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HYDROGEN SULFIDE AS A REGULATOR OF CALCIUM CHANNELS

Luca Munaron[1, 2, 3], Daniele Avanzato[1], Francesco Moccia[4], Daniele Mancardi[5]

[1]Department of Life Sciences & Systems Biology [2]Center for Complex Systems in Molecular Biology and Medicine (SysBioM) [3]Nanostructured Interfaces and Surfaces Centre of Excellence (NIS), University of Torino, Italy, [4]Department of Biology and Biotechnologies "Lazzaro Spallanzani", University of Pavia, Italy, [5]Dept. of Clinical and Biological Sciences, University of Torino, Orbassano (TO), Italy

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*Correspondence to:
Luca Munaron, Ph.D.
Dept. Life Sciences & Systems Biology
University of Torino

Via Accademia Albertina 13

10123 Torino

ITALY

luca.munaron@unito.it

Abstract

An increasing body of evidence suggests the involvement of hydrogen sulfide (H_2S) in different physiological and pathological processes. Similarly to the other gasotransmitters nitric oxide (NO) and carbon monoxide (CO), this bioactive compound is rapidly diffusible through the biological membranes and acts in a paracrine fashion. Despite the large amount of biological actions observed *in vitro* and *in vivo* upon stimulation with H_2S donors, as well as by interfering with its synthesis, the molecular targets and mechanisms through which it exerts its intracellular effects are only partially known. A number of proteins are covalently modified by H_2S through sulfhydration of specific cysteine residues. However, only in few cases their identity has been discovered and the functional role of this post-translational modification needs to be investigated in more detail. Great attention has been devoted to potassium channels, particularly K_{ATP}, as they are considered key mediators of H_2S-induced effects, and their sulfhydration has been clearly demonstrated. Recently, different authors reported the ability of H_2S to interfere with calcium homeostasis in neurons, cardiomyocytes and endothelial cells. Since calcium signaling is involved in all cell processes, these observations attracted increasing attention from basic biology and medicine. Although some effects of H_2S on calcium signals can be ascribed to K_{ATP} modulation, there is growing consensus about the existence of other targets for the gasotransmitter. Some of them are Ca^{2+}-permeable channels. In this review we discuss the state of the art in this specific field, providing an updated report of H_2S interaction with Ca^{2+} channels and its functional outcomes.

Introduction

Hydrogen sulfide (H_2S) is a colorless, flammable gas with a characteristic smell of rotten eggs that has long been regarded as a toxic environmental pollutant with minimal, if any, physiological significance [1]. It is, however, now evident that H_2S may be endogenously synthesized in mammalian tissues from L-cysteine by three pyridoxal-50-phosphate (PLP)-dependent enzymes, namely cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and cysteine aminotransferase (CAT) [2]. The latter, in turn, acts in concert with the zinc-dependent enzyme, 3-mercaptopyruvate sulfurtransferase (3-MST), to release H_2S from L-cysteine and keto acids (e.g., α-ketoglutarate) [1, 3, 4]. The distribution of H_2S-generating enzymes may be tissue specific, whereas CBS is highly expressed in the hippocampus and in the cerebellum within the central nervous system (CNS), and CSE is far more abundant in vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) [1, 3, 5]. Nevertheless, recent studies have detected CSE in microglial cells, spinal cord and cerebellar granule neurons [6]. An additional source of H_2S is provided by bound sulfur, an intracellular reservoir of sulfur, as reported in rodent neurons and astrocytes in the presence of physiologic levels of endogenous reducing substances, i.e. glutathione and cysteine [1]. Interconversion of sulfur-containing amino acids and metabolites is carried out by cysteine CAT, cysteine dioxygenase (CDO), and cysteine lyase (CL) [1]. The assessment of the physiological concentration of free H_2S has engendered a remarkable controversy. It has long been thought that H_2S levels in biological tissues and plasma ranged from 50 μM up to 160 μM [7, 8]. However, recent studies have disclosed that H_2S is rapidly catabolized such that 1) whole tissue concentrations of the free gasotransmitter fall within the low nanomolar range and 2) H_2S may be undetectable in peripheral blood [1, 9, 10]. In order to reconcile this evidence with the notion that H_2S impacts on cellular activities *in vitro* at concentrations that are orders of magnitude larger (100 μM), it has been hypothesized that the equilibrium between H_2S production and consumption results in an intracellular microenvironment with enough H_2S to induce a local signalling cascade without affecting systemic levels of the gas [9, 11].

A number of signal transduction pathways may be recruited by H_2S to finely tune cardiovascular and CNS functions. For instance, H_2S relaxes VSMCs and contributes to the regulation of blood pressure by activating ATP-sensitive K^+ channels (K_{ATP}); it promotes angiogenesis and vascular remodelling *via*

phosphatidylinositol 3-kinase (PI3-K)/Akt/survivin axis in ECs and by augmenting the phosphorylation of extracellular signal-related kinase (ERK) and p38 in VSMCs; moreover, it downregulates a number of pro-inflammatory genes involved in the cardiac ischemic/reperfusion injury by preventing the nuclear translocation of the nuclear factor-κB (NF-κB); it also stimulates long term synaptic potentiation by enhancing the activity of NMDA receptors upon the activation of the cAMP/protein kinase A cascade [1, 3, 5-8, 11].

When considering the impact exerted by intracellular Ca^{2+} concentrations (Ca_i) dynamics on cell physiology, it is not surprising that a growing number of studies are attempting to elucidate the involvement of Ca^{2+}-permeable channels in H_2S-related signalling. The present review aims at providing an updated and concise description of the interaction of H_2S with different types of plasmalemmal Ca^{2+} channels and the associated functional outcomes.

S-sulfhydration is a regulatory mechanism for ion channels

Gasotransmitters, such as nitric oxide (NO), carbon monoxide (CO) and H_2S, selectively interact with different types of ion channels [1, 3, 5, 12-16]. Carbon monoxide modulates (positively or negatively) large-conductance calcium-activated K^+ (BK_{Ca}), voltage-activated K^+ ($K_{V2.1}$) and L-type Ca^{2+} channels, ligand-gated P2X2 and P2X4 receptors, tandem P domain K^+ channels (TREK1) and the epithelial Na^+ channel (ENaC). The detailed mechanisms underlying these effects are not clear. Carbon monoxide activates soluble guanylyl cyclase (sGC), leading to the release of cGMP, but it can also directly modify target proteins such as K_{Ca} α-subunit through interaction with aspartate and histidine residues [17, 18]. Nitric oxide covalently modifies free sulfhydryl (–SH) of cysteine residues via protein S-nitrosylation. Among ion channels, K_{Ca}, ultrarapid delayed rectifier K^+ current ($K_{V1.5}$), K_{ATP}, delayed rectifier K^+, L-type Ca^{2+} channels, and Transient Receptor Potential (TRP) channels are (positively or negatively) modulated by S-nitrosylation [19, 20].

H_2S donors can also modify cysteine residues of different proteins through S-sulfhydration [1, 5]. The –SH from sulfhydryl donor is transferred to free cysteine sulfhydryl and forms covalent persulfide (–

SSH). Sulfhydration can be detected by a modified biotin-switch assay used for nitrosylation as well as by mass spectrometry [5]. A number of H_2S-releasing drugs have been utilized to mimic the endogenous effects of H_2S under experimental conditions [1, 2]. The most popular H_2S donor is sodium hydrosulfide (NaHS), which presents a fast releasing rate in aqueous solution and liberates one third of H_2S compared to the concentration of the salt [8].

Several proteins are sulfhydrated including actin, tubulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), strengthening the idea that this signaling pathways is biologically relevant [5]. Accordingly, a recent report showed that H_2S-linked sulfhydration of NF-kB p65 subunit at cysteine-38 mediates its anti-apoptotic action in macrophages and liver cells [21].

In the vascular tissue, hydrogen sulfide is considered an endothelium-derived hyperpolarizing factor (EDHF) that is released by ECs and affects K^+ channels including intermediate calcium-dependent K^+ (IK_{Ca}), small calcium-dependent K^+ (SK_{Ca}) and K_{ATP} in VSMCs [13]. In VSMCs of rat mesenteric arteries H_2S sulfhydrates Kir 6.1 subunit of K_{ATP} in cysteine-43, both constitutively and during cholinergic simulation [13]. Previous observations by Jiang and co-workers showed that H_2S directly interacts with cysteine-6 and cysteine-26 residues of the extracellular NH_2 terminal of rat vascular sulfonylurea receptor (rvSUR1) subunit of rvKir6.1 K_{ATP} channels [13, 22]. This study, however, did not assess whether H_2S formed persulfides with the exposed free cysteine residues or disassembled the related disulfide bonds [12].

In addition to K^+ channels, also voltage-dependent Na^+ channels (Na_v) can be regulated by H_2S. Native (from jejunum smooth muscle) and recombinant ($Na_v1.5$) Na_v currents are increased by NaHS with an associated positive shift in steady-state activation and inactivation kinetics. Although the high (mM) concentrations of NaHS employed in this study suggest caution about their physiological significance, this effect could extend beyond the jejunum, since Nav1.5 is expressed in other tissues. In the heart, it gives rise to the upstroke of the cardiac action potential [23], whilst in Human Umbilical Vascular Endothelial Cells (HUVECs) Na_v regulates angiogenic calcium signals [24].

S-sulfhydration of calcium channels

Calcium VOCs

Voltage-activated Ca^{2+} channels (Ca_v) are expressed at high density in excitable cells, but are also detectable in some non-excitable tissues. They are typically classified as high voltage-activated (HVA) and low voltage-activated (LVA) channels, based on their electrophysiological features. HVA channels include L-, N-, P/Q-, and R-type, while LVA channels conduct T-type calcium currents [25]. Ca_v are mostly abundant in neurons, cardiac conduction system and smooth muscles.

H_2S modulates cardiovascular homeostasis and exerts cardioprotective effects in different models of *in vitro*, *ex vivo* and *in vivo* ischemia/reperfusion [11, 16, 26-33]. Indeed, whole patch clamp experiments in rat cardiomyocytes revealed that NaHS negatively modulates L-type Ca^{2+} channels composed by the $Ca_V1.2$ subunits [12, 30, 34]. More specifically, NaHS (up to 1 mM) causes a dose-dependent reduction in the Ca^{2+} current peak. This effect is only partial: the current density diminishes by 50% at 1 mM NaHS [34]. The mechanism could involve a direct modification of Ca_v free sulfhydryl groups [34].

The H_2S donor also affects the recovery from depolarization-induced inactivation, without altering the steady state activation and inactivation curves. Accordingly, the shortening of single cardiomyocytes and contraction of isolated rat papillary muscles are depressed. Electric field-induced Ca_i transients in single cardiomyocytes are also reduced by 100 μM NaHS [12, 30]. Consistently, H_2S exerts a negative inotropic effect in isolated perfused rat and papillary muscles when NaHS is administrated at concentrations ranging from 1 μM up to 1 mM [35, 36]. More recently, it has been reported its negative chronotropic action in human atrial fibers by blocking L-type Ca^{2+} channels and an enhancement in the repolarization phase by opening K_{ATP} channels (50-200 μM NaHS) [37]. Interestingly, according to a recent study, H_2S can reverse the negative inotropic effect induced by NO by causing an increase in the peak amplitude of the electrically stimulated Ca_i transients [33]. These apparently discrepant data may be reconciled when considering that, under such conditions, the modulation of the Ca_i toolkit responsible for the positive inotropic effect is not accomplished by H_2S, but by a new thiol-sensitive endogenous modulator deriving from the interaction between the two gasotransmitters [33]. Interestingly, in this report, H_2S was provided by NaHS at low micromolar doses (10 μM).

The negative effect of H_2S on Ca^{2+} influx is not limited to the cardiovascular system. Similarly to rat cardiomyocytes, 100 μM NaHS suppresses voltage-gated Ca^{2+} currents in INS-1E cells (rat insulinoma cell line) and native pancreatic beta-cells: these currents are sensitive to both nifedipine and Bay K-8664, a pharmacological profile consistent with L-type Ca^{2+} channels [12].

On the other hand the effects of NaHS on neurons, that can express both $Ca_V1.2$ and $Ca_V1.3$ subtypes, seem to be opposite [38]. In cultured rat cerebellar granule neurons (CGN), NaHS (50-300 μM) induces cell death as well as Ca_i signals sensitive to nifedipine and nimodipine, L-type Ca^{2+} channel blockers [12]. However, no electrophysiological recordings were conducted and a direct activation of L-type Ca^{2+} channels by NaHS remains to be demonstrated yet. Moreover, there is no evidence about the molecular nature (i.e. $Ca_V1.2$ or $Ca_V1.3$) of L-type channels in these cells. Taken together, these evidences suggest that L-type Ca^{2+} channels are inhibited by H_2S in the myocardium, whereas they are enhanced by the same H_2S doses in the CNS. Future investigations will unveil whether this feature depends on the different molecular make-up of L-type channels, i.e. $Ca_V1.2$ in ventricular cardiomyocytes *vs.* $Ca_V1.3$ in the cerebellum, or on their associated subunits. Alternatively, an intermediate sensor coupled to the channel complex, whose nature varies between the heart and the CNS, might mediate the regulation of L-type Ca^{2+} channels by H_2S.

NaHS increases Ca_i also in astrocytes, hippocampal slices and microglia, through currents sensitive to Ca^{2+} channel inhibitors (La^{3+} and Gd^{3+}) and in a concentration range (100-500 μM) similar to that affecting VOCs [12, 39]. It appears that H_2S-triggered Ca_i waves are due to influx through Ca^{2+} channels on plasma membrane and, to a lesser extent, to the release from intracellular Ca^{2+} stores [12, 39]. In contrast, a recent report showed that NaHS-induced Ca_i increase in isolated rat colonic crypts was not dependent on extracellular Ca^{2+}, but was affected by blockade of either ryanodine receptors (RyRs) or sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) [40].

T-type Ca^{2+} channels are encoded by the three members of the Ca_V3 subfamily and display different biophysical and pharmacological features as compared to L-type Ca^{2+} channels: activation at lower membrane potentials, faster inactivation, slower deactivation, smaller permeability to Ba^{2+}, insensitivity to dihydropyridines and block by $ZnCl_2$ [41]. T-type Ca^{2+} currents are involved in a great number of physiological processes, such as neuronal firing, hormone secretion, smooth muscle contraction, myoblast fusion, and fertilization [41]. Moreover, they play critical roles in mediating either somatic or visceral nociceptive information. Similarly to capsaicin, NaHS, injected intracolonically at 0.5-5 nM per mouse, triggers visceral nociceptive responses *in vivo*, which are completely abolished by mibefradil, an unspecific T-type channel blocker, and insensitive to verapamil and to the K_{ATP} channel blocker glibenclamide [12].

Therefore, H_2S may function as a novel nociceptive messenger through the activation of peripheral T-type Ca^{2+} channels, particularly during inflammatory processes. However, since mibefradil is not selective for T-type channels, this conclusion should be confirmed by future investigations [42].

Furthermore, both intraplantar (1 nM/paw) and intratechal (0.01-0.1 nM/animal) administration of NaHS caused a prompt hyperalgesia in rats, an effect that was abolished by mibefradil, $ZnCl_2$, or antisense oligodeoxynucleotides (ODNs) selectively targeting rat $Ca_V3.2$ [43-45]. The finding that DL-propargylglycine (PPG) and β-cyanoalanine, two CSE inhibitors, abolish the L-cysteine-induced hyperalgesia and attenuate the lipopolysaccharid-induced hyperalgesia, an effect reversed by NaHS, supports these observations [43, 44]. Moreover, mibefradil suppressed the phosphorylation of ERK induced by the infusion of NaHS, a pronociceptive stimulus in the pancreatic duct, albeit at higher concentrations than those reported above (500 nM/rat) [46]. Finally, the neuropathic allodynia/hyperalgesia induced in rats by damaging the right L5 spinal nerve [47] or by systemic injection of paclitaxel [48], an anticancer drug, was strongly attenuated by either mibefradil or CSE inhibitors, or by antisense ODNs against rat $Ca_V3.2$. In addition, $Ca_V3.2$ was significantly up-regulated in the ipsilateral L4, L5, and L6 dorsal root ganglia of rats subjected to spinal nerve injury, but not treated with paclitaxel [48]. A redox modulation of $Ca_V3.2$ has been proposed, since NaHS increases the amplitude of T-type Ca^{2+} currents in a neuroblastoma cell line without affecting their kinetics. This effect was reversed by the oxidizing agent, 5,5′dithio-bis(2-nitrobenzoic acid) (DTNB), and mimicked by the reducing compound, dithiothreitol (DTT) [44]. It should be pointed out that the elevation in the density of T-type Ca^{2+} currents was observed at 0.5-1.5 mM NaHS, i.e. at a concentration considerably higher than that reported to affect L-type VOCs (see above). The enhancement of T-type Ca^{2+} current by the exogenous application of H_2S, in turn, induces neuronal differentiation, as revealed by neurite outgrowth and functional expression of high voltage-activated Ca^{2+} currents, including L-, P/Q-, and N-type channels [49]. Once again, these effects arose when NaHS was administrated at 1.5-13.5 mM. Interestingly, earlier reports demonstrated that L-cysteine selectively potentiates recombinant $Ca_V3.2$-dependent, but not $Ca_V3.1$- and $Ca_V3.3$-, currents [50]. A mechanistic link between H_2S and the onset of the Ca_i waves might be provided by the protein-kinase A (PKA)\cAMP pathway. Accordingly, H-89, a rather selective PKA blocker, hinders NaHS-evoked Ca_i signals in both neurons and microglial cells [51, 52]. Moreover, PKA-dependent phosphorylation may increase the Ca^{2+} permeability of T-type channels, NMDA

receptors, and RyRs (see Discussion in [51]).

Transient receptor potential (TRP) channels

TRP channels can be activated by a variety of stimuli, including Ca^{2+} stores depletion, shear stress, pulsatile stretch, receptor activation, changes in temperature and osmolarity, and intracellular second messengers [53]. Their versatility enables TRP channels to control cellular functions as diverse as proliferation, differentiation, gene expression, migration, cytoskeleton remodelling, apoptosis, transmitter release, and NO synthesis [54]. It has now been widely established that TRP channels may also be modulated by covalent posttranscriptional modifications. For instance, TRP channels are S-nitrosylated at cysteine-553 and cysteine-558, located next to the channel pore [19]. Furthermore, TRPA1 is sensitive to thiol-reactive electrophiles that bind to cysteine residues located in the NH_2-terminus of the channel [55]. In addition, H_2S and its donors trigger TRPV1 opening that mediates chloride secretion in colon, gut motility, acute pancreatitis, airway constriction, and bladder contractility [12, 56-61]. Serosal application of NaHS (0.2-2.5 mM) and L-cysteine stimulates luminal chloride secretion by guinea pig, rat and human colon [12, 40, 58]. This effect is blocked by tetrodotoxin (TTX), by desensitization of afferent nerves with capsaicin, or by TRPV1 antagonist capsazepine [58]. This suggests that H_2S-stimulated mucosal secretion is dependent on TTX-sensitive Na^+ channels and/or TRPV1 channels of sensory nerve endings. Recent works showed that TRPV1-mediated Ca^{2+} entry enhances substance P release from afferent nerves, which, in turn, excite cholinergic secremotor neurons by activating neurokin-1, -2, or -3 (NK1-3) receptors [62, 63]. It should, however, be noted that NaHS-induced chloride secretion in rat distal colon is inhibited by glibenclamide and tetrapentylammonium, suggesting the involvement of different types of K^+ channels, including K_{ATP} and K_{Ca}.

H_2S donors mimick the effect of capsaicin, leading to the release of calcitonin gene-related peptide (CGRP) and substance P from the sensory nerves in the guinea pig airways [12, 57]. More specifically, 50 mM NaHS causes *in vivo* bronchoconstriction and microvascular leakage in a capsazepine-sensitive manner, contributing to the irritant action of H_2S on the respiratory system. NaHS triggers a dose-dependent contraction of isolated bronchial and tracheal rings *in vitro* (IC_{50} about 1.3 mM): this effect is abolished by sensory nerve desensitization with high concentration of capsaicin, by TRPV1 antagonists (ruthenium red, capsazepine and

SB366791), as well as by a mixture of NK1 (substance P receptor) and NK2 receptor (CGRP receptor) antagonists [12]. Interestingly, intraperitoneal injection of NaHS (1-10 mg/kg) to healthy mice induced substantial lung inflammatory reactions. These effects were abolished by a specific NK1 receptor antagonist, but not by NK2 receptor antagonists. In addition, the inflammatory effect of H_2S was abolished by capsazepine and was not observed in mice lacking substance P and neurokinin-A due to the knockout of their common precursor gene, preprotachykinin-A [57].

TRPV1 mediates neurogenic inflammation in pancreatitis, and the effect is blocked by pretreatment with TRPV1 antagonist capsazepine or NK1 receptor antagonist CP96, 345 [12, 59]. Notably, an increase in plasma H_2S levels is induced in caerulein-induced pancreatitis and the therapeutic administration of PAG attenuates the pancreatic inflammation and partially reverses the associated lung injury [56]. Similarly, TRPV1 underpinned the H_2S-dependent neurogenic inflammation in polymicrobial sepsis by increasing substance P production and activating the ERK/NF-kB pathway [64, 65]. In these experiments, NaHS was orally administrated at 10 mg/kg. Conversely, H_2S may prevent ethanol-induced gastric lesions in mice by stimulating TRPV1 channels on the capsaicin-sensitive primary afferent neurons that innervate the gastric mucosa [66]. Indeed, pretreating the animals with capsazepine reversed the gastroprotective action of either L-cysteine or NaHS (75-300 μM/Kg) [66].

In contrast to its vasorelaxant effect, NaHS (30 μM-3 mM) triggers contraction of the detrusor muscle in the rat urinary bladder [60, 61]. As in previously described cases, a direct effect of H_2S on the muscle seems unlikely: it is abolished by the combination of NK1 and NK2 receptor-selective antagonists as well as by high-capsaicin pretreatment, which could desensitize capsaicin-sensitive primary afferent neurons. The response to NaHS is not dependent on Na_v channels since it is mostly resistant to TTX. H_2S could stimulate capsaicin-sensitive primary afferent nerve terminals and the following release of tachykinins, leading to the contractile response. Furthermore, ruthenium red (RR), an unspecific blocker of TRPV1 channels, but not TRPV1 selective antagonist capsazepine, reduces the H_2S-induced contractile response. This opens the possibility that other RR-sensitive channels, such as TRPV1-6 and TRPA1, could be involved. TRPA1 is expressed on capsaicin-sensitive primary sensory neurons where it mediates pain, protective reflexes, and local release of peripheral neurotransmitters [67-69]. This channel is involved in noxious cold- and mechano-

sensations and its activators (allyl isothiocyanate, cinnamaldehyde, allicin, and acrolein) interact with cysteine residues of the protein. In female Sprague-Dawley rat bladder, TRPA1 is located in unmyelinated sensory nerve fibres where it colocalizes with TRPV1 channels [70]. Interestingly, TRPA1-expressing nerve fibres are also detected around blood vessels in the suburothelial region and muscular layer of the bladder [69, 71]. TRPA1 stimulation enhances detrusor activity. After disruption of the urothelial barrier with protamine sulfate, 1 mM NaHS increases maximal bladder pressure, reduces voided and infused volumes, and voiding interval [12, 70]. Recent work extended these observations to the human lower urinary tract, where TRPA1 channels are expressed in the terminal afferents of the urothelium and in basal urothelial cells [72]. After precontraction with phenylephrine, both TRPA1 agonists and NaHS are able to trigger relaxation of urethral strip preparations [72]. NaHS (IC_{50}≈1.2 mM) evokes Ca_i increase in CHO cells expressing mouse or human TRPA1 [70]. Accordingly, in rat Dorsal Root Ganglia (DRG) neurons, NaHS-induced Ca_i signals were abolished by removal of extracellular Ca^{2+} and by selective blockade of TRPA1 channels with HC-030031 [73]. Similar to the finding obtained on urethral strip preparations, the IC_{50} of the Ca_i response to NaHS in DRG neurons was about 1.4 mM. Furthermore, NaHS evoked an HC-030031-sensitive inward current in rat DRG neurons clamped at a holding potential of -80 mV. The current-to-voltage relationship of NaHS-induced current reversed at 0 mV and showed a slight outward rectification at positive potentials: both features are consistent with the biophysical properties of TRPA1 channels [73, 74]. Notably, NaHS-induced inward current was prevented by the reducing agent of disulfide bonds DTT, suggesting that H_2S carries out a covalent modifications of the cysteine residues located at the NH2-terminus of TRPA1 [73].

TRP channels may mediate the proangiogenic calcium influx triggered by H_2S in endothelial cells (ECs) [75-78]. In a recent paper, we investigated the effects of H_2S on microvascular ECs obtained from human breast carcinoma (B-TECs) [79]. Ca^{2+} imaging and patch-clamp experiments revealed that acute perfusion with NaHS activates Ca_i increases, as well as K^+ and non-selective cationic currents. Stimulation with NaHS in the same concentration range (1 nM–200 μM) evoked Ca_i signals also in 'normal' human microvascular ECs (HMVECs), but the amplitude was significantly lower. Conversely, doses lower than 10 μM NaHS did not evoke any detectable elevation in Ca_i in the excised endothelium of rat aorta [79]. Moreover, NaHS failed to promote either migration or proliferation on HMVECs, while B-TEC migration was enhanced at low-micromolar NaHS concentrations (1–10 μM). Remarkably, pretreatment with the

CSE-inhibitor PAG drastically reduced migration and Ca_i signals induced by Vascular Endothelial Growth Factor (VEGF) in B-TECs. These data suggest that H_2S plays a role in proangiogenic signaling of tumor-derived but not normal human ECs. Furthermore, its ability to interfere with B-TEC responsiveness to VEGF suggests that it could be an interesting target for antiangiogenic strategies in tumor treatment.

Although the identity of proangiogenic calcium channels regulated by H_2S in endothelial cells is still unknown, good candidates could be calcium-permeable channels involved in VEGF-dependent signaling. Different reports point to the ability of VEGF to activate TRPC1, TRPC3 and TRPC6 channels in human EC lines [80-86]. In addition, Orai1 and Stim1, components of the so-called CRAC channels, seem to contribute to VEGF-mediated Ca_i signaling in ECs [87-90]. The pattern of endothelial VEGF-activated channels could actually vary among different tissues, especially between small capillaries and large vessels. Remarkably, tumor-derived ECs express several members of TRP channels [91]. TRPV4 is overexpressed and functional in B-TECs, where it mediates arachidonic acid-dependent calcium entry and enhances migration [92]. Future investigations will unveil the potential role of this protein (and/or other TRP-related or unrelated channels) as a molecular target for the H_2S-induced Ca^{2+} entry and its vascular effects.

Conclusion and perspectives

The investigation of cellular and molecular mechanisms underlying physiological and pathological roles of hydrogen sulfide is living an exciting phase that recalls the history of nitric oxide, another gasotransmitter of established and widespread biological relevance. However, despite the growing number of reports, the state of the art is still far from being exhaustive. In particular, the relationship between calcium- and H_2S-dependent cell signaling has been clearly demonstrated in many cell types, including neurons, cardiomyocytes, endothelial cells, and is associated to relevant biological processes such as cardiac contraction, angiogenesis, inflammation and sensory transduction. As noted above, the most striking feature of this relationship is H_2S ability of either inhibiting or activating Ca^{2+} entry depending on the molecular nature of the Ca^{2+} entry pathway. Future investigation will have to address the following issues: 1) does H_2S modulate $Ca_V1.3$-dependent L-type Ca^{2+} channels? 2) Are $Ca_V1.2$, $Ca_V1.3$, $Ca_V3.2$ directly S-sulfydrated and, if so, does this covalent modification differently modulate $Ca_V1.2$ (inhibited) *vs.* $Ca_V1.3$ and $Ca_V3.2$ (activated) gating? 3) Is there any role for Ca_V-associated subunits in the modulation of voltage-gated Ca^{2+} influx by H_2S? 4) Do TRPV1 and TRPA1 proteins undergo any direct covalent modification by H_2S or do they sense its levels via an intracellular sensor?

It should be, finally, pointed out that the concentration of NaHS required to modulate/activate Ca^{2+}-permeable channels is extremely variable. For instance, low- to mid-micromolar doses of NaHS regulate L-type VOCs, whereas T-type and TRPA1 channels are stimulated by low millimolar doses of the donor. Such variability is more evident in vascular endothelium, where the threshold for the onset of the Ca_i signal may vary from the low nanomolar range observed in B-TECs and HMVECs to the low micromolar range reported in RAECs. When considering that approximately one third of the salt concentration is released in form of H_2S in acqueous solution, it turns out that the effective concentration of H_2S may vary from 0.1 nM up to around 400 μM depending on the target channel. As mentioned above, a number of studies have been devoted to ascertain the concentration of endogenous H_2S in biological tissues. A recent paper proposed the existence of at least three discrete H_2S pools in various biological specimens: a free H_2S reservoir, which is in the low nanomolar range, an acid-labile and a bound sulfane-H_2S pools, which are in the low micromolar range. This finding introduces the notion of a reversible sulfide sink into and from which H_2S can be deposited or liberated to exert biologic functions [93]. The possibility that H_2S may reach intracellular levels high enough to activate local signaling pathways should also be taken in account. Nevertheless, the H_2S levels necessary to induce detectable changes in the activity of either T-type VOCs or TRPA1 channels have never been measured, a feature that should be addressed by future investigations.

Although this review mainly focused on L- , T-type Ca^{2+} channels and TRP channels, it is worth of noting that preliminary data hint at additional Ca^{2+}-permeable channels as novel targets of H_2S. For instance, H_2S has been shown to regulate SOCE in both human ECs enzymatically dissociated from saphenous vein and rat aortic endothelium [94, 95]. Furthermore, H_2S may affect intracellular Ca^{2+} mobilization by either inhibiting $InsP_3Rs$ or exciting RyrRs [40, 94]. These findings gain particular relevance when considering that Orai1, the plasmalemmal pore-forming subunit of SOCE, $InsP_3Rs$ and RyRs are all prone to covalent modifications, such as phosphorylations and nytrosilations. Consistently, future studies will be needed to elucidate the possible co-regulations of ion channels by H_2S and NO: similarly to other proteins, some calcium channels contain cysteine residues potentially target for both the gaseous mediators through nytrosilation and sulfhydration.

The interplay between H_2S and calcium signaling is made even more intricate by the fact that CSE activity can be Ca^{2+}-calmodulin-dependent, as shown in bovine aortic endothelial cells (BAECs) [96]. The possibility that H_2S undergoes an auto-regolatory control by either activating (*via* $Ca_V3.2$, TRPA1 or TRPV1) or inhibiting (*via* $Ca_V1.2$) Ca^{2+} entry will deserve future investigations. This feature would add another piece to the growing list of analogies between NO and H_2S,

whereas NO, which is produced by the Ca^{2+}/CaM-dependent endothelial NO synthase, may inhibit SOCE, the main source for eNOS activation, in vascular endothelium [97].

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FIGURE LEGENDS

Figure 1. Modulation of plasmalemmal Ca^{2+} channels by hydrogen sulfide. Hydrogen sulfide (H_2S) regulates the gating of a number of Ca^{2+}-permeable channels, including 1) L-type VOCs formed by either $Ca_V1.2$ or $Ca_V1.3$ subunits, 2) T-type VOCs composed by $Ca_V2.3$ subunits; 3) Transient Receptor Potential Ankyrin 1 (TRPA1); and 4) Transient Receptor Potential Vanilloid 1 (TRPV1). As more extensively described in the test, H_2S inhibits L-type VOCs in cardiac myocytes, whereas it activates them in neurons. Similarly, H_2S stimulates T-type VOCs, TRPA1 and TRPV1 both *in vitro* and *in vivo*. The identity of putative proangiogenic calcium-permeable channels (TRPs?), possibly modulated by H2S, is unknown.

Table 1. Tissue distribution and functional roles of the Ca^{2+}-permeable channels sensitive to hydrogen sulfide.