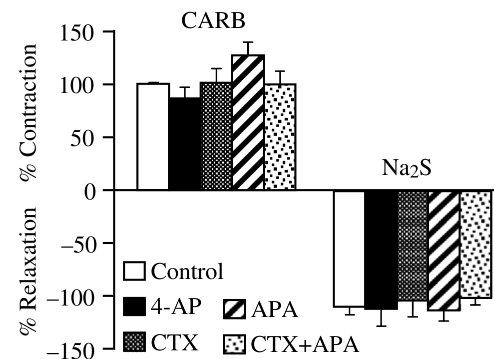

Fig. 3. Effects of K^+ channel inhibitors 4-aminopyridine (4-AP; 100 μmol l^{-1}; N=4), apamin (APA,100 nmol l^{-1}; N=4), charybdotoxin (CTX; 50 nmol l^{-1}; N=4) and APA and CTX in combination (N=4) on a 10 μmol l^{-1} carbachol contraction (left) or on a 1 mmol l^{-1} H_2S (as Na_2S)-induced relaxation of a 10 μmol l^{-1} carbachol prestimulated bladder (right); all controls, N=8. Values are means ± s.e.m. The inhibitors had no significant effect on either the CARB contraction or the H_2S relaxation.

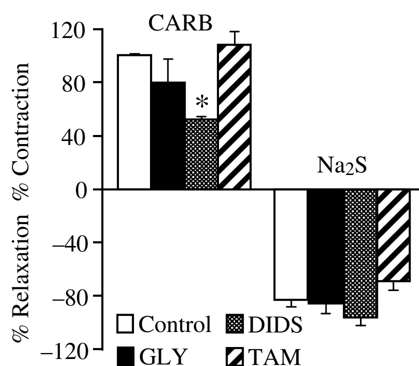

Fig. 4. Effects of Cl^- channel/exchange antagonists, glibenclamide (GLY; 10 μmol l^{-1}; N=4), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS; 400 μmol l^{-1}; N=4), and tamoxifen (TAM; 100 μmol l^{-1}; N=4) on a 10 μmol l^{-1} carbachol contraction (left) or a 1 mmol l^{-1} H_2S (as Na_2S)-induced relaxation of a 10 μmol l^{-1} carbachol-prestimulated bladder (right); all controls, N=8. Values are means ± s.e.m. DIDS significantly (*) reduced the CARB contraction; there were no other significant differences.

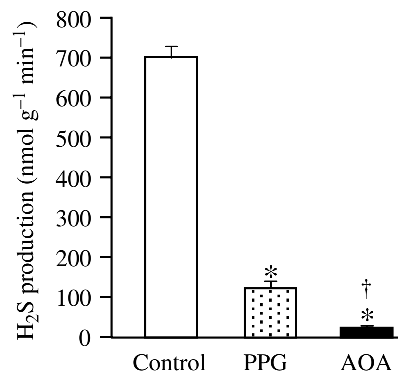

Fig. 5. Twenty-four hour production of H_2S by trout in the presence of no inhibitors (Control), the cystathionine γ-lyase inhibitor, D,L-propargylglycine (PPG; 20 mmol l^{-1}) or the cystathionine β-synthase inhibitor, amino-oxyacetic acid (AOA; 1 mmol l^{-1}). Values are means ± s.e.m. *Significantly different from control; †significantly different from control and PPG; all samples were from pooled bladders and analyzed in triplicate.

did not affect a 10 μmol l^{-1} carbachol contraction, whereas DIDS (400 μmol l^{-1}; N=4) halved ($P\leqslant0.05$) the force developed (Fig. 4). The ability of H_2S to relax carbachol-contracted bladders was unaffected by GLY, TAM or DIDS (Fig. 4). Incubation with *N*-vanillylnonanamide (10 μmol l^{-1}; N=4) did not affect either the magnitude of a carbachol contraction or the ability of H_2S to relax the carbachol contraction (data not shown). Strychnine (10 μmol l^{-1}; N=4), tetrodotoxin (TTX, 10 μmol l^{-1}; N=4) or substituting Cortland buffer for Hepes buffer (N=4) did not affect either KCl or KAc contractions or the ability of H_2S to relax them (not shown).

Effects of hypoxia

Hypoxia (N_2) reduced baseline tone and inhibited spontaneous contractions in otherwise un-stimulated bladders and in 10 μmol l^{-1} carbachol-contracted bladders (Fig. 1C,D). As shown in Fig. 2, hypoxia was significantly less efficacious in relaxing KCl-contracted bladders (49.0±1.6% relaxation) than it was in bladders contracted with carbachol (89.8±2.5% relaxation). Hypoxic relaxation of KAc-contracted bladders (65.6±9.7%) was not significantly different from hypoxic relaxation of either carbachol- or KCl-contracted bladders (Fig. 2; all N=8). Strychnine (10 μmol l^{-1}; N=4), tetrodotoxin (TTX, 10 μmol l^{-1}; N=4) or substituting Cortland buffer for Hepes buffer (N=4) did not affect the ability of N_2 to relax contractions produced by either 80 mmol l^{-1} KCl or 80 mmol l^{-1} KAc (not shown; all N=4).

H_2S production

Homogenates of trout urinary bladders produced H_2S enzymatically (Fig. 5). The cystathionine γ-lyase inhibitor, D,L-propargylglycine (20 mmol l^{-1}), reduced H_2S synthesis by over 80% and the cystathionine β-synthase inhibitor, amino-oxyacetic acid (1 mmol l^{-1}), reduced H_2S synthesis by over 95% (Fig. 5).

Relationship between H_2S and hypoxia

Hypoxia applied after H_2S did not produce any additional relaxation in bladders pre-contracted with carbachol, KCl or KAc (Fig. 2). When H_2S was added after hypoxia in pre-contracted bladders it was similarly ineffective (Fig. 2).

The cystathionine γ-lyase inhibitor, D,L-propargylglycine, caused a slight increase in baseline tone and an increase in the frequency of spontaneous contractions but did not prevent the N_2-induced relaxation or inhibition of spontaneous contractions in un-stimulated bladders (Fig. 1C); this was consistent in bladders from four fish. The cystathionine β-synthase inhibitor, amino-oxyacetic acid (1 mmol l^{-1}) did not appear to have any effect on resting tone, spontaneous contractions, or N_2-induced relaxation (N=4; not shown). Hypoxic relaxation of carbachol-

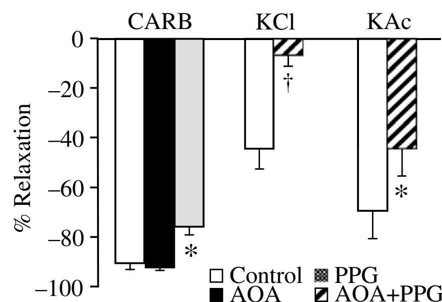

Fig. 6. Effects of inhibitors of H_2S synthesis on hypoxic (N_2) relaxation of pre-contracted bladders. The cystathionine γ-lyase inhibitor, D,L-propargylglycine (PPG; 20 mmol l^{-1}) significantly decreased a N_2 relaxation of 10 μmol l^{-1} carbachol-pre-contracted bladders and a mixture of PPG and the cystathionine β-synthase inhibitor, amino-oxyacetic acid (AOA; 1 mmol l^{-1}) significantly inhibited N_2 relaxation of both 80 mmol l^{-1} KCl- and 80 mmol l^{-1} KAc-pre-contracted bladders (N=4 for all experimental groups; N=8 for controls). Values are means ± s.e.m. *Significantly different from the respective control; †significantly different from all other conditions.

pre-contracted bladders was partially inhibited by amino-oxyacetic acid, and a mixture of D,L-propargylglycine and amino-oxyacetic acid inhibited hypoxic relaxation of both KCl- and KAc-contracted bladders (Fig. 6; N=8 for controls, N=4 for each treatment).

Discussion

The present experiments show that trout bladders synthesize H_2S and that H_2S relaxes and decreases spontaneous contractions in bladders at concentrations slightly above those reported in rainbow trout plasma *in vivo* (Dombkowski et al., 2004). Our experiments also show that both the response of trout bladder to H_2S and the potential mechanism of H_2S action is unlike that observed in the rat. Whereas some of the differences between trout and rats may be due to the different derivation of the tissue, fish bladders are mesodermal and noncloacal in origin whereas tetrapod bladders are derivatives of the cloaca (Kardong, 2005), our results, nevertheless, suggest that H_2S is an endogenous signaling molecule in non-mammalian non-vascular smooth muscle.

Mechanism of H_2S action

H_2S-induced relaxation of mammalian vascular smooth muscle appears to be partially mediated by the activation of ATP-sensitive potassium (K_{ATP}) channels (Zhao et al., 2001; Zhao and Wang, 2002). However, these channels do not seem to contribute to H_2S relaxation of mammalian non-vascular smooth muscle (Teague et al., 2002), nor do they appear to mediate responses in the trout urinary bladder because the sulfonylurea K_{ATP} channel inhibitor, glibenclamide, which partially inhibits H_2S relaxation of rat aortas (Zhao et al., 2001; Zhao and Wang, 2002) is ineffective in trout bladders (Fig. 4). In fact our findings failed to support the involvement of any type of potassium channel because none of a variety of classical potassium channel inhibitors affected the H_2S response (Figs 3, 4), nor did elevation of extracellular [K^+] to 80 mmol l^{-1} with potassium acetate (KAc). The latter would be expected to substantially reduce transmembrane K^+ gradients and obviate K^+ channels.

The reduced H_2S efficacy in bladders contracted with KCl, compared to carbachol and KAc, suggests that the 80 mmol l^{-1} Cl^- may interfere with H_2S. It is possible that HS^-, which at physiological pH accounts for approximately 80% of the total H_2S + HS^- (Dombkowski et al., 2004) interferes with a Cl^- channel or transporter and that this interference is diminished when transmembrane Cl^- gradients are changed. However, we could not find any evidence for a Cl^--dependent mechanism using a variety of inhibitors of Cl^- exchangers and channels (Fig. 4). Glibenclamide, which in addition to inhibiting K_{ATP} channels inhibits the cAMP-activated cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel at micromolar concentrations (Sheppard et al., 1992) was ineffective. A Cl^-/HCO_3^- exchange is also doubtful as the Cl^-/HCO_3^- exchange inhibitor, DIDS, was ineffective (Fig. 4) and H_2S relaxation was unaffected by the absence (Hepes buffer) or presence (Cortland buffer) of extracellular HCO_3^-. Although it is possible that 400 μmol l^{-1} DIDS does not block Cl^-/HCO_3^- exchange in trout bladder, or the nominal absence of extracellular HCO_3^- does not limit the cell's ability to utilize a Cl^-/HCO_3^- exchange, this seems unlikely. This question is further compounded by the lack of specificity of inhibitors of Cl^--dependent mechanisms (Jentsch et al., 2001). Clearly, additional studies with other inhibitors of Cl^--dependent mechanisms and variations in extracellular [Cl^-] are warranted.

Patacchini et al. (Patacchini et al., 2004; Patacchini et al., 2005) reported that rat urinary bladders are indirectly contracted through H_2S stimulation of capsaicin-sensitive nerves. We do not know whether or not the trout bladder has capsaicin-sensitive nerves. However, our results show that trout bladders are relaxed by H_2S and this is not affected by blocking intrinsic neurons with tetrodotoxin, glycine/NMDA receptors with strychnine, or the presence of the capsaicin synthetic, *N*-vanillylnonanamide. Thus both the response and the mechanism of H_2S action in trout urinary bladder are independent of intrinsic nerves, and are therefore unlike those observed in the mammalian urinary bladder.

H_2S synthesis in mammalian vascular smooth muscle has been attributed to the pyridoxal-5′-dependent enzyme, cystathionine γ-lyase, whereas cystathionine β-synthase does not appear to be present (Zhao et al., 2001). Both enzymes are involved in H_2S synthesis in non-vascular tissue (Zhao et al., 2003). Our studies also suggest that both enzymes contribute to H_2S synthesis in the trout urinary bladder (Fig. 5). The increase in baseline tension and frequency of spontaneous contractions following inhibition of cystathionine γ-lyase with D,L-propargylglycine (Fig. 1C) also suggest that H_2S is continuously synthesized by the bladder and has tonic inhibitory activity.

H_2S as an oxygen sensor

We (Olson et al., 2006) recently proposed that H_2S is an oxygen sensor in vascular smooth muscle. This hypothesis is based on our observations that, (1) H_2S and hypoxia produce the same mechanical response in vessels from at least one species in every vertebrate class, even though the response varies from a contraction, to relaxation, to a multi-phasic one; (2) the effects of H_2S and hypoxia are competitive – in the presence of one, the response to the other is greatly reduced or abolished; (3) blood vessels enzymatically generate H_2S and inhibitors of H_2S synthesis inhibit hypoxic responses, whereas the H_2S precursor cysteine augments it. The present study suggests that H_2S is also involved in oxygen sensing/signal transduction in the trout urinary bladder.

In essentially all of the present experiments the effects of hypoxia are similar, if not identical, to those produced by H_2S. Hypoxia relaxes otherwise un-stimulated and pre-contracted bladders (Fig. 1), it becomes less efficacious in KCl-pre-contracted bladders (Fig. 2) and it is unaffected by inhibition of neuronal mechanisms with strychnine, tetrodotoxin or *N*-vanillylnonanamide. In pre-contracted bladders, the presence of either H_2S or hypoxia prevents relaxation by the other. This does not appear to be due to a mechanical inability of the bladders to relax beyond a certain point because, with the

exception of a H_2S relaxation of a carbachol contraction, neither H_2S nor hypoxia produced 100% relaxation when applied initially (Fig. 2). Furthermore, hypoxic relaxation is reduced by inhibitors of bladder H_2S synthesis (Fig. 6).

Inhibitors of cystathionine β-synthase (amino-oxyacetic acid) and cystathionione γ-lyase (D,L-propargylglycine) appeared more efficacious in inhibiting H_2S synthesis (Fig. 5) than they were in inhibiting hypoxic relaxation (Fig. 6). This may be due to inhibitor accessibility to the enzyme; H_2S production was measured in homogenized tissues whereas the hypoxia effects were examined in intact bladder rings. Other studies have also suggested that amino-oxyacetic acid is not readily taken up by smooth muscle cells (Zhao et al., 2003). It is also possible that there are other pathways for H_2S synthesis (Julian et al., 2002; Maclean and Kraus, 2004; Stipanuk, 2004) or that H_2S effects are compartmentalized within the cell (Dombkowski et al., 2005).

Phylogeny of H_2S and hypoxic responses

The similarity of H_2S and hypoxic responses in vertebrate vascular smooth muscle (Olson et al., 2006) may be a property of non-vascular smooth muscle as well. This suggests that H_2S signaling in smooth muscle is a phylogenetically ancient mechanism. Clearly, additional tissues need to be examined. The primordial role of H_2S in smooth muscle, or as a signal molecule in general is unknown, but certainly it could be coupled to O_2 availability. The effect of hypoxia (and H_2S) in blood vessels is often commensurate with function: systemic vessels dilate to increase blood flow and match perfusion to metabolism, and respiratory vessels constrict to couple ventilation to perfusion. H_2S seems ideally positioned to mediate these hypoxic responses as the constitutive vascular synthesis of H_2S would be offset by H_2S oxidation during normoxia, but progressively unabated as oxygen levels fall. The benefit of hypoxic relaxation of non-vascular smooth muscle is less obvious. Perhaps it is a mechanism to reduce oxygen demand in less critical tissues.

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