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REVIEW ARTICLE

Hydrogen sulphide as a signalling molecule regulating physiopathological processes in gastrointestinal motility

Correspondence Dr Marcel Jiménez, Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Barcelona, Spain. E-mail: marcel.jimenez@uab.es

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M Jimenez[1,2], V Gil[1], M Martinez-Cutillas[1], N Mañé[1] and D Gallego[1,2]

[1]*Department of Cell Biology, Physiology and Immunology and Neuroscience Institute, Universitat Autònoma de Barcelona, Barcelona, Spain, and* [2]*Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Instituto de Salud Carlos III, Barcelona, Spain*

The biology of H_2S is a still developing area of research and several biological functions have been recently attributed to this gaseous molecule in many physiological systems, including the cardiovascular, urogenital, respiratory, digestive and central nervous system (CNS). H_2S exerts anti-inflammatory effects and can be considered an endogenous mediator with potential effects on gastrointestinal motility. During the last few years, we have investigated the role of H_2S as a regulator of gastrointestinal motility using both animal and human tissues. The aim of the present work is to review published data regarding the potential role of H_2S as a signalling molecule regulating physiopathological processes in gastrointestinal motor function. H_2S is endogenously produced by defined enzymic pathways in different cell types of the intestinal wall including neurons and smooth muscle. Inhibition of H_2S biosynthesis increases motility and H_2S donors cause smooth muscle relaxation and inhibition of propulsive motor patterns. Impaired H_2S production has been described in animal models with gastrointestinal motor dysfunction. The mechanism (s) of action underlying these effects may include several ion channels, although no specific receptor has been identified. At this time, even though there is much experimental evidence for H_2S as a modulator of gastrointestinal motility, we still do not have conclusive experimental evidence to definitively propose H_2S as an inhibitory neurotransmitter in the gastrointestinal tract, causing nerve-mediated relaxation.

Abbreviations

3-MPST, 3-mercaptopyruvate sulfurtransferase; AOAA, amino-oxyacetic acid; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; Ethe1, sulphur dioxygenase [ethylmalonic encephalopathy 1]; GI, gastrointestinal; HA, hydroxylamine; ICC, interstitial cells of Cajal; K_{ATP} channels, ATP-sensitive potassium channels; KO, knockout; L-NNA, N^{ω}-nitro-L-arginine; MP, myenteric plexus; nNOS, neuronal NOS; NSAIDs, non-steroidal anti-inflammatory drugs; ODQ, 1*H*-[1,2,4]oxadiazolo [4,3-*a*]quinoxalin-1-one; PAG, L-propargylglycine; PLP, pyridoxal phosphate; RMP, resting membrane potential; sGC, soluble guanylyl cyclase; SK_{Ca}, small-conductance calcium-activated potassium channels; SMCs, smooth muscle cells; SMP, submuscular plexus; SQR, sulphide quinone reductase; TTX, tetrodotoxin

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Introduction

Hydrogen sulphide (H_2S) is a toxic gas that may lead to inhibition of the mitochondrial cytochrome c oxidase (Reiffenstein *et al.*, 1992). However, it is also an endogenous gasomediator with potential physiological roles in a wide range of systems, including the cardiovascular, urogenital, respiratory, digestive systems and the CNS (Abe and Kimura, 1996; Patacchini *et al.*, 2004; Trevisani *et al.*, 2005; Yang *et al.*, 2008; d'Emmanuele *et al.*, 2009; Wallace *et al.*, 2010; Gur *et al.*, 2015). In the vascular system, H_2S acts as an inhibitory endothelium-derived factor with similar functions to **NO**, causing smooth muscle relaxation and hypotension (Skovgaard *et al.*, 2011). Regarding the gastrointestinal (GI) tract, H_2S has been proposed as an anti-inflammatory mediator (Fiorucci and Distrutti, 2011; Vandiver and Snyder, 2012; Takeuchi *et al.*, 2015; Wallace *et al.*, 2015) and as an endogenously synthesized molecule through-specific enzymic pathways with potential effects on GI motility (Jimenez, 2010). H_2S produced by luminal bacteria has the potential to modify GI function and participates in motility disorders when intestinal microbiota is altered. The epithelium plays an important role as a barrier between the internal and external milieu. Nowadays, many authors consider H_2S to be an inhibitory neurotransmitter in the GI tract with functions similar to those of NO. However, this needs a discussion based on experimental data. During the last years, our research group has been investigating the role of H_2S as a regulator of GI motility using both animal and human tissues. The aim of the present review is to analyse published data regarding the potential role of H_2S as a signalling molecule regulating physiopathological processes in GI motility.

Synthesis of H_2S in the GI tract

In mammalian cells, two **pyridoxal phosphate** (PLP)-dependent enzymes are responsible for the synthesis of H_2S from the amino acid **L-cysteine**: **cystathionine β-synthase (CBS)** and **cystathionine γ-lyase (CSE)** (Cavallini *et al.*, 1962; Braunstein *et al.*, 1971; Stipanuk and Beck, 1982; Yang *et al.*, 2008). A third route of H_2S synthesis involving L-cysteine is the one performed by the enzyme **2-oxoglutarate aminotransferase** in cooperation with **3-mercaptopyruvate sulfurtransferase (3-MPST)** (Stipanuk and Beck, 1982; Shibuya *et al.*, 2009a, b) (Table 1). Recently, a new pathway for H_2S biosynthesis has been reported using **D-cysteine** as a substrate (Shibuya *et al.*, 2013). Although the mechanisms regulating H_2S release remain unclear, it has been proposed that H_2S might be synthesized on demand or, alternatively, released from sulphur stores in response to physiological signals. Selective activation of CSE by calcium–**calmodulin** has been suggested (Yang *et al.*, 2008) although opposite results have also been published (Mikami *et al.*, 2013). Release of H_2S in response to reducing conditions has been reported as well (Ishigami *et al.*, 2009; Kimura, 2010). In the latter, H_2S might be stored in the cytoplasm as bound sulphane sulphur, a divalent sulphur bound with other sulphur atoms present in intracellular proteins (Ishigami *et al.*, 2009; Kimura, 2010).

Both CBS and CSE are localized along the entire GI tract in mammals (Table 2). CSE is expressed in neurons of both the submucosal (SMP) and myenteric plexuses (MPs) as well as in certain subclasses of interstitial cells of Cajal (ICC) (Linden *et al.*, 2008; Schicho *et al.*, 2006). Both enzymes are expressed in the epithelium and muscle wall in the rat colon (Hennig and Diener, 2009; Gil *et al.*, 2011). CBS immunoreactivity is detected in enteric neurons from guinea pigs and humans (Schicho *et al.*, 2006; Quan *et al.*, 2015). Similar results have been reported in the murine colon with expression of these two enzymes in a wide variety of cellular types (Linden *et al.*, 2008; Hennig and Diener, 2009; Martin *et al.*, 2010; Liu *et al.*, 2013). 3-MPST and CSE are expressed in smooth muscle cells (SMCs) isolated from the rabbit stomach, suggesting that both enzymes might participate in H_2S

Table 1

Enzymes responsible for H_2S production in mammalian cells

Nomenclature (EC number)	Common Abbreviation	Endogenous substrates	Cofactors	Inhibitors
Cystathionine β-synthase (4.2.1.22)	CBS	L-cysteine L-homocysteine	Pyridoxal phosphate	AOAA
Cystathionine γ-lyase (4.4.1.1)	CSE	L-cysteine	Pyridoxal phosphate	**Aminoethoxyvinylglycine** AOAA **β-cyano-L-alanine** **Propargylglycine**
L-cysteine : 2 oxoglutarate aminotransferase (4.4.1.13)	CAT	L-cysteine	Pyridoxal Phosphate	Compound 3[a]
3-mercaptopyruvate sulfurtransferase (2.8.1.2)	3-MPST	**3-mercaptopyruvic** acid	Zn^{2+}	–

CAT and 3-MPST function in combination to generate H_2S.
Adapted from (Alexander *et al.*, 2015a).
CAT, Cysteine:2-oxoglutarate aminotransferase.
[a]Toutle *et al.* 2013.

Table 2

Distribution of CSE, CBS and 3-MPST in the GI tract

Species	Organ	Enzyme	Cell type/layer	Method	References
Mouse	Stomach	CBS/CSE	SMC	IHC/WB	Han *et al.*, 2011 Huang *et al.*, 2013
	Small intestine	CBS/CSE	SMC (tunica muscularis)	WB	Guo *et al.*, 2012
	Colon	CBS	Lamina propia	RT-PCR/IHC	Linden *et al.*, 2008
		CSE	MP (lamina propia and SMC diffuse)		
Guinea Pig	Ileum and Colon	CBS	MP,SMC	IHC	Schicho *et al.*, 2006
		CSE	MP, SMP, ICC		
Human	Colon	CBS/CSE	SMP	IHC	Schicho *et al.*, 2006
		CBS/CSE	Epithelium	WB	Martin *et al.*, 2010
Rat	Stomach	CBS/CSE	Epithelium (mucosa)	RT-PCR	Fiorucci *et al.*, 2005
	Jejunum	CBS/CSE	MP	IHC	Kasparek *et al.*, 2012
	Colon	CBS/CSE	Epithelium, SMC	IHC	Hennig and Diener, 2009
		CBS	Muscularis mucosa, SMP, lamina propia	IHC/WB	Martin *et al.*, 2010
		CSE	General diffuse		
		CBS	Epithelium, SMC (diffuse)	IHC	Gil *et al.*, 2011
		CSE	MP, SMP, SMC (mucosa and submucosa diffuse)		
		CBS	MP, epithelium (SMC diffuse)	IHC	Liu *et al.*, 2013
		CSE	MP, SMC (mucosa and submucosa diffuse)		
Rabbit	Stomach	CSE/3-MPST	SMC	RT-PCR/WB	Nalli *et al.*, 2015

Enzymes: CSE, 3-MPST. Techniques: IHC, immunohistochemistry; RT-PCR; WB, Western blot. Cells/layers: SMC, MP, SMP, ICC.

synthesis in smooth muscle (Nalli *et al.*, 2015). According to these data, several types of intestinal cells possess the enzymic machinery to produce H_2S (Table 2).

Experimental approaches to investigate the functional role of H_2S in GI function

An important limitation in the characterization of the role played by H_2S in GI motility is the lack of a clearly identified receptor to be targeted as a possible pharmacological approach. For NO, in contrast, although it has many signalling pathways, **soluble guanylyl cyclase (sGC)** has been identified as its primary target leading to smooth muscle hyperpolarization and relaxation in the GI tract (Lies *et al.*, 2013, 2014; Mane *et al.*, 2014b). However, several experimental approaches have been used to investigate the putative role of H_2S in GI physiology.

H_2S synthesis inhibition

The first approach is to characterize the role of endogenous H_2S by blocking its production. This can be achieved through the use of H_2S synthesis inhibitors (Table 1). **L-propargylglycine** (PAG), an inhibitor of CSE; **amino-oxyacetic acid** (AOAA), an inhibitor of both CBS and CSE; and hydroxylamine (HA), a CBS inhibitor (Wang, 2002; Szabo, 2007; Linden *et al.*, 2010), are the most commonly used inhibitors of H_2S biosynthesis. AOAA and HA are non-selective PLP-dependent enzyme inhibitors, whereas PAG is an irreversible inhibitor of CSE (John and Charteris, 1978; Sun *et al.*, 2009; Linden *et al.*, 2010). These compounds have been widely used in experiments with tissue homogenates and at a cellular level (Stipanuk and Beck, 1982; Hosoki *et al.*, 1997; Szabo, 2007; Linden *et al.*, 2010). This experimental approach has the limitation of the selectivity of the pharmacological tools available and the presence of multiple pathways of H_2S synthesis (Whiteman *et al.*, 2011; Asimakopoulou *et al.*, 2013).

Genetically modified animals

Another experimental approach that blocks H_2S production is the use of genetically modified animals that lack a specific synthesis pathway. This is an interesting approach since CSE knockout (KO) mice have been used to demonstrate that endogenous H_2S maintains a smooth muscle relaxation and hypotension in the vascular system (Yang *et al.*, 2008), although hypertension was not observed in CSE KO mice used in a similar study (Ishii *et al.*, 2010). This might be due to the fact that different H_2S synthesis pathways are present

in the GI tract and the three gasomediators [NO, **carbon monoxide** (CO) and H_2S] might have overlapping and interacting functions.

H_2S donors

A third approach is the use of compounds that increase the concentration of H_2S. This can be achieved by using H_2S donors such as **sodium hydrosulphide (NaHS)** or H_2S slow-releasing organic compounds and H_2S precursors such as L-cysteine or by blocking the degradation pathway of H_2S. NaHS is widely used to study the biological effects of H_2S (Hosoki *et al.*, 1997; Wang, 2002; Szabo, 2007; Linden *et al.*, 2010). In the case of NaHS and L-cysteine, one of the crucial points of discussion is the concentration of the compound. The limit between physiological, pharmacological and even toxic concentrations is unknown. Moreover, the effects obtained with NaHS incubation are not always equivalent to those obtained with promotion of endogenous H_2S synthesis (Figure 1). L-cysteine might be binding to other receptors in the plasma membrane of different cell types.

H_2S and smooth muscle contractility

Experiments performed with colonic samples in which the mucosa and submucosa were removed demonstrated that H_2S can be enzymically produced from L-cysteine in the mouse and rat colon (Linden *et al.*, 2008; Gil *et al.*, 2011). PAG and AOAA significantly reduced H_2S production (Linden *et al.*, 2008; Gil *et al.*, 2011). Therefore, these experiments demonstrate that under these experimental conditions, H_2S is endogenously produced by defined enzymic pathways in the colonic wall.

In vitro, intestinal preparations have the ability to 'spontaneously' release inhibitory neurotransmitters from enteric motor neurons (Gil *et al.*, 2010). Therefore, incubation with the neuronal blocker **tetrodotoxin** (TTX) causes smooth muscle depolarization and enhances the frequency and amplitude of spontaneous contractions due to the inhibition of the neuronal inhibitory tone. Similar results are observed with the inhibitor of neuronal NOS (**nNOS**), N^{ω}-nitro-l-arginine (**L-NNA**) or the sGC blocker **ODQ**, showing that NO is the responsible for the inhibitory neuronal tone (Gil *et al.*, 2010). If H_2S also contributes to smooth muscle inhibition, smooth muscle depolarization and increase of tone and/or an increase in spontaneous contractions should be observed after inhibition of H_2S synthesis. Interestingly, PAG causes smooth muscle depolarization and increases the frequency of spontaneous contractions in rat colonic circular muscle (Figure 1), whereas AOAA caused a mild increase in muscle contraction without major changes in the membrane potential (Gil *et al.*, 2013). A second study reported that both PAG and AOAA increased spontaneous contractions in both circularly and longitudinally oriented rat colonic preparations (Liu *et al.*, 2013). This suggests that H_2S, synthesized by CSE and possibly also by CBS, is tonically inhibiting colonic motility. Interestingly, the depolarization and motility increase observed with PAG are still observed after neuronal blockade with TTX (Gil *et al.*, 2011), suggesting that H_2S synthesis is not dependent on sodium-mediated action potentials in neurons and, therefore, a potential non- neuronal source of H_2S might be present in the GI tract.

In human colonic samples, the presence of an inhibitory neuronal tone is still under discussion (Jimenez *et al.*, 2014). Several issues such as regional differences or differences in sample handling (i.e. time interval from extraction to experimentation) can be important to detect a functional inhibitory neuronal tone *in vitro*. Incubation with PAG and AOAA causes a smooth muscle depolarization and a transient increase in tone and amplitude of spontaneous contractions (Martinez-Cutillas *et al.*, 2015), suggesting that, as previously shown in rats, H_2S contributes to an endogenous neuronal tone in human colonic samples. However, these compounds are non-selective inhibitors of CSE and CBS and show effects on several other enzymes and receptors (John and Charteris, 1978; Teague *et al.*, 2002; Szabo, 2007; Whiteman *et al.*, 2011). Therefore, the interpretation of the results obtained with these pharmacological tools must always be carried out with the support of other experimental findings (Szabo, 2007; Jimenez, 2010; Whiteman *et al.*, 2011).

It is important to note that HA is not only a H_2S-producing enzyme inhibitor but also has been described as an NO donor (Iversen *et al.*, 1994; Correia *et al.*, 2000). For instance, HA causes smooth muscle hyperpolarization leading to an inhibition of spontaneous contractility in both the rat and human colons (Gil *et al.*, 2011; Martinez-Cutillas *et al.*, 2015). This response is sensitive to the sGC inhibitor ODQ. Accordingly, we strongly recommend not using HA as an inhibitor of CBS in biological processes involving the GI tract where the role of NO is extremely relevant.

Participation of H_2S in the transwall gradient of smooth muscle membrane potential

The resting membrane potential (RMP) of the colonic muscle is graded through the colonic muscle wall; that is, SMCs located near the SMP are more hyperpolarized than the cells near the MP (Sha *et al.*, 2010). Several factors such as ICC-SMP themselves that are electrically coupled to smooth muscle or inhibitory mediators released by SMP neurons may contribute to setting this gradient in the RMP of the circular muscle. An excellent work using haem oxygenase-2-KO (Sha *et al.*, 2010) and CSE KO mice (Sha *et al.*, 2014) demonstrated that the transwall gradient is probably due to CO and potentiated by H_2S. Both NO and CO are possibly released by SMP neurons. CO and H_2S produced by the mucosa itself might also contribute to inhibit motility in the circular layer (Martin-Cano *et al.*, 2014). The consequence of this organization is that SMCs near the SMP are considerably more hyperpolarized than the cells near the MP and have the ability to oscillate at a frequency paced by ICC-SMP (Mane *et al.*, 2014b).

Effect of NaHS on GI function

In the GI tract, NaHS exerts pro-secretory effects both through neuronal mechanisms involving afferent neurons and by direct stimulation of the intestinal epithelium (Schicho *et al.*, 2006; Hennig and Diener, 2009; Krueger *et al.*, 2010; Pouokam *et al.*, 2011). Both anti-nociceptive and pro-nociceptive effects have been observed in response to NaHS when administrated

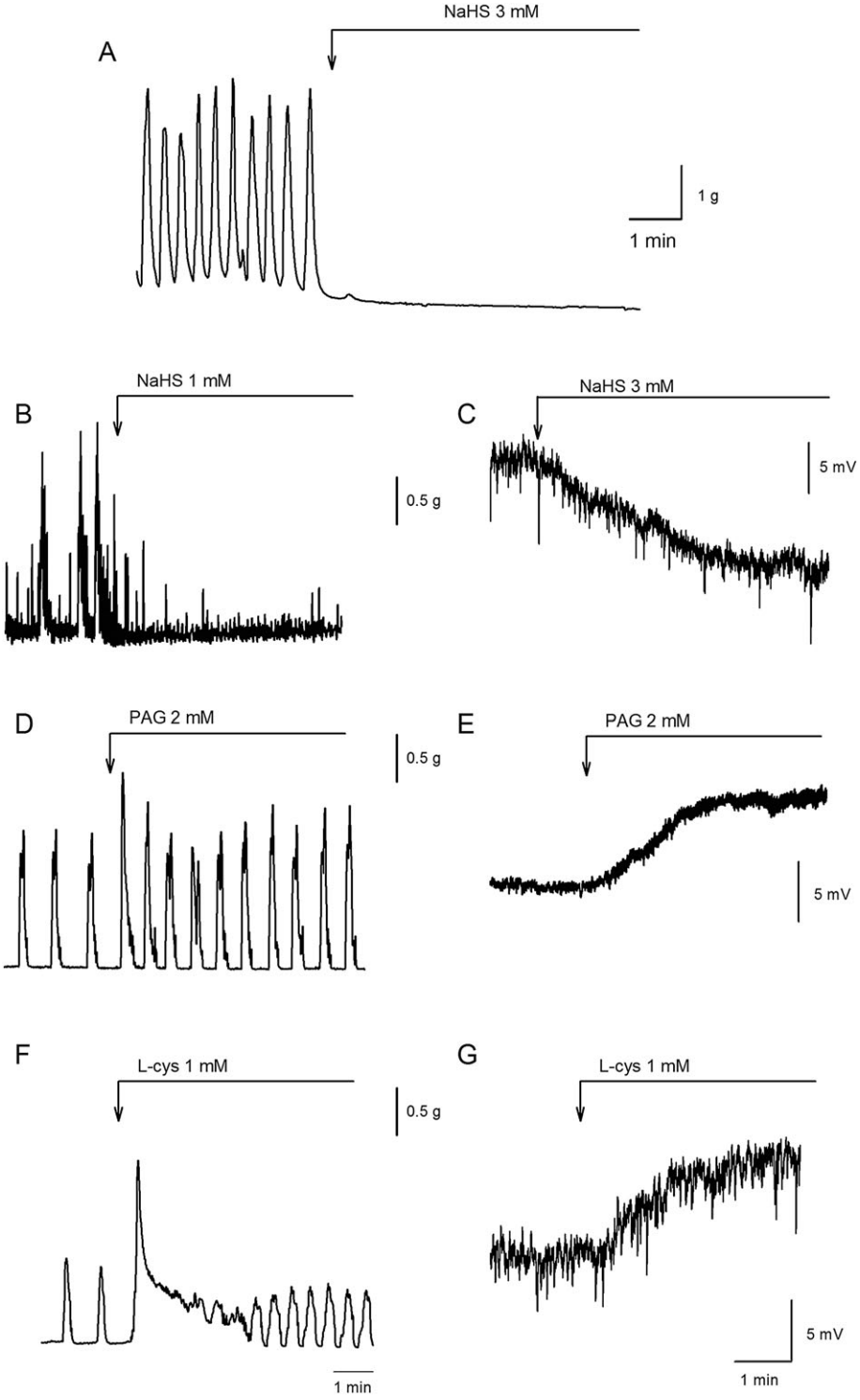

Figure 1

Muscle bath recordings showing the effect of NaHS on spontaneous contractions in the human colon [(A) NaHS at 3 mM; Martinez-Cutillas *et al.*, 2015] and rat colon [(B) NaHS at 1 mM; Gil *et al.*, 2013]. (C) Intracellular microelectrode recording showing the effect of NaHS (1 mM) on the RMP of the rat mid-colon (obtained from Gil *et al.*, 2013). Mechanical (left) and intracellular recording (right) showing the increase of spontaneous motility and the depolarization of the RMP elicited by PAG (2 mM) (D, E) and L-cysteine (L-cys; 1 mM) (F, G) in the rat colon (unpublished results).

intraperitoneally and intracolonically respectively (Distrutti *et al.*, 2006; Matsunami *et al.*, 2009; Schemann and Grundy, 2009). Anti-inflammatory properties have also been described for H_2S as administration of NaHS accelerates healing of gastric ulcers and significantly contributes to the resolution of colitis (Wallace *et al.*, 2007, 2009, 2012).

Regarding its role in modulating smooth muscle activity, contractile but more often inhibitory responses have been reported. For example, in the guinea pig and mouse stomach, NaHS causes a dual effect, producing contraction at low concentrations and relaxation at high concentrations (Zhao *et al.*, 2009; Han *et al.*, 2011). Spontaneous circular smooth muscle contractions recorded *in vitro* in rat and human colonic preparations are concentration-dependently inhibited by NaHS (Gallego *et al.*, 2008) (Figure 1). NaHS concentration-dependently relaxed circular muscle strips of mouse fundus and distal colon, contracted by PG**$F_{2\alpha}$** (Dhaese and Lefebvre, 2009; Dhaese *et al.*, 2010). NaHS also exerted relaxant effects on guinea pig, rabbit and rat ileum and jejunum preparations (Hosoki *et al.*, 1997; Teague *et al.*, 2002; Nagao *et al.*, 2011, 2012; Kasparek *et al.*, 2012). However, the concentrations of NaHS used to induce relaxation in the GI tract are high and the physiological relevance of this action is still unknown (Figures 1 and 2).

Inorganic sulphide salts such as NaHS induce a short-lasting increase in H_2S concentration that can reach non-physiological concentrations, and furthermore, they can be easily oxidized. For these disadvantages to be solved, organic slow-releasing H_2S agents such as GYY4137 have been developed. However, the effect of these compounds on GI motility has not yet been tested.

Effect of NaHS on intestinal motor patterns

NaHS inhibits peristaltic activity in the mouse small intestine and colon (Gallego *et al.*, 2008). In rats, video recordings of spontaneous active colonic segments reveal two types of movements: (i) low-frequency high-amplitude aboral propulsive motor movements and (ii) high-frequency non-propulsive low-amplitude contractions (ripples) (Huizinga *et al.*, 2011). Propulsive movements cause outflow of intraluminal contents, and consequently, their most likely function is to propel pellets in an aboral direction. In contrast, ripples probably participate in segmentation motor patterns responsible for mixing movements (Huizinga *et al.*, 2011). Both motility patterns are probably related to the presence of two pacemaker systems in the colon (Pluja *et al.*, 2001; Alberti *et al.*, 2005; Mane *et al.*, 2014b). NaHS produces a decrease of propulsive contractions without major changes on ripples (Gil *et al.*, 2013). It is important to notice that both rhythmic activities are differently affected by *smooth muscle hyperpolarization*, which is a potential effect of H_2S. A second potential effect of NaHS is *inhibition of neurally mediated excitatory responses* involving post-junctional mechanisms. A third potential effect of NaHS is a *direct effect on pacemaker activity.*

Smooth muscle hyperpolarization

Activation of ATP-sensitive potassium (**K_{ir} 6.1 and 6.2**; K_{ATP}) channels by H_2S has been proposed in a wide variety of studies with vascular SMCs (Zhao *et al.*, 2001; Cheng *et al.*, 2004; Dombkowski *et al.*, 2004; d'Emmanuele *et al.*,

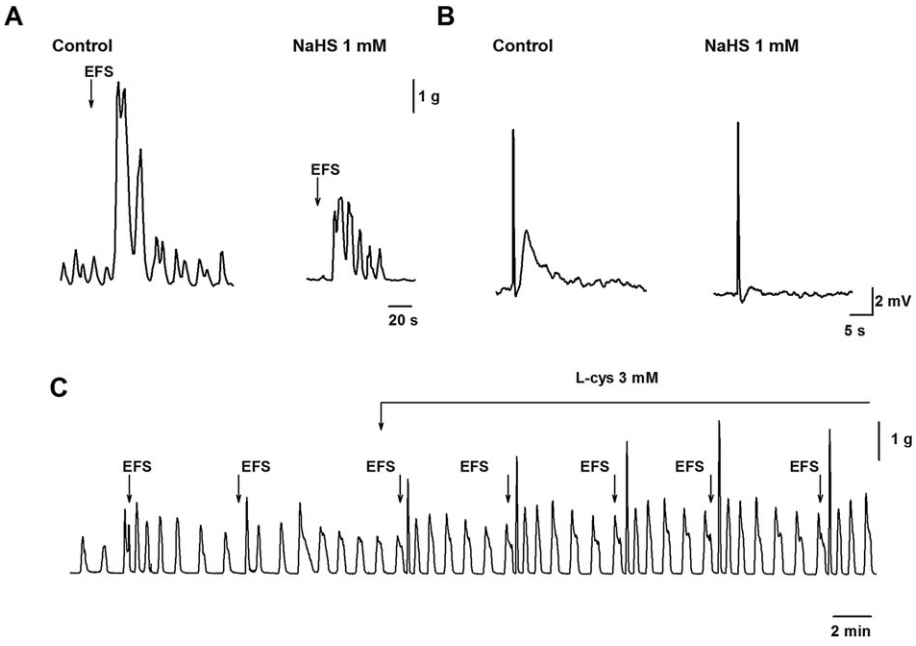

Figure 2

(A) Effect of NaHS (1 mM) on cholinergic contractions in the human colon (Martinez-Cutillas *et al.*, 2015) and on the excitatory junction potential (B) in the rat colon (Gil *et al.*, 2013). (C) Increase of the amplitude of cholinergic contractions elicited by L-cysteine (L-cys; 3 mM) in the rat colon (*unpublished results*). EFS, electrical field stimulation.

2009). Participation of these channels in the relaxant effect of NaHS has also been reported in the colon (Zhao *et al.*, 2001; Distrutti *et al.*, 2006; Gallego *et al.*, 2008; Nagao *et al.*, 2012; Liu *et al.*, 2013). Both K_{ATP} and small-conductance calcium-activated potassium (**K_{Ca}2.2/K_{Ca}2.3**; SK_{Ca}) channels might participate in smooth muscle hyperpolarization in both human and rat colonic tissues (Gallego *et al.*, 2008; Gil *et al.*, 2013). sGC may indirectly participate in the mediation of NaHS responses in the colon by releasing NO from nitrosothiols, as observed in the brain (Ondrias *et al.*, 2008). Also, H_2S inhibits phosphodiesterase activity and, therefore, accumulation of **cGMP** takes place in post-junctional cells (Bucci *et al.*, 2010). All these mechanisms might account for the crosstalk between NO and H_2S pathways. A complex interaction between H_2S and NO also occurs in the vascular system (Dunn *et al.*, 2016) where the crosstalk between the two gaseous compounds has been more extensively studied (Figure 1).

Inhibition of excitatory neurally mediated responses

In addition to muscle hyperpolarization, H_2S might also produce its inhibitory effects by inhibiting excitatory neuromuscular transmission. In the rat colon, NaHS is able to inhibit atropine-sensitive excitatory junction potentials and contractions elicited by electrical field stimulation (Gil *et al.*, 2013) (Figure 2). Similar results have been observed in the human colon where NaHS reduced both cholinergic (Figure 2) and tachykinergic neural responses. In contrast, purinergic inhibitory junction potentials were not affected (Martinez-Cutillas *et al.*, 2015). NaHS also reduced **carbachol**- and **neurokinin A**- evoked responses, suggesting that NaHS effects are at a post-junctional level (Martinez-Cutillas *et al.*, 2015). Smooth muscle contractions are calcium-calmodulin dependent and due to the activation of myosin light-chain kinase and inhibition of the myosin-light chain phosphatase (MLCP). **Rho kinase** and **protein kinase C** (PKC) inhibit MLCP, causing a sustained contraction. Exogenous (NaHS) and endogenous (L-cysteine) H_2S reduced carbachol-induced contractions in isolated rabbit gastric SMCs. This reduction is due to the activation of MLCP and inhibition of Rho kinase and PKC activities leading to the dephosphorylation of the myosin light chain and inhibition of contraction (Nalli *et al.*, 2015). These results suggest that H_2S might be inhibiting contractions by targeting specific post-junctional pathways (Figure 2).

Effect on pacemaker activity

Electrophysiological experiments and intracellular calcium analysis demonstrated that high concentrations of NaHS (0.5–1 mM) are needed to inhibit pacemaker currents in cultured ICC isolated from the mouse small intestine (Parajuli *et al.*, 2010). However, with low concentrations of NO donors, low concentrations of NaHS potentiate the inhibitory effect exerted by NO on the pacemaker system, suggesting a possible interaction between mediators (Yoon *et al.*, 2011). Despite the effects observed in isolated ICC, NaHS at a concentration of 1 mM does not modify the amplitude, duration or frequency of slow-wave activity originated in ICC-SMP in whole thickness preparations from rat colon (Gil *et al.*, 2013). In fact, neither hyperpolarization nor dihydropyridines modify electrical slow-wave activity in intact tissue (Mane *et al.*, 2014b). In contrast, propulsive contractions are reduced by NaHS (Gallego *et al.*, 2008; Gil *et al.*, 2013). This inhibition of low-frequency contractions has been attributed to the hyperpolarization of the smooth muscle (Gil *et al.*, 2013) and/or a direct effect on L-type calcium channels (**Ca_v1.2**) (Quan *et al.*, 2015) needed for the generation of the pacemaker, which is nifedipine-sensitive.

Mechanism of action

NaHS exerts its biological effects through a wide variety of mechanisms of action that include activation of cAMP-dependent pathways (Kimura, 2000); activation of the MLCP (Dhaese and Lefebvre, 2009; Nagao *et al.*, 2012); opening of K_{ATP} channels (Gallego *et al.*, 2008; Zhao *et al.*, 2009; Nagao *et al.*, 2012), SK_{Ca} channels (Gallego *et al.*, 2008), **Na_v1.5** voltage-dependent sodium channels (Strege *et al.*, 2011), **Ca_v3.2**-T-type channels (Matsunami *et al.*, 2009), **TRPV1** and TRP**A1** cation channels (Schicho *et al.*, 2006; Macpherson *et al.*, 2007; Krueger *et al.*, 2010); and inhibition of phosphodiesterase activity (Bucci *et al.*, 2010). Recently, it has been demonstrated using patch clamp experiments that NaHS inhibits L-type calcium channels in rat colonic SMCs. This effect might also be responsible for its inhibitory effect on spontaneous contractions. However, inhibition of large-conductance calcium-activated potassium channels (**K_{Ca}1.1**) has also been reported (Quan *et al.*, 2015). *Why is the effect of H_2S so diverse?* It has been hypothesized that sulfhydration of different proteins modulating a wide variety of cellular functions might explain the 'promiscuity' of H_2S (Mustafa *et al.*, 2009a, b; Paul and Snyder, 2015).

Comparison of responses to L-cysteine and to NaHS

L-cysteine is the precursor of H_2S synthesis, and as previously mentioned, it is often used to stimulate endogenous H_2S production. Ideally, the response obtained with endogenous H_2S production should be similar to the response obtained with exogenous NaHS (Nalli *et al.*, 2015). Although this might be the case in some studies, in others, different or even opposite results have been reported (Figures 1 and 2). Although NaHS inhibited contractile activity in the rat small intestine, L-cysteine did not (Nagao *et al.*, 2011; Kasparek *et al.*, 2012). Furthermore, in the rat colon, recent experiments performed in our laboratory show that, whereas NaHS inhibits motility, L-cysteine increases spontaneous contractions (Figure 1). Moreover, L-cysteine increased atropine sensitive nerve-mediated contractions (Figure 2), whereas NaHS decreased them (Figure 2). One possible explanation is that, because of the high concentrations of L-cysteine (i.e. 1 to 10 mM) needed to measure H_2S production (Gil *et al.*, 2013) and to observe an inhibitory effect (Yamane *et al.*, 2014), this amino-acid could target many receptors and channels (Kendig *et al.*, 2014).

H_2S degradation

Enzymes involved in the degradation of H_2S are crucial in the termination of H_2S signalling. H_2S can be metabolized to thiosulphate by the serial action of three mitochondrial enzymes: sulphide quinone reductase (SQR), sulphur dioxygenase [ethylmalonic encephalopathy 1 (Ethe1)] and sulphur transferase (Hildebrandt and Grieshaber, 2008; Tiranti *et al.*, 2009). This functional unit of enzymes has been described in the mitochondria of colonic epithelial cells and is probably responsible for the degradation of luminal H_2S (Mimoun *et al.*, 2012). Interestingly, SQR has been identified in the muscle layer and MP of the mouse colon. In addition, pharmacological blockade of SQR induces an increase of the tissue levels of H_2S (Linden *et al.*, 2012). However, Ethe1 and sulphur transferase have not been detected in colonic muscle cells. Therefore, it is possible that there are other downstream enzymes for the degradation of H_2S in this tissue (Linden *et al.*, 2012) although the level of H_2S degradation in the musculature is negligible, when compared with that in the mucosa (Flannigan *et al.*, 2013).

Bacteria as a potential source of H_2S in the GI tract

In the large intestine, luminal bacteria also represent a potential source of H_2S (Blachier *et al.*, 2010). However, despite the fact that high concentrations of H_2S are present in the colon (mM range), the vast majority of this H_2S is bound to luminal contents (Jorgensen and Mortensen, 2001; Levitt *et al.*, 2002). Thus, low levels (~60 μM in the human colon, measured with spectrophotometry) of free H_2S are available in the colonic lumen (Jorgensen and Mortensen, 2001; Mimoun *et al.*, 2012). Furthermore, luminal H_2S is quickly oxidized to thiosulphate by colonic epithelial cells (Furne *et al.*, 2001; Ramasamy *et al.*, 2006; Goubern *et al.*, 2007; Mimoun *et al.*, 2012). Therefore, under physiological conditions, the concentration of H_2S that reaches the submucosa and the muscle layers could be much lower. Accordingly, NaHS infused into the lumen is not able to cause motor changes in the colonic mechanical activity in rats (Gil *et al.*, 2013). Therefore, it can be hypothesized that this source of H_2S will not be able to modify colonic functions when the integrity of the barrier is preserved. Further studies are needed to evaluate if under pathological conditions that imply barrier disruption or impairment of epithelium metabolism, the H_2S produced in the lumen can reach the effector cell and consequently modify motility. Interestingly, instability in microbiota has been recently reported in patients with flatulence. In these patients, *Bacteroides fragilis* or *Bilophila wadsworthia* correlated with number of gas evacuations or volume of gas evacuated respectively. *Bilophila wadsworthia* has strong catalase activity and produces H_2S from sulphur-containing amino acids. Excessive gas including H_2S production can participate in physiopathological abdominal symptoms including distention and pain (Pozuelo *et al.*, 2015). Figure 3 is a schematic overview of the potential role of H_2S on GI function.

H_2S in motility dysfunction

Few data are available on a possible role for endogenous H_2S in GI motility dysfunction. Both central and peripheral

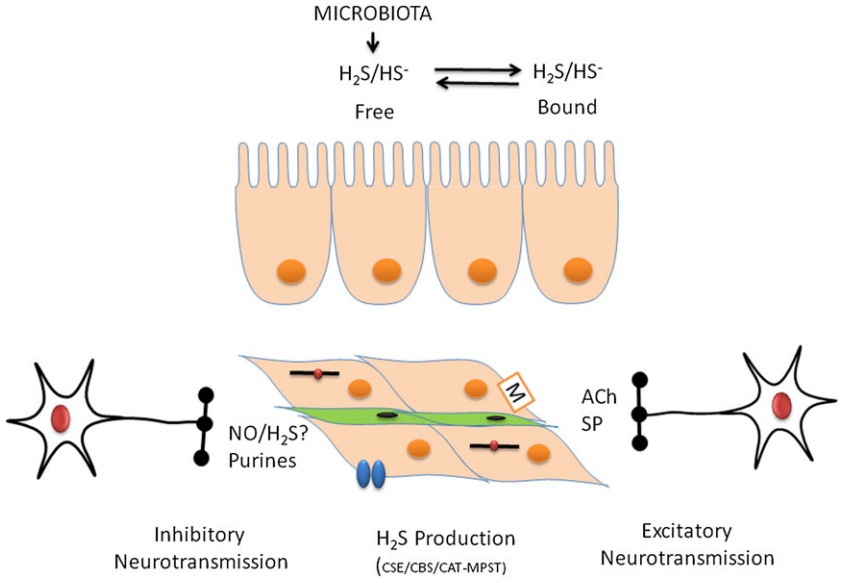

Figure 3

H_2S is produced by luminal bacteria. Enterocytes participate in H_2S detoxification. H_2S can be produced by different cell types including neurons, SMCs or interstitial cells. H_2S causes smooth muscle relaxation possibly acting on different mechanisms including the contractile apparatus, channels ($K_{ir}6$, K_{Ca}, Ca_v) and receptors. Smooth muscle hyperpolarization and inhibition of nerve-mediated contractions are potential mechanisms to inhibit propulsion. Neurally mediated relaxation is mediated by NO and purines. More experiments are needed to determine if H_2S is an inhibitory neurotransmitter in the GI tract. CAT, L-cysteine: 2-oxoglutarate aminotransferase; SP, substance P.

mechanisms may contribute to the physiopathological processes underlying esophageal motility, gastric emptying or colonic hypermotility.

Achalasia is an oesophageal motor disorder characterized by aperistalsis of the oesophageal body and impaired relaxation of the lower oesophageal sphincter. Accordingly, mechanisms that participate in pre- or post-junctional nerve-mediated relaxation could be impaired in achalasia. Lack of functional nNOS has been described in the lower oesophageal sphincter (Mearin *et al.*, 1993; Shteyer *et al.*, 2015) and a mutation in sGC disrupts NO signalling, causing achalasia (Wallace *et al.*, 2016). Regarding the H_2S pathway, reduced expression of both CBS and CSE has been reported in patients with achalasia (Zhang *et al.*, 2015). However, it is unknown if the loss of H_2S producing enzymes is the consequence of the loss of myenteric neurons (De Giorgio *et al.*, 1999).

H_2S enhances gastric emptying in rats through a peripheral mechanism that involves pyloric relaxation (Medeiros *et al.*, 2012). Neurons expressing CBS have been detected in the dorsal motor nucleus of the vagus, and central administration of NaHS inhibits gastric motility and enhances gastric secretion (Sun *et al.*, 2015). Recently, decreased H_2S production has been associated with gastroparesis in an experimental model of diabetic rats (Mard *et al.*, 2016). This is consistent with a dual effect of NaHS on gastric contractility producing contraction at low concentrations and relaxation at high concentrations (Zhao *et al.*, 2009; Han *et al.*, 2011; Mard *et al.*, 2016).

Colonic hypermotility has been associated with decreased H_2S synthesis in an experimental model of stress in rats. Under these experimental conditions, both lower H_2S production and CBS/CSE down-regulation were observed. This lower production was also accompanied by lower NaHS smooth muscle sensitivity associated with up-regulation of K_{ATP} channels (Liu *et al.*, 2013). CBS and CSE were also down-regulated in a model of partial ileal obstruction with ICC loss, although these changes have been associated with inflammation with **TNFα** as the central mediator (Guo *et al.*, 2012). Increase of H_2S during inflammation has shown to decrease the proliferation of smooth muscle during ulcerative colitis in rats (Wallace *et al.*, 2009), which will definitely also affect GI motility. More studies should be conducted to ascertain if there is a role for H_2S in abnormal GI motility and whether this gaseous mediator is a key factor in diseases affecting the GI tract or not.

H_2S as a potential therapeutic agent

The aim of the present review is not to discuss the role of H_2S as a potential therapeutic molecule. However, there is solid experimental evidence to suggest that H_2S is as a potential anti-inflammatory mediator, in combination with non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs that release H_2S have enhanced activity and/or improved safety profiles. Gaseous mediators improve blood flow, reduce oxidative stress, prevent GI mucosa injury, enhance anti-inflammatory effects of NSAIDS and promote resolution of inflammation, angiogenesis and epithelialization (see Sulaieva and Wallace, 2015).

Final remarks: can we consider H_2S an inhibitory neurotransmitter in the GI tract?

In spite of many reports of H_2S as an inhibitory gasotransmitter in the enteric nervous system, with functions similar to those of NO, we strongly believe that we do not have enough experimental evidence to support this conclusion. For H_2S to be considered an inhibitory neurotransmitter, it should be demonstrated that stimulation of inhibitory motor neurons releases H_2S and that the release is blocked by Na^+ channel blockers such as TTX. Pre-junctional calcium channel blockers such as **ω-Conotoxin GVIA** that block nerve-mediated relaxation should also block H_2S release. Moreover, it is well known that NOS inhibitors such as L-NNA decrease nerve-mediated relaxation, and to our knowledge, this has never been reported for H_2S synthesis inhibitors. Another important limitation to demonstrate the putative role of H_2S as an inhibitory gasotransmitter is the lack of a specific post-junctional receptor. A classical experimental approach with *in vitro* preparations is tissue incubation with ODQ (sGC inhibitor) that blocks nitrergic inhibitory responses, and animals with cell-specific deletion of sGC have impaired nitrergic neurotransmission (Lies *et al.*, 2014). This experimental approach identifies the receptor and possible post-junctional pathways (ICC vs. SMCs) that contribute to nitrergic nerve-mediated relaxation (Lies *et al.*, 2015). None of these experiments can be carried out if post-junctional receptors are not identified. We have recently performed a variety of experiments by using different voltage and frequencies of stimulation and by measuring electrophysiological post-junctional responses. In these, L-NNA and **MRS2500** totally blocked inhibitory responses in a wide variety of experimental conditions, and therefore, inhibitory neurotransmission in the GI tract can be said to involve NO and a purine acting on **$P2Y_1$ receptors** (Mane *et al.*, 2014a,b; Mane *et al.*, 2016). In the context of H_2S, we do not have sufficient clear experimental evidence to demonstrate that H_2S is an inhibitory gasotransmitter in the GI tract leading to nerve-mediated smooth muscle relaxation (Figure 3). Further experiments with more selective pharmacological tools are needed to identify the exact physiological role of H_2S in motor function and dysfunction.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b,c).

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Conflict of interest

The authors declare no conflicts of interest.

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