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ORIGINAL PAPER

Comparative Effects of Hydrogen Sulfide-Releasing Compounds on [^{3}H]D-Aspartate Release from Bovine Isolated Retinae

Pratik Bankhele[1] · Ankita Salvi[1] · Jamal Jamil[1] · Fatou Njie-Mbye[2] · Sunny Ohia[2] · Catherine A. Opere[1]

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Abstract

We investigated the pharmacological actions of a slow-releasing H_2S donor, GYY 4137; a substrate for the biosynthesis of H_2S, L-cysteine and its precursor, *N*-acetylcysteine on potassium (K^+; 50 mM)-evoked [^{3}H]D-aspartate release from bovine isolated retinae using the Superfusion Method. GYY 4137 (10 nM–10 μM), L-cysteine (100 nM–10 μM) and *N*-acetylcysteine (10 μM–1 mM) elicited a concentration-dependent decrease in K^+-evoked [^{3}H]D-aspartate release from isolated bovine retinae without affecting basal tritium efflux. At equimolar concentration of 10 μM, the rank order of activity was as follows: L-cysteine > GYY 4137 > *N*-acetylcysteine. A dual inhibitor of the biosynthetic enzymes for H_2S, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), amino-oxyacetic acid (AOA; 3 mM) reversed the inhibitory responses caused by GYY 4137, L-cysteine and *N*-acetylcysteine on K^+-evoked [^{3}H]D-aspartate release. Glibenclamide (300 μM), an inhibitor of K_{ATP} channels blocked the inhibitory action of GYY 4137 and L-cysteine but not that elicited by *N*-acetylcysteine on K^+-induced [^{3}H]D-aspartate release. The inhibitory effect of GYY 4137 and L-cysteine on K^+-evoked [^{3}H]D-aspartate release was reversed by the non-specific inhibitor of nitric oxide synthase (NOS), L-NAME (300 μM). Furthermore, a specific inhibitor of inducible NOS (iNOS), aminoguanidine (10 μM) blocked the inhibitory action of L-cysteine on K^+-evoked [^{3}H]D-aspartate release. We conclude that both donors and substrates for H_2S production can inhibit amino acid neurotransmission in bovine isolated retinae, an effect that is dependent, at least in part, upon the intramural biosynthesis of this gas, and on the activity of K_{ATP} channels and NO synthase.

Keywords Retinae · Aspartate · Hydrogen sulfide · Cystathionine β-synthase · Cystathionine γ-lyase · K_{ATP} channels

Introduction

Although hydrogen sulfide (H_2S) was previously known as a toxic gas, it is now widely accepted as a signaling molecule that has—physiological roles in the central nervous, cardiovascular and the immune systems [1]. H_2S is endogenously biosynthesized from L-cysteine and D-cysteine by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and cysteine aminotransferase (CAT) and D-aminotransferase in combination with 3-mercaptosulfurtransferase (3MST). In the central nervous system, H_2S has been shown to facilitate the induction of hippocampal long-term potentiation by enhancing the activity of *N*-methyl-D-aspartate (NMDA) receptors in neurons and by the stimulation of calcium waves in astrocytes [2, 3]. H_2S can protect neurons from the deleterious action of oxygen-derived free radicals by enhancing the activity of glutathione synthase, scavenging reactive oxygen species and preventing the excessive increase in intracellular calcium concentration [4–6]. H_2S has also been reported to exhibit neurotransmitter-like activities because of its ability to regulate synaptic activities induced by steroid hormones and other neurotransmitters [7, 8]. Evidence of a pathophysiological role for H_2S has been demonstrated in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, vascular dementia, Huntington's disease and amyotrophic lateral sclerosis [9–11]. In the eye, the presence of enzymes of the trans-sulfuration pathway (CBS, CSE and 3MST) have been localized in tissues of

✉ Catherine A. Opere
copere@creighton.edu

[1] Department of Pharmacy Sciences, School of Pharmacy and Health Professions, Creighton University, 2500 California Plaza, Omaha, NE 68178, USA

[2] Department of Pharmaceutical & Environmental Health Sciences, College of Pharmacy & Health Sciences, Texas Southern University, 3100 Cleburne Street, Houston, TX 77004, USA

both anterior and posterior segments [12–14]. There is evidence that deficiency of CBS due to a mutation in the gene encoding the enzyme can lead to several eye disorders such as glaucoma and retinal detachment [15]. High levels of homocysteine, a substrate for H_2S biosynthesis have been observed in the aqueous humor, tear fluid and plasma of patients with primary open-angle glaucoma [16, 17]. Furthermore, elevated concentrations of H_2S have been demonstrated in the vitreous body and plasma of patients with proliferative diabetic retinopathy [18]. The ability of H_2S to exert pharmacological actions on mammalian ocular tissues from both anterior and posterior segments has been reported by us and other investigators [6, 19–27]. For instance, we have evidence that H_2S donors such as sodium hydrosulfide (NaHS) can relax both isolated mammalian irides and long posterior ciliary arteries [19–22]. Moreover, H_2S donors enhanced cyclic AMP production in bovine and porcine isolated neural retinae and retinal pigment epithelial cells, an effect that was dependent upon the biosynthesis of H_2S by CSE and CBS and, on the activity of K_{ATP} channels [23, 24]. In other studies, we demonstrated that H_2S donors can inhibit both electrically-evoked [^{3}H]-norepinephrine release and endogenous catecholamine concentrations from isolated porcine iris-ciliary bodies in a concentration-related manner [25]. Interestingly, the inhibitory action of H_2S donors on norepinephrine release was antagonized by inhibitors of CSE and CBS suggesting that the observed response was due, at least in part, on the intramural biosynthesis of this gas [25]. In bovine and porcine isolated retinae, the H_2S donors, NaHS and Na_2S inhibited K^+-evoked [^{3}H]D-aspartate release by a mechanism that was dependent upon the intramural biosynthesis of the gas [26]. Since an increase in retinal glutamate concentrations have been linked to excitotoxicity, the ability of H_2S to inhibit glutamate release suggests a potential neuroprotective action of this gas in the retinae. Indeed, evidence for a neuroprotective effect of H_2S in the retinae has been reported by other investigators [6, 27]. Thus, we sought to delineate the role of other H_2S producing compounds on excitatory neurotransmission. The aim of the present study was twofold: (a) to investigate the pharmacological actions of a slow-releasing H_2S donor, GYY 4137 and L-cysteine, a substrate for H_2S biosynthesis on K^+-evoked [^{3}H]D-aspartate release, and (b) to examine the role of K_{ATP} channels and NO in the responses elicited by these compounds on neurotransmitter release.

Methods

Tissue Preparation

Freshly harvested bovine eyeballs were obtained from a local slaughterhouse (Greater Omaha Packing or J. F. O'Neil packing company) within 3 h after enucleation and delivered to the laboratory in an ice bucket. The anterior segment tissues and the vitreous humor were carefully removed and the eyecup everted to isolate the retinae.

[^{3}H]D-Aspartate Release Studies

The method used for assessment of [^{3}H]D-aspartate release studies was similar to the one previously described by us [26, 28–30] and others [31, 32]. Briefly, bovine retinae were incubated in oxygenated (95% O_2/5% CO_2) Krebs buffer solution containing 200 nM [^{3}H]D-aspartate at 37 °C for 1 h. The Krebs buffer solution was prepared as follows (mM): NaCl 118; KCl 4.8; $CaCl_2$ 2.5; KH_2PO_4 1.2; $NaHCO_3$ 25; $MgSO_4$ 2.0; dextrose 10 (pH 7.4). The cyclooxygenase inhibitor, flurbiprofen (3 μM) was added to the Krebs buffer solution to inhibit the endogenous production of prostanoids [30]. Following the incubation period, the radiolabeled retinae were rinsed three times with 25 mL of Krebs buffer solution for 5 min each. The radiolabeled tissues were then mounted between nylon mesh cloths and placed in thermostatically-controlled Superfusion Chambers. In order to establish a stable baseline of spontaneous tritium efflux, retinal tissues were superfused with oxygenated Krebs buffer solution at the rate of 1.5 mL/min for 60 min. Fractional collection was commenced at the same rate (1.5 mL/min) and effluent collected every six min. Neurotransmitter ([^{3}H]D-aspartate) release was evoked by the application of iso-osmotic concentrations of KCl (50 mM) stimuli applied between 84 and 96 min (S_1) and 132–144 min (S_2) after onset of superfusion. Superfusates were assessed for radioactivity by liquid scintillation spectrometry (LS 6500 Multipurpose Scintillation Counter, Beckman Coulter) and [^{3}H]D-aspartate release was estimated by subtraction of the extrapolated basal tritium efflux from total tritium released during the 20 min period after the onset of stimulation. The basal tritium efflux declined linearly between pre-stimulation and post-stimulation fractions.

To determine the effects of a H_2S-releasing compound on K^+-evoked [^{3}H]D-aspartate release, the compound was applied 12 min before S_2 (120 min after start of superfusion). To examine the effect of enzyme inhibitors and ion channel blockers on responses to H_2S-releasing compounds, the inhibitors/blockers were added 40 min from onset of Superfusion and were present during both S_1 and S_2 stimulation periods.

Data Analysis

Results are expressed as absolute S_2/S_1 ratios of the K^+-evoked [^{3}H]D-aspartate release in control and treated preparations. Values are given as the arithmetic means ± standard error of the mean (SEM). Significance of

differences between control and test values was tested using the analysis software by analysis of variance (ANOVA) test followed by Dunnett's test (Graph Pad Prism software San Diego, CA). The level of significance selected was at least $P < 0.05$.

Results

Effects of Compounds on [^{3}H]D-Aspartate Release

Application of an iso-osmotic high potassium chloride (K^+; 50 mM) stimuli to isolated retinae loaded with [^{3}H] D-aspartate and mounted in Superfusion Chambers elicited an overflow of tritium over basal levels, a response that can be repeated more than twice (S_1, S_2, S_3, etc). In control experiments in which no compound was present, application of two subsequent K^+ stimuli yielded two peaks of [^{3}H] D-aspartate overflow, as depicted in Fig. 1 (Panel a). The area under curve (AUC) calculated for both the peaks (S_1 and S_2) yielded S_2/S_1 ratios of 1.033 ± 0.03 ($n = 12$). As illustrated in Fig. 1 (Panel b), application of GYY 4137 (10 µM) 12 min before the second train of field stimulation (S_2) caused a marked inhibition of K^+-evoked [^{3}H]D-aspartate release from bovine retinae. A similar profile of inhibitory response was observed with L-cysteine and *N*-acetylcysteine on stimulated [^{3}H]D-aspartate release from isolated bovine retinae (data not shown).

Since there is evidence that fast-releasing H_2S donors (such as NaHS and Na_2S) can alter the release of norepinephrine from sympathetic nerves in the anterior uvea and excitatory amino acids from iris-ciliary bodies and mammalian retinae, in vitro [25, 26], we investigated the effect of the slow-releasing H_2S donor, GYY 4137 (0.01–10 µM) on K^+-induced [^{3}H]D-aspartate release from isolated bovine retinae (Fig. 2, Panel a). GYY 4137 exhibited a concentration-dependent inhibition of [^{3}H]D-aspartate release yielding a maximum inhibition of $21.48 \pm 2.53\%$ ($n = 5$; $p < 0.05$) at a concentration of 10 µM.

We next examined the effect of L-cysteine, a substrate for endogenous biosynthesis of H_2S via the enzymes, CSE and CBS, on [^{3}H]D-aspartate release. In the concentration range 0.1–10 µM, L-cysteine inhibited K^+-induced [^{3}H]D-aspartate release in a concentration-dependent manner, achieving a maximum inhibition of $54.28 \pm 3.61\%$ ($n = 4$; $p < 0.001$) at 10 µM and an IC_{50} value of 9.2 µM (Fig. 2, Panel b).

To further delineate the role of endogenous production of H_2S on the inhibitory effect on neurotransmission, we examined the effect of *N*-acetylcysteine, an acetylated derivative of the amino acid L-cysteine and a precursor to L-cysteine on K^+-induced [^{3}H]D-aspartate release. *N*-acetylcysteine (10 µM–1 mM) also elicited a concentration-dependent inhibition of [^{3}H]D-aspartate release from bovine isolated retinae yielding a maximum inhibition of $29.63 \pm 14.7\%$ ($n = 5$; $p < 0.05$) at the 1 mM concentration and an IC_{25} value of 843.5 µM (Fig. 2, Panel c). At an equimolar concentration of 10 µM, the rank order of inhibitory activity elicited by the H_2S producing compounds was as follows: L-cysteine > GYY 4137 > *N*-Acetylcysteine.

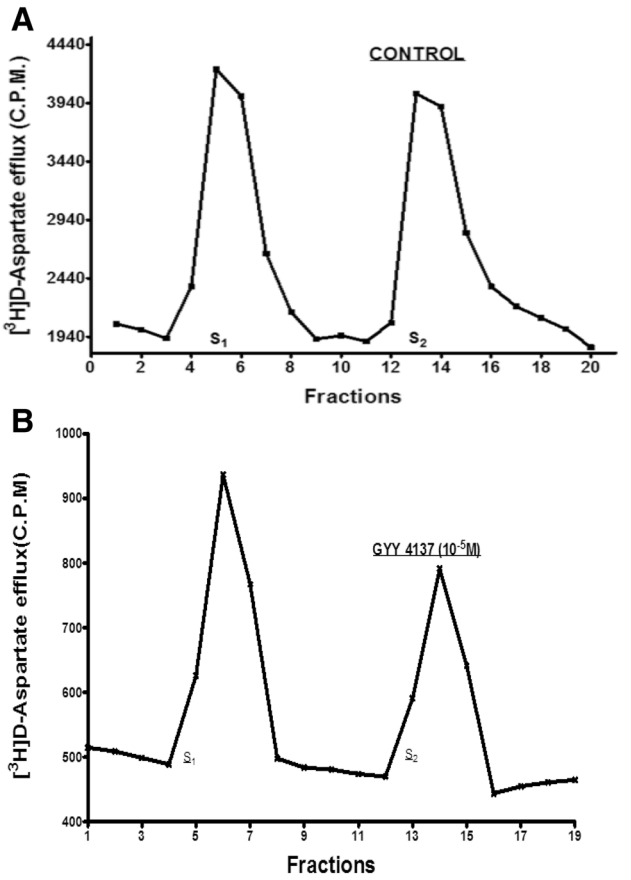

Fig. 1 Effect of slow hydrogen sulfide (H_2S) releasing compound, GYY 4137 (10 µM) on KCl (K^+, 50 mM)-induced release of [^{3}H] D-aspartate from isolated, superfused bovine retinae. K^+ stimuli were applied at fractions 5/6 (S_1) and 13/14 (S_2). **a** control (no compound present); **b** GYY 4137 (10 µM) applied 12 min before S_2. Fractions of the superfusate were collected at 6 min. intervals and analyzed for radioactivity as described under Methods

Effect of an Inhibitor of H_2S-Biosynthesis

There is evidence that amino-oxyacetic acid (AOA) can inhibit both CBS and CSE, enzymes involved in the biosynthesis of H_2S [33]. We examined the effect of AOA on the inhibitory action of the H_2S producing compounds, L-cysteine, *N*-acetylcysteine and GYY 4137. Whereas AOA (3 mM) had no effect on K^+-induced [^{3}H]D-aspartate release, it reversed the inhibitory action of GYY 4137, L-cysteine and *N*-acetylcysteine on neurotransmitter release (Fig. 3, Panels a–c).

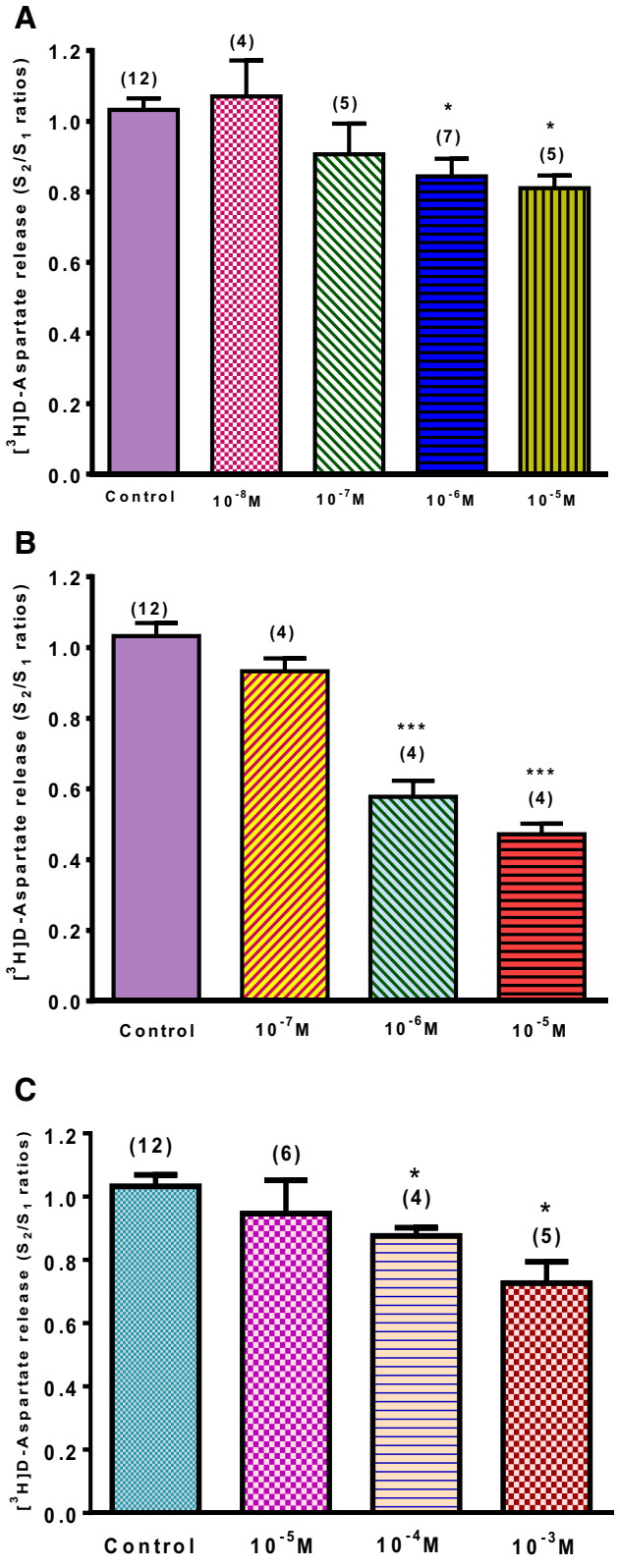

Fig. 2 Inhibitory effect of hydrogen sulfide (H_2S) releasing compounds on K^+-evoked [^{3}H]D-aspartate release from isolated, bovine retinae. **a** the slow H_2S donor, GYY 4137 (0.01–10 μM); **b** the endogenous substrate for H_2S synthesis, L-cysteine (0.1–10 μM); **c** L-cysteine precursor, N-acetylcysteine (0.01–1 mM). Vertical bars represent means ± SEM. Number of observations is in parenthesis. *P < 0.05; ***P < 0.001 significantly different from the control

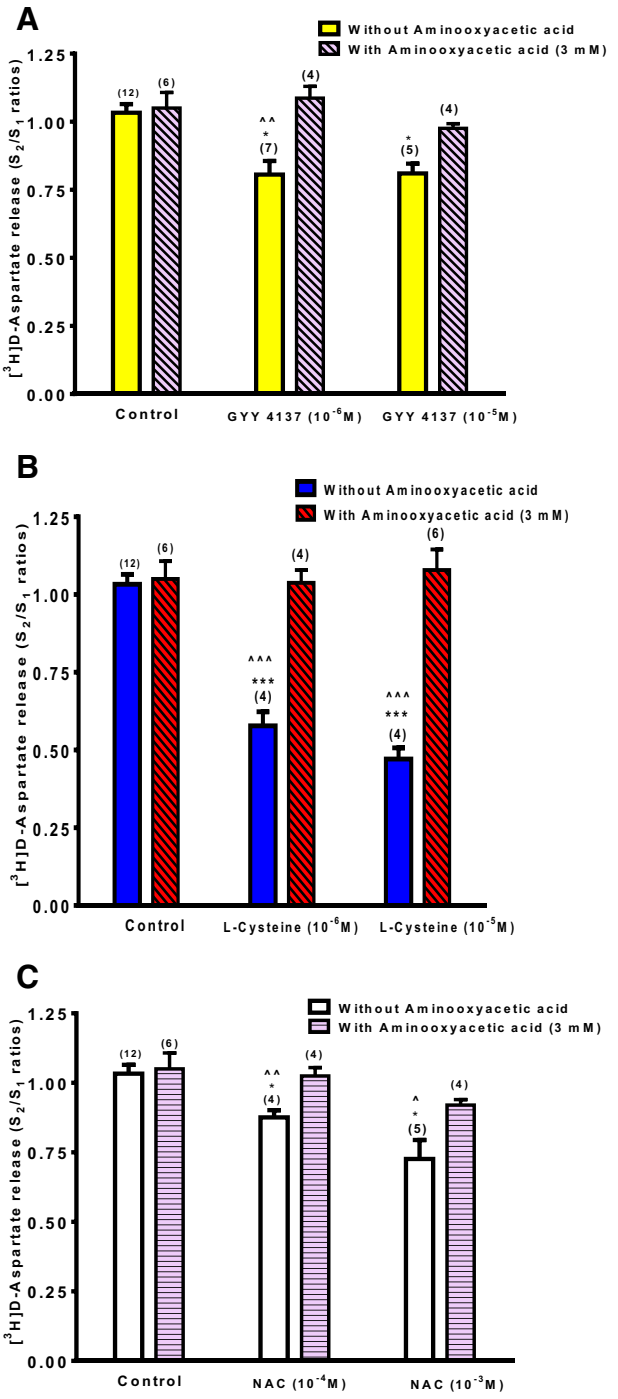

Fig. 3 Effect of the cystathionine β-synthase inhibitor, aminooxyacetic acid (AOA; 3 mM) on the inhibitory effect of hydrogen sulfide (H_2S) releasing compounds on K^+-evoked [^{3}H]D-aspartate release from isolated, bovine retinae. **a** control or GYY 4137 (10^{-6} and 10^{-5} M) in presence or absence of AOA (3 mM); **b** control or L-cysteine (10^{-6} and 10^{-5} M) in presence or absence of AOA (3 mM); **c** control or *N*-acetylcysteine (10^{-4} and 10^{-3} M) in presence or absence of AOA (3 mM). Vertical bars represent means ± SEM. Number of observations is in parenthesis. *P < 0.05; ***P < 0.001 significantly different from the control; ^P < 0.05; ^^^P < 0.001 significantly from H_2S releasing compound in presence of AOA (3 mM)

Effect of a Potassium Channel (K_{ATP}) Blocker

In the next series of experiments, we examined the potential involvement of K_{ATP} channels on the inhibitory action of the H_2S donors on K^+-evoked [^{3}H]D-aspartate release. There is evidence that glibenclamide, a K_{ATP} channel inhibitor can antagonize the pharmacological actions of H_2S producing compounds in several tissues [34]. Consequently, we investigated the effect of glibenclamide (300 µM) on the inhibitory responses elicited by the H_2S producing compounds. Although glibenclamide (300 µM) had no effect on K^+-induced [^{3}H]D-aspartate release, it reversed the inhibitory action of L-cysteine and GYY 4137 on neurotransmitter release (Fig. 4, Panels a, b). Interestingly, glibenclamide had no significant ($P > 0.05$) effect on the inhibitory action of *N*-acetylcysteine on neurotransmitter release (data not shown).

Effect of Inhibitors of Nitric Oxide Synthase

The gaseous transmitter, nitric oxide (NO) has been reported to interact with H_2S in various mammalian tissues and organs as reviewed by [35]. Therefore, in a series of experiments, we examined the effect of a non-specific inhibitor of nitric oxide synthase (NOS), L-NAME on the inhibitory responses elicited by L-cysteine and GYY 4137 on K^+-evoked [^{3}H]D-aspartate release. On its own, L-NAME (300 µM) had no effect on K^+-induced [^{3}H]D-aspartate release. However, L-NAME (300 µM) completely reversed the inhibitory effects of L-cysteine and GYY 4137 on [^{3}H] D-aspartate release (Fig. 5, Panels a, b) .

To further elucidate the role of NO in the inhibitory effect of H_2S-releasing compounds, we investigated the effect of the specific inhibitor of inducible NOS (iNOS), aminoguanidine on the inhibitory response elicited by L-cysteine [36, 37]. Although aminoguanidine (10 µM) had no effect on the neurotransmitter release, it reversed the inhibitory effects of L-cysteine (1 and 10 µM) on [^{3}H]D-aspartate release (Fig. 6).

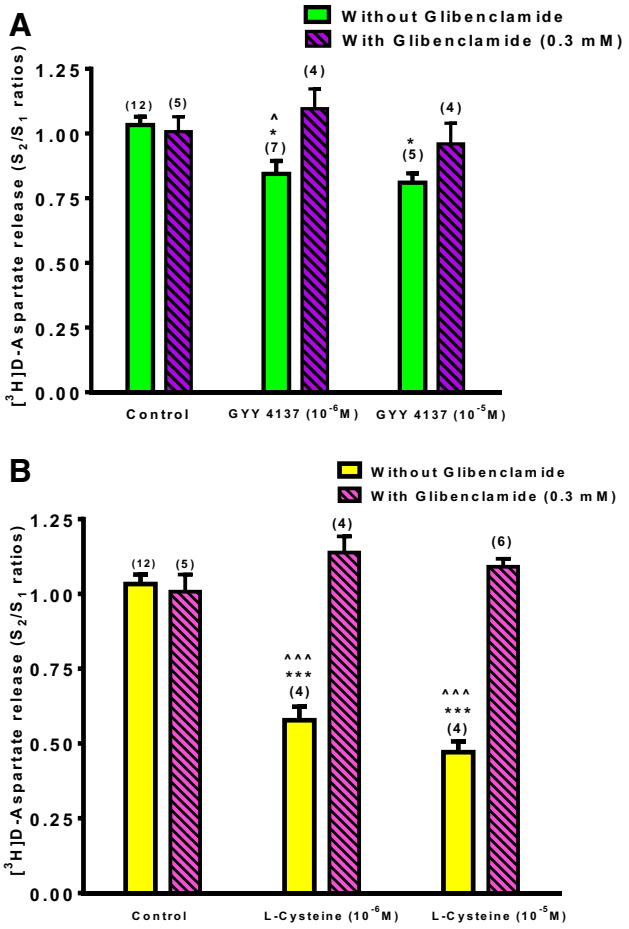

Fig. 4 Effect of the K_{ATP} channel blocker, glibenclamide (0.3 mM) on the inhibitory effect of hydrogen sulfide (H_2S) releasing compounds on K^+-evoked [^{3}H]D-aspartate release from isolated, bovine retinae. **a** control or GYY 4137 (10^{-6} and 10^{-5} M) in presence or absence of glibenclamide (0.3 mM); **b** control or L-cysteine (10^{-6} and 10^{-5} M) in presence or absence of glibenclamide (0.3 mM). Vertical bars represent means ± SEM. Number of observations is in parenthesis. *$P < 0.05$; ***$P < 0.001$ significantly different from the control; ^$P < 0.05$; ^^^$P < 0.001$ significantly different from H_2S releasing compound in presence of glibenclamide (0.3 mM)

Discussion

In the central nervous system, H_2S has been reported to play neuro-modulatory and neuroprotective roles by virtue of its effect on synaptic transmission [2, 3] and its preventive action against toxic insults such as oxidative stress, excitotoxicity and neuronal injury [4–6, 38]. H_2S has also been shown to play a role in the pathophysiology of neurodegenerative disease such as Alzheimer's disease, Parkinson's disease, vascular dementia, Huntington's disease and amyotrophic lateral sclerosis [9–11]. The three enzymes responsible for the biosynthesis of H_2S from amino acids, L-cysteine and D-cysteine (i.e., CBS, CSE and 3MST) have been localized in tissues of the eye [12–14]. A deficiency in the activity of CBS has been implicated in some eye diseases such as glaucoma and retinal detachment [15]. On the other hand, high concentrations of H_2S have been reported in the vitreous body and plasma of patients with diabetic retinopathy [18]. Taken together, these observations support a pathophysiological role for H_2S in the eye. Studies to determine the pharmacological actions of H_2S in biological systems have utilized sulfide salts such as NaHS and Na_2S as gas donors because of their ability to generate H_2S in vivo [2]. These sulfide salts have been shown to release large amounts of H_2S in a short duration of time. In contrast, the release of endogenous H_2S from cells may occur at a much slower rate than that from sulfide salts [39, 40]. Indeed, compounds

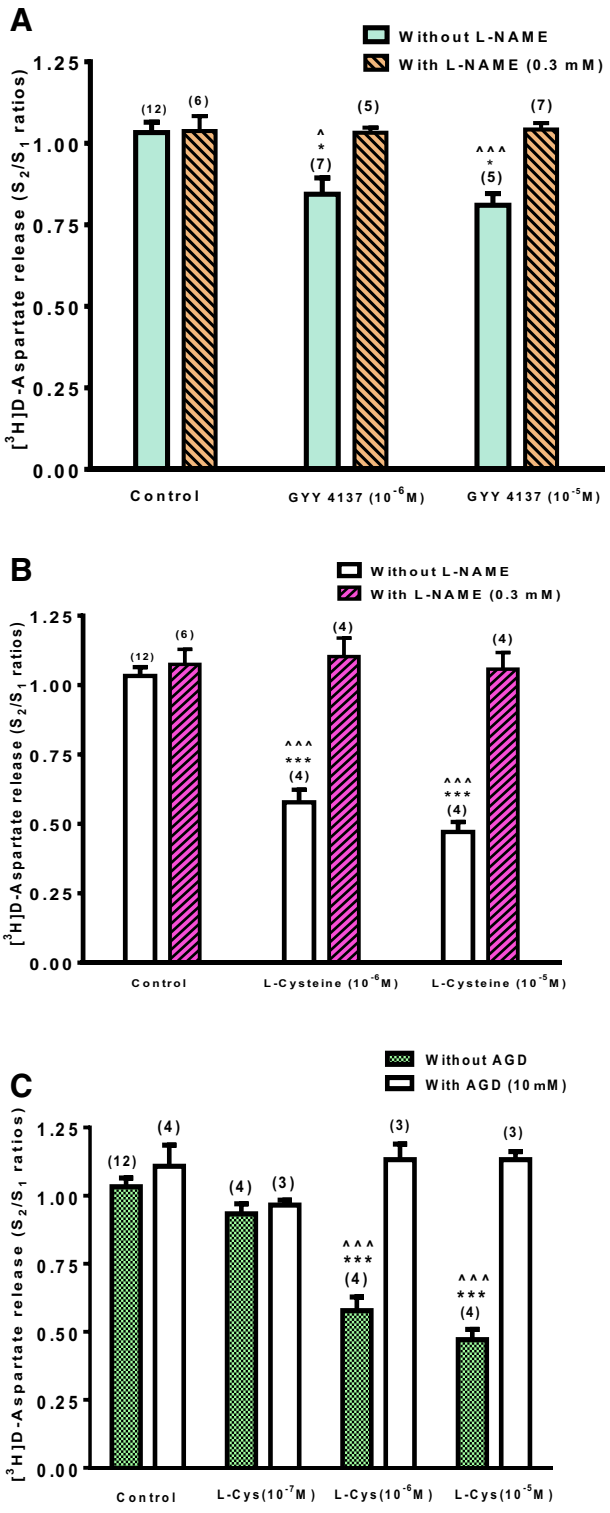

Fig. 5 Effect of the non-specific nitric oxide synthase (NOS) inhibitor, L-NAME (0.3 mM) and the inducible NOS (iNOS) inhibitor, aminoguanidine (10 μM) on the inhibitory effect of hydrogen sulfide (H_2S) releasing compounds on K^+-evoked [^{3}H]D-aspartate release from isolated, bovine retinae. **a** control or GYY 4137 (10^{-6} and 10^{-5} M) in presence or absence of L-NAME (0.3 mM). **b** control or L-cysteine (10^{-6} and 10^{-5} M) in presence or absence of L-NAME (0.3 mM). **c** control or L-cysteine (10^{-7} to 10^{-5} M) in presence or absence of aminoguanidine (10 μM). Vertical bars represent means ± SEM. Number of observations is in parenthesis. *P < 0.05; **P < 0.001 significantly different from the control; ^P < 0.05; ^^^P < 0.001 significantly different from H_2S releasing compound in presence of NOS inhibitor

have been synthesized (e.g., GYY 4137, AP67, AP72) that can release H_2S slowly in biological systems [40, 41]. Using NaHS and Na_2S as gas donors, we have evidence that H_2S can relax both mammalian irides and long posterior ciliary arteries [19–22] and can increase cyclic AMP production in bovine and porcine isolated retinae and retinal pigment epithelial cells [23, 24]. Both NaHS and Na_2S inhibited electrically-induced [^{3}H]-norepinephrine release and reduced basal catecholamine levels in porcine isolated irides [25] and K^+-evoked [^{3}H]D-aspartate overflow from bovine and porcine retinae, in vitro [26]. In the present study, we examined the effects of three compounds on K^+-depolarization induced release of [^{3}H]D-aspartate from bovine isolated retinae: a slow-releasing H_2S donor, GYY 4137; a substrate for H_2S biosynthesis, L-cysteine; and a precursor of L-cysteine, *N*-acetylcysteine. We employed [^{3}H]D-aspartate as a marker for glutamate because this amino acid has been reported to utilize the same transport system as L-glutamate and L-aspartate and, therefore, it has been presumed to be an appropriate marker for neurons that employ glutamate and/or aspartate as neurotransmitters in the retinae [31, 32, 42].

In the present study, all three compounds tested (GYY 4137, L-cysteine and *N*-acetylcysteine) inhibited K^+-evoked [^{3}H]D-aspartate release from bovine isolated retinae without affecting basal tritium efflux. At an equimolar concentration of 10 μM, the rank order of inhibitory activity on K^+-evoked [^{3}H]D-aspartate release was: L-cysteine > GYY 4137 > *N*-acetylcysteine. The ability of the slow-releasing H_2S compound, GYY 4137 to attenuate [^{3}H]D-aspartate release is consistent with earlier observations made using fast-releasing gas compounds such as NaHS and Na_2S in bovine and porcine isolated neural retinae [26]. It is pertinent to note that H_2S-releasing compounds have also been shown to inhibit [^{3}H]-norepinephrine from porcine isolated iris-ciliary bodies [25]. It is, however, unclear whether the mechanisms involved in the inhibition of neurotransmitter release by H_2S-releasing compounds in noradrenergic and glutamatergic neurons are identical. Be that as it may, the finding that both the slow-releasing H_2S compounds and substrates of the H_2S biosynthetic pathways (L-cysteine and *N*-acetylcysteine) inhibited K^+-depolarization evoked [^{3}H]D-aspartate overflow suggest that this gas exerts a negative regulatory role on glutamatergic transmission in the retinae. Interestingly, the ability of H_2S to regulate neurotransmission has been described in various biological systems such as the cat carotid body, pig intravesical ureter, brain and endocrine system [7, 8, 43–45].

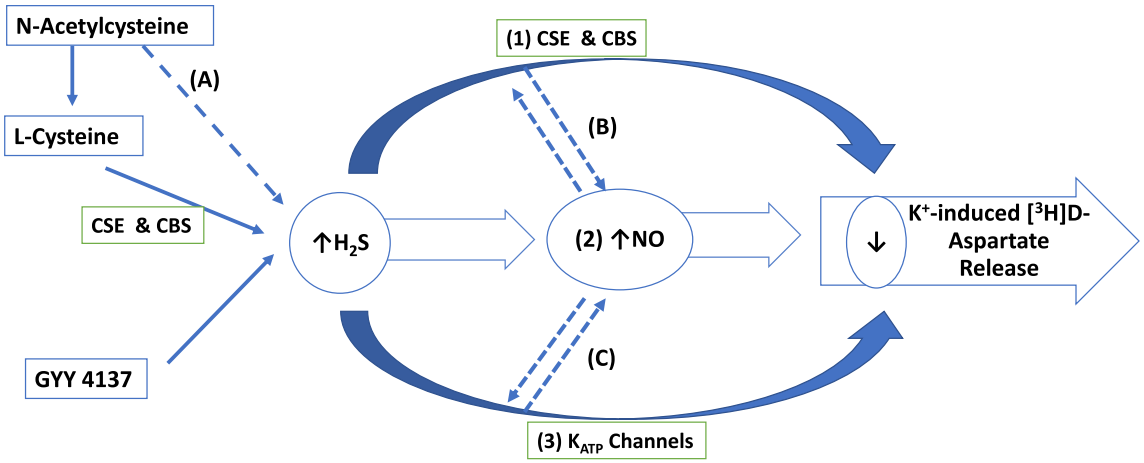

Fig. 6 Schematic representation of putative mechanisms by which hydrogen sulfide (H_2S) regulates excitatory neurotransmitter release in isolated bovine retina. The slow releasing H_2S donor, GYY 4137, and the substrate for endogenous production of H_2S, L-cysteine and its prodrug, *N*-acetylcysteine produce the gasotransmitter to attenuate K^+-induced [^{3}H]D-aspartate release in bovine retina. This effect is facilitated by mechanisms that involve either (1) the H_2S biosynthetic enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) enzymes; (2) endogenous generation of nitric oxide (NO) gasotransmiter; or (3) opening of potassium (K)ATP (K_{ATP}) channels. *N*-Acetylcysteine is presumed to release L-cysteine, in situ, which then attenuates neurotransmitter release. However, the K_{ATP} antagonist did not reverse its effect, suggesting that other mechanisms may be involved in the effect elicited by *N*-acetylcysteine (A). The interaction between NO and CSE/CBS (B) and/or K_{ATP} channels (C) has not been completely elucidated in these tissues

It has been well established that H_2S can be produced by three enzymes, CBS, CSE and 3MST, along with cysteine aminotransferase (CAT), which is identical to aspartate aminotransferase (as reviewed by [46]). In 2013, Asimakopoulou et al. showed evidence that, amino-oxyacetic acid (AOA) can inhibit both CBS and CSE activities [33]. In the present study, we found that while AOA had no effect on basal tritium efflux, it blocked the inhibitory action of GYY 4137, L-cysteine and *N*-acetylcysteine on K^+-induced [^{3}H] D-aspartate release without affecting basal tritium efflux. A blockade of NaHS-induced attenuation of K^+-evoked [^{3}H] D-aspartate release in isolated mammalian retinae and field-stimulated [^{3}H]-norepinephrine overflow in isolated porcine irides by the CSE inhibitor, propargylglycine has also been reported by Opere et al. [26] and Kulkarni et al. [25]. It is noteworthy that AOA blocked responses elicited by both the H_2S-releasing compound and substrates in the present study indicating that endogenously produced H_2S could account, at least in part, for the observed inhibitory action of these compounds on glutamatergic neurotransmission in the bovine retinae.

A potential mechanism of action of H_2S in mammalian tissues and organs is via its action on ion channels such potassium or calcium channels [47, 48]. In the vasculature, ATP-sensitive K^+ channels or voltage-dependent K^+ channels have been implicated in the relaxations induced by H_2S donors such as NaHS [21, 22, 49]. In the present study, the K_{ATP} channel inhibitor, glibenclamide blocked the inhibitory effect of GYY 4137 and L-cysteine on K^+-evoked [^{3}H] D-aspartate release from bovine isolated retinae without affecting basal tritium efflux. Glibenclamide has also been reported to block NaHS-induced decrease in insulin release from Syrian hamster pancreatic β-cells (HIT-T15 cells) [50]. These authors found that NaHS, by increasing K^+ efflux will lead to hyperpolarization which then prevents the opening of voltage-gated calcium channels. The subsequent prevention of calcium influx by NaHS leads to a decrease in the release of insulin from the HIT-T15 cells [50]. The observation in the present study that the inhibitory action of a H_2S-releasing compound and its substrate on glutamatergic neurotransmission can be antagonized by glibenclamide supports the data reported by Ali et al. [50] that K_{ATP} channels are involved in the effects of H_2S on neurotransmission. Glibenclamide has also been shown by us and other investigators to block the pharmacological actions of H_2S-releasing compound in ocular and non-ocular tissues [21, 22, 49–52]. Surprisingly, we found that the inhibition of K^+-induced [^{3}H]D-aspartate overflow by the L-cysteine precursor, *N*-acetylcysteine was not blocked by glibenclamide. It is unclear why glibenclamide had no effect on the inhibition of evoked excitatory amino acid transmission caused by *N*-acetylcysteine. It may well be that at the concentration of *N*-acetylcysteine employed in the present study, this compound may involve other non-specific actions such as those affecting the cystine-glutamate transporter [53].

It is well known that H_2S can interact with other gaseous molecules such as NO and CO to produce physiological and pharmacological actions in biological tissues and organs [34]. In addition to interaction at the level of enzyme activity between gaseous molecules such as H_2S and NO, Whiteman

et al. proposed that a chemical reaction between these gases can occur leading to the formation of nitrosothiols [54]. In the eye, an interaction between H_2S and NO has been reported in rabbit ophthalmic artery [55] and bovine isolated posterior ciliary arteries [26]. In the present study, the NOS inhibitor, L-NAME blocked the inhibitory actions of GYY 4137 and L-cysteine on K^+-evoked [^{3}H]D-aspartate release without affecting basal tritium efflux. Furthermore, the specific inhibitor of iNOS, aminoguanidine antagonized the inhibitory effect of L-cysteine on K^+-depolarization-induced [^{3}H]D-aspartate release. A similar observation was made by Moustafa and Habara in rat pancreatic acini where NaHS induced an increase in intracellular calcium release was inhibited by L-NAME [56]. These authors also showed evidence that H_2S can directly stimulate NO production in a dose-dependent manner [56]. Taken together, our findings support the view that H_2S and NO can act synergistically to inhibit glutamatergic neurotransmission in the bovine isolated retinae. Data obtained in experiments with aminoguanidine suggests that the iNOS could be involved in the response elicited by L-cysteine in the bovine isolated retinae. Since excessive glutamate release has been implicated in neurotoxic processes leading to neurodegeneration, it is conceivable that H_2S and NO donors could find therapeutic application in ocular neuropathies such as glaucoma and age-related macular degeneration. Figure 6 provides a schematic representation of the possible H_2S interactions that lead to attenuation of [^{3}H]D-aspartate release in isolated bovine retinae.

There is evidence that K^+-stimulated release of [^{3}H]D-aspartate in mammalian retinae dependent on calcium homeostasis [31, 57]. In a previous study, we reported that the fast releasing H_2S donor, NaHS inhibited glutamatergic neurotransmission in bovine and porcine isolated retinae, a response that was dependent, at least in part, on the intramural biosynthesis of H_2S [26]. The present observation that both a slow-releasing H_2S donor and L-cysteine (a substrate for H_2S biosynthesis) can inhibit K^+-depolarization induced [^{3}H]D-aspartate release suggests that this gas may serve a protective role in preventing damage to retinal neurons under conditions that involve excessive glutamate production leading to calcium overload.

We conclude that both the slow-releasing H_2S donor, GYY 4137 and a substrate for the production of this gas, L-cysteine can inhibit K^+-depolarization-induced release of [^{3}H]D-aspartate from the bovine isolated retinae. The pharmacological actions elicited by these compounds on glutamatergic neurotransmission is dependent, at least in part, upon the intramural biosynthesis of this gas, and on the activity of K_{ATP} channels and NOS activity. The exact mechanism/s that underlie the effect of H_2S on the pathway leading to the release of glutamate from retinal neurons merits further investigation.

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