



Themed Section: Chemical Biology of Reactive Sulfur Species

REVIEW ARTICLE

Effects of sulfide and polysulfides transmitted by direct or signal transduction-mediated activation of TRPA1 channels

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Received 8 March 2018; **Revised** 22 June 2018; **Accepted** 24 September 2018

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Hydrogen sulfide (H₂S) is a gaseous mediator in various physiological and pathological processes, including neuroimmune modulation, metabolic pathways, cardiovascular system, tumour growth, inflammation and pain. Now the hydrogen polysulfides (H₂S_n) have been recognised as signalling molecules modulating ion channels, transcription factors and protein kinases. Transient receptor potential (TRP) cation channels can be activated by mechanical, thermal or chemical triggers. Here, we review the current literature regarding the biological actions of sulfide and polysulfide compounds mediated by TRP channels with special emphasis on the role of TRPA1, best known as ion channels in nociceptors. However, the non-neuronal TRPA1 channels should also be considered to play regulatory roles. Although sulfide and polysulfide effects in different pathological circumstances and TRPA1-mediated processes have been investigated intensively, our review attempts to present the first comprehensive overview of the potential crosstalk between TRPA1 channels and sulfide-activated signalling pathways.

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Abbreviations

AITC, allyl isothiocyanate; AMPK, AMP-activated protein kinase; apoE, apolipoprotein E; ARDs, ankyrin repeat domains; CBS, cystathionine β-synthase; CK2, casein kinase 2; cryo-EM, single-particle electron cryomicroscopy; DADS, diallyl disulfide; DAS, diallyl sulfide; DATS, diallyl trisulfide; DMTS, dimethyl trisulfide; DRG, dorsal root ganglion; HIF-1α, hypoxia inducible factor 1 alpha; HNO, nitroxyl; IKK, IκB kinase complex; IP6, myo-inositol-1,2,3,4,5,6-hexakisphosphate; phytic acid; Nrf2, nuclear factor erythroid 2-related factor 2; SP, substance P; SSNO-, nitrosopersulfide; SULFI/NO, dinitrosulfite; TRG, trigeminal root ganglion

Introduction

The superfamily of the transient receptor potential (TRP) ion channels consists of six subfamilies: TRP canonical, TRP melastatin, TRP vanilloid (TRPV), TRP ankyrin (TRPA), TRP mucolipin and TRP polycystin (TRPP or PKD). TRP receptors form non-selective cation channels, preferentially high calcium ion permeability. Calcium influx triggers several intracellular pathways. TRP channels are sensitive to a variety of stimuli including mechanical or thermal triggers, or chemical ligands. Based on such interactions, they are likely to be sensors for several physiological or pathophysiological stimuli. (Khalil *et al.*, 2018). The main goal of this review paper is to discuss the TRP-mediated effects of sulfide and polysulfide compounds, with particular emphasis on the role of **TRPA1** channels.

H₂S as a gaseous transmitter

At the beginning of the 1990s, **H₂S** appeared as a new example of agaseous molecule modulating a number of biological functions, such as nociception, inflammation and vascular responses. Its potential biological effect on capsaicin-sensitive sensory nerves was first suggested in the lung (Prior *et al.*, 1990). In 2004, Patacchini *et al.* identified the H₂S donor **sodium hydrosulfide** (NaHS) as the activator of the capsaicin-sensitive neurons in the rat isolated urinary bladder. They concluded that H₂S either acts on **TRPV1** channels or stimulates a still unidentified TRP-like channel co-expressed with TRPV1 channels on sensory neurons (Patacchini *et al.*, 2004). Four years later, Streng *et al.* (2008) suggested that NaHS acts on TRPA1 channels in the urinary bladder. The next key findings were published by Miyamoto *et al.* (2011) presenting evidence that the NaHS-evoked increase in [Ca²⁺]_i was inhibited by Ca²⁺ free condition and by a selective antagonist for TRPA1 channels, **HC030031**, suggesting that H₂S stimulates sensory neurons via activation of these channels. Generation of Ca²⁺ signals in response to H₂S were first described in astrocytes, but the authors could not conclude that TRPA1 channels were the mediators (Nagai *et al.*, 2004). TRPA1 ion channels were first described as being sensitive to cold and later to be receptors for **mustard oil** (Story *et al.*, 2003; Jordt *et al.*, 2004). Several papers also suggest that TRPA1 channels may also be expressed by non-neuronal cells (Chen and Hackos, 2015). Vasodilator effects of H₂S were decreased in TRPA1 knockout (TRPA1^{-/-}) mice or in wild-type mice after treatment with HC030031 (Pozsgai *et al.*, 2012), indicating a primary role of TRPA1 channels in mediating the vascular effects of H₂S in the skin. Activation of TRPA1 receptors by sulfide-released **CGRP** with consequent vasodilation (Pozsgai *et al.*, 2012; Eberhardt *et al.*, 2014; Hajna *et al.*, 2016).

Structure and function of TRPA1 channels

TRPA1 channels were originally called ANKTM1 channels, as they were identified by a homology search for ankyrin repeats and six transmembrane domains. TRPA1 channels have a

particularly long ankyrin repeat region within the N-terminus and contain calcium-sensitive regions in the N-terminal EF-hand motif and in the S4 transmembrane segment. The flexible ankyrin domains may serve the structural basis for protein–protein interactions (Brewster and Gaudet, 2015). In the TRP superfamily, TRPA1 channels have unique aspects and, reflecting a wide range of ligands; it is a promiscuous receptor. Low and high temperature, osmotic changes, natural and synthetic irritants are all known to activate TRPA1 channels. Several TRPA1 channel agonists are reactive electrophilic ligands. Channel gating by these compounds is based on covalent modification of cysteine and lysine residues within the N-terminus and the transmembrane domain. Regarding sulfide-evoked TRPA1 channel activation, the formation of an intramolecular C422–C622 disulfide bond has been proposed (Kimura, 2015). TRPA1 channels can also be activated by non-reactive compounds that bind with non-covalent interactions as well as low or high pH or polyunsaturated fatty acids (Viana, 2016). Activated TRPA1 channels interact with signalling downstream mechanisms of several GPCRs, such as **bradykinin B₂** receptors or **PAR2**. TRPA1 channels behave as integrators of endogenous and exogenous activating stimuli, including temperature, light, bacterial toxins, mechanical and chemical stimuli (Baraldi *et al.*, 2010). As TRPA1 channels can be triggered by endogenous compounds generated during tissue injury and inflammation, the main trend of their development treats these channels as novel potential drug targets for analgesics and anti-inflammatory drug candidates. Recent papers have presented evidence that TRPA1 channels are also expressed in the brain and play pivotal roles in neurodegenerative disorders and neuroinflammation, such as multiple sclerosis and Alzheimer's disease, opening another potential benefit of TRPA1 channel antagonists (Sághy *et al.*, 2016; Lee *et al.*, 2016a).

Generally, TRPA1 channel subunits constitute homotetrameric complexes on the cell membrane, but there is evidence that these subunits also co-localize with TRPV1 subunits into heterotetrameric complexes to form a single channel in sensory neurons (Garrison and Stucky, 2011). The structure of the TRPA1 channel at near-atomic (4 Å) resolution has been determined by single-particle electron cryomicroscopy (cryo-EM) (Paulsen *et al.*, 2015). This is essential for understanding TRPA1 channel function and determining the binding sites for drug interactions. Another comprehensive study of the structural characteristics and potential binding sites of agonists and antagonists of TRPA1 channels has been published recently (Brewster and Gaudet, 2015). The cryo-EM analysis confirmed that TRPA1 channels are tetrameric proteins formed by the assembly of four subunits (1119 amino acids in humans) with six transmembrane α -helices (S1–S6). Their structure includes a series (14–18 depending on species) of ankyrin repeat domains (ARDs) within the long intracellular N-terminus (Zygmunt and Högestätt, 2014; Brewster and Gaudet, 2015). At the structural level, the permeation pathway shows two major constriction sites (Paulsen *et al.*, 2015). The channel is highly Ca²⁺ permeable and the permeating divalent cations contribute to channel regulation, characterized by initial potentiation followed by desensitization (Kádková *et al.*, 2017; Zimova *et al.*, 2018). Recently, Brewster and Gaudet (2015) have edited a novel

schematic map of TRPA1 channels demonstrating and proposing the functions of different parts of the receptor protein. They also reveal an unexpected novel ligand-binding site and an unusual C-terminal coiled coil stabilized by myo-inositol-1,2,3,4,5,6-hexakisphosphate (phytic acid, IP6). They suggest that endogenous soluble intracellular ligands, such as IP6, are required to maintain the receptor in an agonist-receptive state (Brewster and Gaudet, 2015). Following channel activation, removal of IP6 by the influx of Ca^{2+} could act a molecular kill switch to trigger inactivation. In spite of much experimental effort, the exact role of TRPA1 channels in thermo- and mechanosensation has not been elucidated yet (Chen and Hackos, 2015). On the basis of recent studies, these channels might not be primary cold sensors, but are associated with cold allodynia and mechanical hyperalgesia where endogenous activation mechanisms are present (Brewster and Gaudet, 2015; Viana, 2016) (Figure 1A, B).

Neuronal and non-neuronal TRPA1 channels

Expression of TRPA1 channels by somatic and visceral primary sensory neurones is well known. Peptidergic nociceptors, also known as capsaicin-sensitive sensory neurones, co-express TRPV1 and TRPA1 channels (Story *et al.*, 2003). Activation of these channels induces membrane depolarization by Na^+ influx, while the inward Ca^{2+} current increases $[\text{Ca}^{2+}]_i$, which releases neuropeptides such as **CGRP**, **substance P** (SP) or **neurokinin A** from large dense-core vesicles via Ca^{2+} -dependent exocytosis (Kádková *et al.*, 2017). Pro-inflammatory neuropeptides amplify nociception and mediate neurogenic inflammation through the local efferent function of the **capsaicin**-sensitive sensory nerve endings. Neurogenic inflammation results in vascular changes such as vasodilatation and plasma protein extravasation followed by recruitment of inflammatory and immune cells (Pintér *et al.*, 2006; Bodkin and Brain, 2011; Szolcsányi, 2014; López-Requena *et al.*, 2017). Other events involving TRPA1 channels modulate a number of chronic pain conditions associated with inflammation. Thus, pro-inflammatory and algogenic mediators released within the injured or inflamed tissue, such as PGs, **bradykinin**, **5-HT** and proteases, modulate TRPA1 channels via GPCRs and **phospholipase C**-coupled signalling cascades. These mediators, including lipid peroxidation products such as **4-oxo-2-nonenal**, **4-hydroxy-2-nonenal**, 4-hydroxyhexenal as well as **15-deoxy-12,14-prostaglandin J2**, stimulate **PKA**, **PKC** and phospholipase C (PLC) pathways to induce phosphorylation of TRPA1 channels (Chen and Hackos, 2015; Viana, 2016; Kádková *et al.*, 2017). The putative roles of the PKA- and PLC-dependent signalling pathways in TRPA1 channel-mediated nociception have been very well reviewed (Zygmunt and Högestätt, 2014; Viana, 2016).

Following the activation of TRPA1 channels, not only pro-inflammatory but anti-inflammatory peptides are also released from the nerve ending, such as **somatostatin** (Pozsgai *et al.*, 2017). Somatostatin is a cyclic peptide found in the CNS and peripheral tissues. Neuronally derived somatostatin could exert systemic antinociceptive and anti-

inflammatory effects (Pintér *et al.*, 2006), mediated by **sst4 receptors** (Pintér *et al.*, 2002; Helyes *et al.*, 2009). This could explain why activation of TRPA1 channels in wild-type mouse diminishes mild heat injury-induced thermal hyperalgesia; nevertheless, it cannot be observed in TRPA1 receptor knock out animals. Alkylated polysulfide compounds, such as dimethyl trisulfide (DMTS), activate TRPA1 channels of primary sensory neurons inducing release of the neuropeptide somatostatin that might elicit analgesic effect in animal models of nociception via sst4 receptors (Pozsgai *et al.*, 2017).

Several non-neuronal cell types express TRPA1 channels. In human and murine lung, apart from the sensory fibres, non-neuronal cells including fibroblasts, alveolar epithelial cells, and smooth muscle cells express these channels. TRPA1 channels were also detected in melanocytes, keratinocytes and fibroblasts and, therefore, these channels have been suggested to regulate keratinocyte differentiation and inflammation in the skin. In addition to sensory fibres innervating the urinary tract, TRPA1 channels have been found in epithelial cells and might play a role in urinary micturition (Khalil *et al.*, 2018). Activation of TRPA1 channels in **insulin** producing beta cells of the pancreatic islet results in insulin secretion (Cao *et al.*, 2012). Astrocytes also express these channels in different brain structures, which may play a role in calcium homeostasis, influence GABA transporter **GAT-3** and regulate the oligodendrocyte apoptosis with consequent exacerbation of demyelination (Shigetomi *et al.*, 2012; Sághy *et al.*, 2016). Hydrogen polysulfides were first reported to activate TRPA1 channels of astrocytes more potently than H_2S (Kimura *et al.*, 2013). There are few data on the expression of these channels in immune cells. The functional role of TRPA1 channels in macrophages was recently investigated in the pathogenesis of atherosclerosis by Zhao *et al.*, 2016. Expression of these channels was increased in macrophage foam cells in atherosclerotic aortas of apolipoprotein E (apoE)-deficient mice. Atherosclerotic lesions, hyperlipidaemia and systemic inflammation were worsened with chronic administration of the channel antagonist HCO30031 or genetic ablation of TRPA1 channels in apoE knockout mice. These findings suggest that TRPA1 channels may serve to regulate the pathogenesis of atherosclerosis and cholesterol metabolism of macrophage foam cells (Zhao *et al.*, 2016). TRPA1 immuno-positive macrophages have also been identified by merged fluorescent double staining in the human oral submucosa. The number of TRPA1 positive macrophages was significantly elevated in samples from patients suffering oral lichen planus (Kun *et al.*, 2017). Expression of TRPA1 channels was also detected by Northern blot, Western blot and immunohistochemical methods in Jurkat T cells and in human splenocytes. Expression of these channels has been confirmed in murine and human T cells at mRNA and protein level (Bertin *et al.*, 2017). While the pro-inflammatory effects of the activation of TRPA1 channels are well established, there is emerging evidence for its protective effects. Deletion of TRPA1 channels enhanced inflammatory responses in various colitis animal models (Kun *et al.*, 2014; Bertin *et al.*, 2017). Kemény *et al.* (2018) provided clear evidence that psoriasisform dermatitis induced by topically applied **imiquimod** was enhanced in TRPA1 KO mice and after treatment with the TRPA1 channel antagonist **A967079**, compared to the wild-type controls. Their

immunohistochemical data revealed that CD4+ T helper cells expressed TRPA1 channels in murine skin. They concluded that protective effects in psoriasiform dermatitis could be mediated by the activation of neuronal and non-neuronal TRPA1 channels (Kemény *et al.*, 2018). Although the best known site of TRPA1 channels is the nociceptive primary sensory neuron, these channels in non-neuronal sites should also be considered when assessing the regulatory role of TRPA1 channel activation. As TRPA1 channel antagonists are under active development for pain and inflammation, it is important to allow for the possibility of on-target side effects mediated by non-neuronal TRPA1 channels.

Activators of TRPA1 channels

In contrast with other chemoreceptors, which are usually stimulated by ligands with rather conserved structures, a unique feature of TRPA1 channels is that they appear to be activated by a variety of structurally unrelated compounds.

Exogenous compounds

Plant-derived organosulfur compounds

Allium sativum is commonly known as garlic and its active compounds include thiosulfinates that are responsible for the distinctive pungent and spicy aroma of garlic. **Allicin** is an unstable organosulfur component generated from alliin by the vacuolar enzyme alliinase after the clove has been cracked. Like other highly reactive thiosulfinates, allicin is converted to more stable organosulfur compounds such as diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS). While alliin is odourless, these derivatives are volatile compounds responsible for the pervasive garlic aroma and flavour. Thiosulfinates have structural similarities with allyl isothiocyanate (AITC), the pungent agent of wasabi, horseradish and mustard oil. These compounds activate TRPA1 channels on primary sensory neurons causing the release of pro-inflammatory neuropeptides with consequent pain sensation and neurogenic inflammation (Bautista *et al.*, 2005). Highly reactive isothiocyanates such as propargyl isothiocyanate, benzyl isothiocyanate and phenethyl isothiocyanate also act on TRPA1 channels, but their experimental use is strongly limited due to their toxicity. AITC activates other TRPs (including TRPV1). These organosulfur compounds could be conjugated with cysteines in the TRPA1 channel structure to form disulfide bridges (Baraldi *et al.*, 2010; Viana, 2016).

Irritant chemicals from air pollution or cigarette smoke

TRPA1 channels are activated by almost all oxidizing industrial electrophiles, including aldehydes (e.g. formaldehyde and acetaldehyde), alkenals (e.g. **acrolein** and **crotonaldehyde**), hypochlorites, toluene diisocyanate and tear gases, most likely through covalent protein modification (Bessac and Jordt, 2008).

Endogenous substances

TRPA1 channels act as sensors of toxic signals and molecular integrators of cellular stress, including ROS. Allogenic activators of these channels are released from the sites of inflammation or tissue injury. They are lipid peroxidation products such as 4-oxo-2-nonenal, 4-hydroxy-2-nonenal and 4-hydroxyhexenal, and oxidized lipids such as the cyclopentenone PG (PGA₁, **PGA₂**, 8-isoPGA₂, **15-deoxy-Δ^{12,14}-PGJ₂** and Δ¹²-PGJ₂), formed by non-enzymic dehydration of the respective PGs (**PGD₂**, **PGE₂** and **PGE₁**). These compounds are TRPA1 channel ligands and directly gate the channel to cause acute nociception (Chen and Hackos, 2015). Activators of the inflammasome, such as monosodium urate crystals, stimulate TRPA1 channels by an indirect mechanism involving production of **H₂O₂** (Trevisan *et al.*, 2014).

Neuronal and non-neuronal expression patterns of TRPA1 channels allow a broad activation profile and enable it to be a versatile sensor of tissue injury. Inflammatory mediators and reactive electrophilic agents bind to TRPA1 channels covalently as electrophilic attack can damage cellular components by Michael addition. Nitrated fatty acids such as nitro-oleic acid that are generated during inflammation by phospholipids and **NO** are other examples endogenous lipidergic TRPA1 activators. DAG and **arachidonic acid**, generated through bradykinin B₂-receptor/**PLA2**/PLC pathways, are also activators of these channels and may represent a downstream mechanism of bradykinin-induced pain (Brewster and Gaudet, 2015) (Table 1).

Sulfide (H₂S)-NO, polysulfides

Several papers provided evidence suggesting the signalling pathways that are shared by NO and hydrogen sulfide (H₂S) (Takahashi *et al.*, 2012). The synergistic effect of the two gasotransmitters was first identified in the late 1990s (Hosoki *et al.*, 1997). The chemical basis of this interaction has not been elucidated properly. Moreover, polysulfides (H₂S_n) recently emerged as potential mediators of H₂S/sulfide signalling, but their biosynthesis and relationship to NO are still under intensive investigation (Kimura, 2017). Some recent papers have suggested that the reaction between sulfide and NO leads to formation of different bioactive intermediates [including nitrosopersulfide (SSNO), H₂S_n and dinitrososulfite (SULFI/NO)] capable of scavenging, transporting and releasing NO and generating its redox congeners, nitroxyl (HNO), nitrous oxide (N₂O) and sulfane sulfur (Eberhardt *et al.*, 2014; Cortese-Krott *et al.*, 2015; Kimura, 2017; Miyamoto *et al.*, 2017). These reports conclude that SSNO is a potent NO donor, resistant to the reducing milieu of the cell, and able to release both NO and H₂S_n. SULFI/NO is a weak combined NO/HNO donor and generator of N₂O with potent effects on the heart. Formation of its precursor sulfite and generation of sulfur and/or oxygen-centred free radicals may be responsible for the scavenging effects of sulfide on NO bioavailability. Polysulfides may be formed secondary to the reaction of sulfide with NO, either through HSNO or after decomposition of SSNO, and may also contribute to NO scavenging and sulfane sulfur signalling. These findings open new areas of

Table 1

Publications on TRPA1 channel-mediated effects of inorganic sulfide donors

Sulfide source	Cell type, organ, <i>in vivo</i> setting	Readout	References
Hydrogen sulfide donors			
NaHS	Rat TRG neurons	Electrophysiology activation inhibited by HC030031	Koroleva <i>et al.</i> (2017)
NaHS	Chicken thoracic aorta epitheloid cells	5-HT release by HPLC inhibited by TRPA1 antagonist	Delgermurun <i>et al.</i> (2016)
NaHS	Human ureter	Precontraction by electrical stimulation was relaxed.	Weinhold <i>et al.</i> (2017)
NaHS	RIN14B cells	Ca ²⁺ imaging and 5-HT release activation inhibited by HC030031	Ujike <i>et al.</i> (2015)
NaHS	Murine cerulein-induced pancreatitis	Elevated spinal cFOS expression by immunohistochemistry inhibited by AP18 mechanical abdominal allodynia inhibited by AP18 and Ca _v 3.2 channel inhibitor	Terada <i>et al.</i> (2015)
NaHS	CHO hTRPA1	Ca ²⁺ imaging	Hajna <i>et al.</i> (2016)
NaHS	CHO hTRPA1 Murine TRG neurons	Ca ²⁺ imaging activation inhibited by HC030031 and TRPA1 knockout	Hajna <i>et al.</i> (2016)
NaHS	Murine skin	Increased blood flow by laser Doppler imaging inhibited by RTX pretreatment, CGRP and NK1 antagonists, glibenclamide	Hajna <i>et al.</i> (2016)
NaHS	RIN14B cells	Ca ²⁺ imaging and 5-HT release response enhanced by acidosis	Takahashi and Ohta (2013)
NaHS	Rat lung vagal afferents	Ca ²⁺ imaging and respiratory changes respiratory effect inhibited by HC030031	Hsu <i>et al.</i> (2013)
NaHS	Murine DRG neurons	Ca ²⁺ imaging and electrophysiology inhibited by TRPA1 knockout potentiated by acidosis	Andersson <i>et al.</i> (2012)
NaHS	CHO mTRPA1	Electrophysiology	Andersson <i>et al.</i> (2012)
NaHS	Murine hind paw	Mechanical hyperalgesia inhibited by AP18 and TRPA1 knockout	Andersson <i>et al.</i> (2012)
NaHS	Murine colon	Nocifensive behaviour present in TRPA1 knockout	Andersson <i>et al.</i> (2012)
NaHS	Rat mesenteric artery	Dilatation prevented by capsaicin pretreatment, HC030031, CGRP receptor antagonist and Cl ⁻ channel inhibitor	White <i>et al.</i> (2013)
NaHS	Murine colon	Nocifensive behaviour inhibited by AP18 and Ca _v 3.2 channel inhibitor	Tsubota-Matsunami <i>et al.</i> (2012)
NaHS	Rat trachea nerve endings	CGRP release by RIA inhibited by HC030031	Pozsgai <i>et al.</i> (2012)
NaHS	Murine skin	Elevated blood flow by laser Doppler imaging inhibited by HC030031 and TRPA1 knockout	Pozsgai <i>et al.</i> (2012)
NaHS	Murine DRG neurons	Ca ²⁺ imaging activation inhibited by HC030031 and TRPA1 knockout	Ogawa <i>et al.</i> (2012)
NaHS	HEK293 mTRPA1	Ca ²⁺ imaging activation inhibited by mutation of key cysteines potentiated by acidosis	Ogawa <i>et al.</i> (2012)
NaHS	Murine hind paw	Nocifensive behaviour inhibited by TRPA1 knockout potentiated by acidosis	Ogawa <i>et al.</i> (2012)
NaHS	Murine hind paw	Nocifensive behaviour inhibited by AP18, gene silencing of TRPA1, inhibition and silencing of Ca _v 3.2 channels	Okubo <i>et al.</i> (2012)
NaHS	Rat DRG neurons	Ca ²⁺ imaging activation inhibited by HC030031	Miyamoto <i>et al.</i> (2011)
NaHS	Human prostate	Relaxation of precontracted smooth muscle	Gratzke <i>et al.</i> (2010)

continues

Table 1

(Continued)

Sulfide source	Cell type, organ, <i>in vivo</i> setting	Readout	References
NaHS	Rat urinary bladder	Intravesicular NaHS increased micturition frequency and lowered voiding volume	Streng <i>et al.</i> (2008)
NaHS	CHO m/hTRPA1	Ca ²⁺ imaging	Streng <i>et al.</i> (2008)
Na ₂ S	RIN14B cells	Ca ²⁺ imaging activation inhibited by HC030031	Ujike <i>et al.</i> (2018)
Na ₂ S	CHO hTRPA1	Ca ²⁺ imaging	Hajna <i>et al.</i> (2016)
Na ₂ S	Rat TRG neuron, brainstem slice, <i>dura mater</i>	CGRP release by ELISA	Wild <i>et al.</i> (2015)
GY4137	Porcine bronchioles	Relaxation electrical stimulation-induced relaxation inhibited by CSE-inhibitor and TRPA1 antagonist	Fernandes <i>et al.</i> (2014)
GY4137	Porcine intravesical ureter	Relaxation of precontracted smooth muscle prevented by HC030031, CGRP and PACAP receptor antagonists	Fernandes <i>et al.</i> (2014)

Ca_v3.2, a member of voltage-gated T-type calcium channels; CSE, cystathionine-γ-lyase; NK₁, neurokinin 1 receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; RTX, resiniferatoxin.

research with SSNO, HS_n and SULFI/NO as biologically important mediators of both the NO and H₂S transduction pathways. These interactions could be important in the cardiovascular, neuronal and immune systems (Cortese-Krott *et al.*, 2015) (Tables 2 and 3). Endogenous polysulfides were produced in the mouse brain by **3-mercaptopyruvate sulfurtransferase**, making this pathway physiologically highly relevant (Kimura *et al.*, 2015, 2017). Reports of the effects of various products of the sulfide/NO interaction are shown in Tables 2 and 3 and Figure 2.

Effects of sulfur species mediated by TRPA1 channels

As neurons are the main cell type expressing TRPA1 channels, apart from astrocytes, numerous scientific accounts describe activation of the ion channel by sulfide in these cells (Kimura *et al.*, 2013). The most abundantly investigated expression systems are *Xenopus* oocytes, RIN14B rat pancreatic islet cells, CHO and HEK293 cells. Murine and rat trigeminal and dorsal root ganglion (TRG and DRG) neurons as well as isolated rat vagal afferents and neurons were also investigated. NaHS and sodium sulfide nonahydrate (Na₂S) were mostly used as sulfide donors. Ion channel opening was either detected as a Ca²⁺ signal indicated by Ca²⁺-sensitive fluorescent dyes, electrophysiology in case of nerve fibres, patch-clamp or serotonin release from RIN14B pancreatic islet cells. Involvement of TRPA1 channels in the increased [Ca²⁺]_i and cation currents was confirmed with selective receptor antagonists **AP18**, HC030031 or genetic deletion of the gene encoding the ion channel (Miyamoto *et al.*, 2011; Ujike *et al.*, 2015; Hajna *et al.*, 2016). Some papers report a potentiating effect of acidic pH on sulfide-evoked responses of TRPA1 channels (Andersson *et al.*, 2012; Ogawa *et al.*, 2012; Takahashi and

Ohta, 2017). Others identified critical cysteine residues involved in activation of TRPA1 channels by sulfide, using cells expressing mutated ion channel (Ogawa *et al.*, 2012). A relatively recent publication detected activation of TRPV1 channels in rat TRG neurons by NaHS based on inhibition by **capsazepine**. However, the majority of investigators in the sulfide field consider the gasotransmitter as selective for TRPA1 channels and evidence for activation of TRPV1 channels is scarce (Trevisani *et al.*, 2005; Ang *et al.*, 2010; Medeiros *et al.*, 2012; Lu *et al.*, 2014; Koroleva *et al.*, 2017; Yu *et al.*, 2017). No individual excitatory action but potentiation of the capsaicin-induced response by NaHS was reported in isolated vagal neurons from rat lungs. Even in this model, respiratory effects of sulfide could be abolished by HC030031 (Hsu *et al.*, 2013). CGRP release from peptidergic nerves of rat tracheae was reported in response to stimulation with NaHS. Peptide release was prevented by the TRPA1 channel antagonist (Pozsgai *et al.*, 2012).

Contribution of TRPA1 channels to sulfide-induced smooth muscle relaxation was examined in rat mesenteric arteries, pig bronchioles and intravesical ureter, as well as human ureter and prostate. Besides the obvious inhibition by defunctionalization of peptidergic nociceptors or HC030031, dilator effects of sulfide in rat mesenteric arteries was diminished by a CGRP receptor antagonist or a Cl⁻ channel inhibitor (White *et al.*, 2013). Precontracted porcine bronchiole rings were relaxed by a slow-release sulfide donor, **GY4137**. Sulfide-induced relaxation was ameliorated by elevated extracellular K⁺ concentration and inhibition of **large conductance Ca²⁺-activated K⁺ channels**. Endogenous sulfide and TRPA1 channel activation were involved in electrical stimulation-evoked relaxation (Fernandes *et al.*, 2016). Porcine intravesical ureter precontracted with a TXA₂ analogue was relaxed by GY4137. The response was prevented by the selective antagonist HC030031. Release and

Table 2

Publications on TRPA1-mediated effects of reaction products of sulfide and NO

Products of sulfide and NO interactions			
Na ₂ S	Rat middle meningeal artery	Vasodilatation inhibited by HC030031, L-NMMA and CGRP receptor antagonist	Dux <i>et al.</i> (2016)
Na ₂ S	Rat <i>dura mater</i>	CGRP release by ELISA potentiation by DEA-NONOate inhibition by L-NMMA	Dux <i>et al.</i> (2016)
Na ₂ S	Rat meningeal artery	Vasodilatation inhibited by HC030031, L-NMMA, L-NAME and CGRP receptor antagonist	Eberhardt <i>et al.</i> (2014)
Na ₂ S	Murine BP	Hypotensive effect inhibited by knockout of TRPA1, CGRP and L-NMMA	Eberhardt <i>et al.</i> (2014)
Na ₂ S	Murine mesentery	CGRP release by ELISA inhibited by L-NMMA	Eberhardt <i>et al.</i> (2014)
Na ₂ S	Rat mesenteric artery	Vasodilatation inhibited by L-NMMA, capsaicin pretreatment, CGRP receptor antagonist and HC030031	Eberhardt <i>et al.</i> (2014)
DEA-NONOate	Rat middle meningeal artery	Vasodilatation inhibited by oxamic acid and ODQ	Dux <i>et al.</i> (2016)
Na ₂ S + DEA-NONOate	Rat spinal trigeminal nucleus	Electrophysiology potentiating effect	Teicher <i>et al.</i> (2017)
Na ₂ S + DEA-NONOate	Rat DRG neurons	Ca ²⁺ imaging potentiation activation inhibited by HC030031	Miyamoto <i>et al.</i> (2017)
Na ₂ S + DEA-NONOate	Murine DRG neurons	Ca ²⁺ imaging activation inhibited by HC030031, DTT and mutation of key cysteines	Eberhardt <i>et al.</i> (2014)
Na ₂ S + DEA-NONOate	Murine heart	CGRP release by ELISA inhibited by knockout of TRPA1	Eberhardt <i>et al.</i> (2014)
Na ₂ S + DEA-NONOate	Human skin	Flare by laser Doppler imaging pain and itch potentiation	Eberhardt <i>et al.</i> (2014)
Na ₂ S + DEA-NONOate	Rat TRG neuron, brainstem slice, <i>dura mater</i>	CGRP release by ELISA potentiation	Wild <i>et al.</i> (2015)
HNO	Murine DRG neurons	Ca ²⁺ imaging activation inhibited by HC030031, TRPA1 knockout and mutation of key cysteines	Eberhardt <i>et al.</i> (2014)
HNO	Rat <i>dura mater</i>	CGRP release by ELISA inhibited by HC030031	Eberhardt <i>et al.</i> (2014)
HNO	Murine <i>dura mater</i>	CGRP release by ELISA inhibited by HC030031 and knockout of TRPA1	Eberhardt <i>et al.</i> (2014)
HNO	Murine sciatic nerve	CGRP release by ELISA inhibited by HC030031 and knockout of TRPA1	Eberhardt <i>et al.</i> (2014)
HNO	Rat meningeal artery	Vasodilatation inhibited by HC030031 and CGRP receptor antagonist	Eberhardt <i>et al.</i> (2014)
HNO	Mouse BP	Hypotensive effect inhibited by knockout of TRPA1	Eberhardt <i>et al.</i> (2014)
HNO	Human skin	Vasodilatation by laser Doppler imaging	Eberhardt <i>et al.</i> (2014)

DEA-NONOate, diethylamine NONOate; L-NMMA, *N*^G-monomethyl-L-arginine; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one.

participation of neuropeptides is indicated by inhibitory effect of either **pituitary adenylate cyclase-activating peptide** or CGRP receptor antagonists. In this model, relaxation produced by GYY4137 was also inhibited by a TRPV1 channel antagonist (Fernandes *et al.*, 2014). NaHS prevented contractions evoked by electrical stimulation in human ureter and prostate smooth muscle (Weinhold *et al.*, 2017). *In vivo* studies on TRPA1 channel-mediated effects of sulfide focused on vasodilatation, somatic and visceral nociception, as

well as bladder function. Vasorelaxant effect of NaHS was investigated in the mouse ear. The sulfide donor was applied topically, and blood flow changes were detected by laser Doppler imaging. Sulfide-evoked elevated blood flow could be lowered by the TRPA1 channel antagonist, desensitization of peptidergic nerve endings by **resiniferatoxin** pretreatment and genetic lack of the ion channel. The role of vasodilator peptides CGRP and SP was implied by the inhibitory action of the corresponding receptor antagonists. Inhibition

Table 3

Publications on TRPA1 channel-mediated effects of inorganic and organic polysulfides

Inorganic polysulfide			
Na ₂ S ₂ and Na ₂ S ₃	Rat DRG neurons	Ca ²⁺ imaging	Miyamoto <i>et al.</i> (2017)
Na ₂ S ₃	Chicken thoracic aorta epitheloid cells	5-HT release by HPLC inhibited by TRPA1 antagonist	Delgermurun <i>et al.</i> (2016)
Na ₂ S ₃	RIN14B cells	Ca ²⁺ imaging activation inhibited by HC030031	Ujike <i>et al.</i> (2018)
Na ₂ S ₃	HEK293 mTRPA1	Ca ²⁺ imaging and electrophysiology activation prevented by mutation of key cysteines and DTT	Hatakeyama <i>et al.</i> (2015)
Na ₂ S ₃	Murine hind paw	Nocifensive behaviour and oedema formation inhibited by TRPA1 knockout	Hatakeyama <i>et al.</i> (2015)
Na ₂ S ₃	RIN14B cells	Ca ²⁺ imaging and electrophysiology	Hatakeyama <i>et al.</i> (2015)
Na ₂ S ₃ and Na ₂ S ₄	Murine DRG neurons	Ca ²⁺ imaging activation inhibited by HC030031 and TRPA1 knockout	Hatakeyama <i>et al.</i> (2015)
Na ₂ S ₃ and Na ₂ S ₄	Rat astrocytes	Ca ²⁺ imaging inhibition by AP18 and HC030031	Kimura <i>et al.</i> (2013)
Sodium polysulfide	Murine hind paw carrageenan-induced inflammation	Antihyperalgesic effect inhibited by TRPA1 and sst4 knockout	Bátaï <i>et al.</i> (2018)
Organic polysulfides			
Ajoene	<i>Xenopus</i> oocyte	Electrophysiology enhances activation by TRPA1 agonists	Yassaka <i>et al.</i> (2010)
Allicin	Rat TRG neurons	Ca ²⁺ imaging	Bautista <i>et al.</i> (2005)
Allicin	HEK293 hTRPA1	Ca ²⁺ imaging	Bautista <i>et al.</i> (2005)
Allicin	<i>Xenopus</i> oocyte hTRPA1	Ca ²⁺ imaging	Bautista <i>et al.</i> (2005)
Allicin	Rat mesenteric artery	Relaxation prevented by capsaicin pretreatment and CGRP receptor antagonist	Bautista <i>et al.</i> (2005)
Asadisulfide	HEK293 rTRPA1	Ca ²⁺ imaging	Shokoohinia <i>et al.</i> (2013)
DAS	CHO hTRPA1	Ca ²⁺ imaging activation inhibited by HC030031	Koizumi <i>et al.</i> (2009)
DADS	CHO hTRPA1	Ca ²⁺ imaging activation inhibited by HC030031	Koizumi <i>et al.</i> (2009)
DADS	Rat TRG neurons	Ca ²⁺ imaging	Bautista <i>et al.</i> (2005)
DADS	HEK293 hTRPA1	Ca ²⁺ imaging	Bautista <i>et al.</i> (2005)
DADS	<i>Xenopus</i> oocyte hTRPA1	Ca ²⁺ imaging	Bautista <i>et al.</i> (2005)
DADS	Rat mesenteric artery	Relaxation prevented by capsaicin pretreatment and CGRP receptor antagonist	Bautista <i>et al.</i> (2005)
DATS	CHO hTRPA1	Ca ²⁺ imaging activation inhibited by HC030031	Koizumi <i>et al.</i> (2009)
DMS	CHO hTRPA1	Ca ²⁺ imaging, automated patch-clamp activation inhibited by HC030031	Pozsgai <i>et al.</i> (2017)
DMS	Murine TRG neurons	Ca ²⁺ imaging activation inhibited by HC030031 and TRPA1 knockout	Pozsgai <i>et al.</i> (2017)
DMS	Murine skin	Somatostatin release by RIA inhibited by HC030031	Pozsgai <i>et al.</i> (2017)
DMS	Murine hind paw heat injury	Anti-hyperalgesic effect inhibited by TRPA1 and sst4 knockout	Pozsgai <i>et al.</i> (2017)
DMS	Murine hind paw carrageenan-induced inflammation	Anti-hyperalgesic effect inhibited by sst4 knockout; lowered oedema formation inhibited by sst4 knockout; decreased MPO activity	Bátaï <i>et al.</i> (2018)

MPO, myeloperoxidase.

of **ATP-dependent K⁺ channels** reversed vasodilatation (Pozsgai *et al.*, 2012; Hajna *et al.*, 2016). Intraplantar administration of NaHS leads to nocifensive behaviour in mice shown as mechanical and cold hyperalgesia. Such responses are weakened by *trpa1*-gene deficiency, gene silencing of TRPA1 channels and a TRPA1 channel antagonist. Several papers

claim relieving activity of inhibitors or gene silencing of T-type Ca²⁺ channels, especially **Ca_v3.2** channels. A contribution of endogenous sulfide to intraplantar **LPS**-evoked pain was indicated by amelioration after the TRPA1 channel antagonist AP18 or inhibition of sulfide synthesis. Acidic pH potentiated nocifensive reaction to sulfide (Andersson

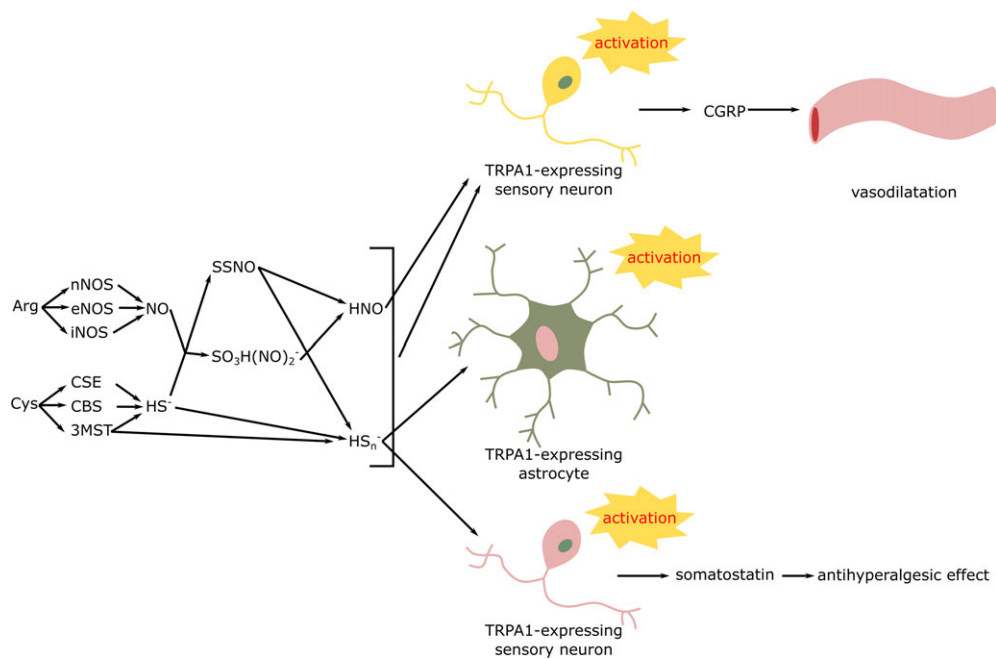


Figure 2

Products of the interaction of sulfide and NO and their documented effects in neurons and astrocytes. Related publications are listed in Tables 2 and 3. 3MST, 3-mercaptopyruvate sulfurtransferase; Arg, L-arginine; CSE, cystathionine- γ -lyase; Cys, L-cysteine; HS⁻, sulfide; HS_n⁻, hydrogen polysulfide; n/e/iNOS, neuronal/endothelial/inducible NO synthase; SO₃H(NO)₂⁻, dinitrososulfite.

et al., 2012; Ogawa *et al.*, 2012; Okubo *et al.*, 2012). Application of NaHS into the pancreatic duct in mice undergoing cerulein-induced pancreatitis led to elevated cFos expression in the spinal cord and mechanical allodynia. Visceral pain was relieved by a TRPA1 channel antagonist and a Ca_v3.2 channel inhibitor (Terada *et al.*, 2015). Intracolonic administration of NaHS induced visceral nociception, which was similarly inhibited by a TRPA1 channel antagonist and Ca²⁺ channel blocker (Tsubota-Matsunami *et al.*, 2012). However, another study detected visceral pain in TRPA1 knockout mice after intracolonic sulfide treatment (Andersson *et al.*, 2012; Tsubota-Matsunami *et al.*, 2012). Intravesical administration of NaHS in rats produced increased micturition frequency and decreased voiding volume as observed with known TRPA1 channel agonists. Expression of TRPA1 channels was demonstrated by immunohistochemistry in the urothelium (Streng *et al.*, 2008) (Table 1).

An emerging field of sulfide research proposes that products of the reaction of sulfide with NO are responsible for various biological actions, including those mediated by TRPA1 channels. The most investigated derivatives are HNO, polysulfides, SSNO and SULFI/NO (Cortese-Krott *et al.*, 2015). Combined application of sulfide and NO activated TRPA1 channels in rat TRG and DRG neurons, astrocytes and brainstem slices, as well as murine DRG cells. Responses were abolished by TRPA1 channel antagonists or reciprocal inhibition of sulfide or NO synthesis or downstream signalling. CGRP release in response to the sulfide/NO combination was detected in rat neurons and dura mater. A functional role of HNO in elevated dura skin blood flow, as well as its effect on murine BP was demonstrated by Eberhardt and colleagues in very elegant experiments. They also detected CGRP release

in response to HNO from various tissues including murine heart, mesentery, sciatic nerve and rat dura (Eberhardt *et al.*, 2014; Wild *et al.*, 2015; Dux *et al.*, 2016; Miyamoto *et al.*, 2017; Teicher *et al.*, 2017). Interaction of inorganic polysulfide with TRPA1 ion channels was examined in RIN14B, HEK293, chicken thoracic aorta epitheloid cells and murine DRG neurons. Calcium signal readouts were inhibited by a TRPA1 channel antagonist. In some reports, inhibitors of L- and N-type Ca²⁺ channels as well as NO synthesis ameliorated activation of TRPA1 channels. Critical cysteine residues of these channels involved in the activation by polysulfide were identified using cells expressing mutant receptor protein (Hatakeyama *et al.*, 2015; Delgermurun *et al.*, 2016; Ujike *et al.*, 2018). Intraplantar injection of sodium polysulfide evoked reduced pain and oedema formation in TRPA1 gene deficient mice compared to wild-type controls (Hatakeyama *et al.*, 2015). Repeated i.p. administration of sodium polysulfide was reported to relieve mechanical hyperalgesia due to carrageenan-induced paw inflammation in mice. The effect was inhibited if TRPA1 channels or somatostatin sst4 receptors were knocked out (Bátaí *et al.*, 2018) (Table 2).

Plant-derived asadisulfide produced Ca²⁺ signals in TRPA1-expressing HEK293 cells. Ajoene from garlic did not produce membrane currents in *Xenopus* oocytes alone but potentiated the action of various TRPA1 channel agonists. Organic sulfide compounds from garlic – DAS, DADS, DATS and DMTS – activated TRPA1 channels in CHO cells. Effects of diallyl compounds were not only diminished by a TRPA1 channel antagonist but also by capsazepine. Similar activity of allicin and DADS was found in rat TRG neurons. DMTS activated TRPA1 channels in murine TRG cells but did not have any effect on neurons from knockout animals. DMTS

activated nerve endings in mouse isolated skin and provoked somatostatin release. This effect was abolished in skin samples dissected from TRPA1 channel knockout mice. Allicin and DADS dilate rat mesenteric arteries and this vasodilation was inhibited either by desensitization of peptidergic nerve fibres by capsaicin pretreatment or a CGRP receptor antagonist. *In vitro*, DMTS reduced the mechanical hyperalgesia due to mild heat injury in a manner dependent on TRPA1 channels and sst4 receptors. In contrast, repeated i.p. administration of DMTS inhibited nociception, oedema formation and myeloperoxidase activity in murine carrageenan-evoked paw inflammation. These inhibitory effects were still present in TRPA1 knockout mice, indicating actions independent of TRPA1 channels (Bautista *et al.*, 2005; Koizumi *et al.*, 2009; Yassaka *et al.*, 2010; Shokoohinia *et al.*, 2013; Pozsgai *et al.*, 2017; Bártai *et al.*, 2018) (Table 3).

Putative mechanisms of sulfide-TRPA1 channel interactions

Functional modulation of TRP channels – including TRPA1 channels – by phosphorylation is a well-known phenomenon (Kádková *et al.*, 2017). In this section, we attempt to give a summary of indirect sulfide-TRPA1 channel interactions

based on the effect of sulfide on kinases. Reports of the sensitization or activation of TRPA1 channels by signalling kinases should be assessed cautiously, as elevated neuronal $[Ca^{2+}]_i$ can also reflect PLC activation or simply being included in external patch-clamp solutions could activate the channel (Figure 3).

Protein kinase A

The activation of TRPA1 channels by agonists is potentiated by bradykinin. This effect is partly mediated via PKA, as it is reproduced by PKA activators and prevented by its inhibitors (Kádková *et al.*, 2017). The most likely serine and threonine phosphorylation sites of human TRPA1 channels are located in ARD 1 (S86 and S87), 2 (T274), 8 (S317) and C-terminal cytoplasmic domain (S972 and S1101) (Kádková *et al.*, 2017). A wide range of reports claim modulation of PKA by sulfide or polysulfides. Sulfide treatment increases **cAMP** concentration in neuronal and glial cell lines, as well as in *Xenopus* oocytes (Kimura, 2000). The sulfide donor NaHS leads to persulfidation and activation of PKA in rat hippocampal neurons (Li *et al.*, 2016). NaHS raised $[Ca^{2+}]_i$ in human neuroblastoma SH-SY5Y cells partly by PKA-mediated phosphorylation of plasma membrane Ca^{2+} channels. The effect was blocked by a PKA inhibitor (Yong *et al.*, 2010). Sulfide-induced PKA activation was also described in microglial cells (Lee *et al.*,

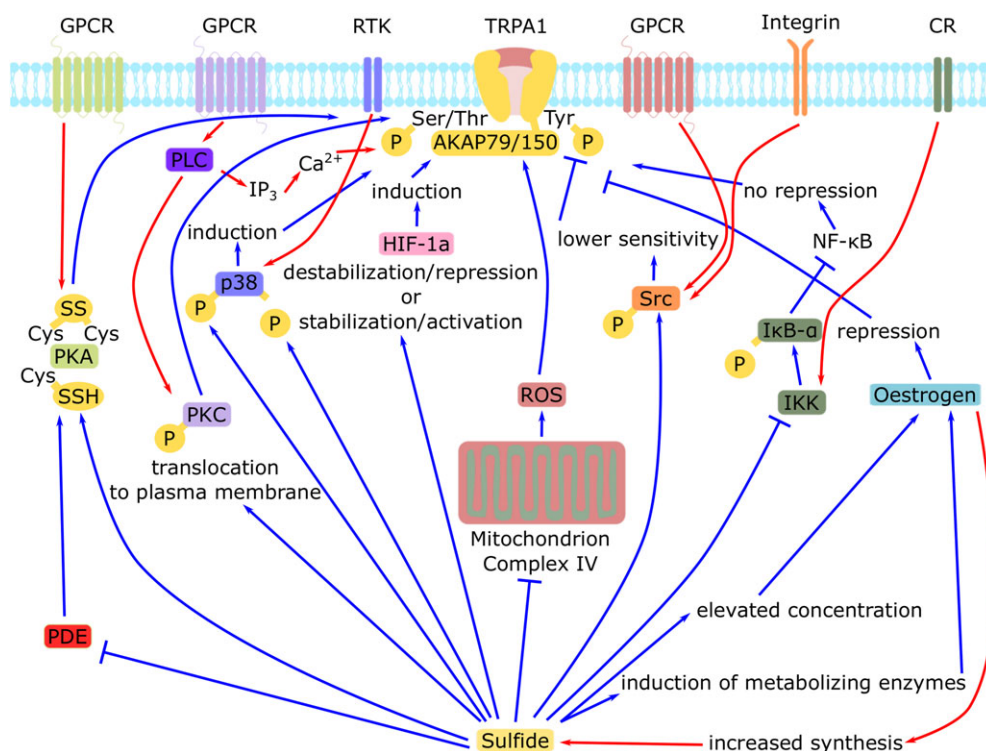


Figure 3

Putative mechanisms of the modulation of TRPA1 function or expression by sulfide. Modulation of the activation or expression of TRPA1 ion channel by the signalling kinases, transcription factors, hormones and reactive species is documented in the literature. Effect of sulfide on these signalling mechanisms was proven independently. Modulation of TRPA1 channels via these pathways has never been confirmed directly. In case of PKA persulfidation and disulfide formation in the are indicated. In case of IκB-α, p38, PKC, Src and TRPA1 phosphorylation or the lack thereof are shown. Arrows mean activation, and capped lines mean inhibition. CR, cytokine receptor; RTK, receptor TK.

2006). Apart from neuronal cells, similar effects were observed in hepatocytes (Untereiner *et al.*, 2016). Cysteine persulfides created in reactions with polysulfides might be reduced in a reaction with thiols of the same or other proteins leading to disulfide formation. Disulfide bond formation between two regulatory subunits of PKA involving cysteines C17 and C38 leads to enzyme activation in rat tissues (Burgoyne and Eaton, 2009). Sulfide might not only activate PKA by direct interaction but by inhibition of **PDE** enzymes. Sulfide-induced relaxation of rat aortic smooth muscle is mediated by elevated cGMP and is prevented by a pretreatment with a PDE inhibitor (Bucci *et al.*, 2010). Besides being an alternative electron acceptor in oxidative phosphorylation in a certain concentration range, inhibition of **PDE2A** in the mitochondrial matrix contributes to protective effects of sulfide, too (Módis *et al.*, 2013). Dimethyl disulfide, an organic disulfide compound, leads to pulmonary vasodilatation in rats by activating NOS via phosphorylation by PKA (Han *et al.*, 2017).

PKC

The interaction of PKC and TRPA1 channels is only supported by indirect data and still awaits direct evidence. PLC inhibitors – preventing PKC activation – ameliorate TRPA1 channel sensitization in response to activation of bradykinin or protease-activated receptors (Dai *et al.*, 2007; Pethő and Reeh, 2012). This effect might be simply mediated by lower Ca^{2+} efflux from the endoplasmic reticulum and consequent lower activation of TRPA1 channels. The A kinase anchor protein (AKAP79/150) that is able to bind PKA and PKC was reported to attach to TRPA1 channels. Several possible phosphorylation sites of PKC were identified in the sequence of TRPA1 protein (Kádková *et al.*, 2017). In order to provide a realistic view of the interaction of sulfide and PKC, it has to be noted that at least as many papers report inhibitory effect of the gasotransmitter on enzyme activity as those proposing activation. Activation of PKC by sulfide was documented in various cell types and tissues. Chronic pancreatitis leads to increased expression of **cystathionine β -synthase (CBS)** – the key enzyme of sulfide synthesis – and **PKC γ** in the arcuate nucleus. (Zheng *et al.*, 2016). Inhalation of H_2S gas activated PKC in the affected brain area and ameliorated the damage (Wei *et al.*, 2015). The protective effect of NaHS in an *in vitro* model of Parkinson's disease was mediated by the induction of **PKC α** and **PKC ϵ** in SH-SY5Y neuronal cells (Yong *et al.*, 2008). NaHS induced PKC phosphorylation and activation in the rat hippocampus (Li *et al.*, 2016). The PKC inhibitor **chelerythrine** blunted Ca^{2+} responses to NaHS in SH-SY5Y cells (Yong *et al.*, 2010). The protective effect of NaHS against hypoxic damage in SH-SY5Y cells was prevented by various PKC inhibitors (Tay *et al.*, 2010). Outside the nervous system, murine bone marrow mesenchymal stem cells heterozygous for CBS or with the enzyme being inhibited showed smaller PKC activity. PKC activity was elevated by NaHS treatment (Liu *et al.*, 2014). Beneficial effect of ischaemic postconditioning on rat cardiac myocytes was blunted by the inhibition of endogenous H_2S synthesis due to diminished activity of PKC α and PKC ϵ (Yong *et al.*, 2008). Preconditioning of rat cardiac cells with NaHS produced translocation of PKC ϵ and **PKC δ** into the plasma membrane (Hu *et al.*, 2008; Pan *et al.*, 2008).

Src kinase

Unlike the signalling kinases discussed above, **Src** kinase is a non-receptor, protein-tyrosine kinase (TK). Src integrates growth factor signalling, including that from **nerve growth factor**, as it can either switch on **Ras/Raf/MEK/ERK** or the **PI3K/Akt** signalling routes (Roskoski, 2015). Brain-derived neurotrophic factor and glial cell-derived neurotrophic factor were reported to induce TRPA1 channels in rat DRG neurons and increase the responsiveness to agonists (Ciobanu *et al.*, 2009). There is no direct evidence of phosphorylation of the TRPA1 channel by Src. On the other hand, sensitivity of neuronal SH-SY5Y cells to a TRPA1 channel agonist – lost during several passages of the cells – could be restored by an Src kinase inhibitor (Kádková *et al.*, 2017). The site of interaction might be tyrosine 69 (Y69) in the first ARD (Morgan *et al.*, 2015). Based on *in silico* models, there are two other tyrosines in TRPA1 channels proposed for phosphorylation by Src: number 22 and 680 in the N-terminal cytoplasmic domain. Few accounts on the interaction of sulfide and Src are available. NaHS increased neuriteogenesis in NG108-15 cells. The effect was blocked by Src inhibition. It has to be mentioned that supraphysiological concentrations of NaHS were applied (Tarui *et al.*, 2010). Interaction of sulfide and Src was also reported in non-neuronal cells. Phosphorylation of Src due to NaHS treatment contributes to migration of RAW264.7 murine macrophages (Miao *et al.*, 2016). Activation of Src upon NaHS treatment was described in murine pancreas acini (Tamizhselvi *et al.*, 2010).

p38 MAPKs

Odontoblast cells respond to stimulation by **TNF- α** with increased reaction of TRPA1 channels to agonist ligands and membrane stretch. The effects were mediated by **MAPKs 38** (p38)-evoked induction of TRPA1 channels (El Karim *et al.*, 2015). There is limited evidence that sulfur compounds are able to increase phosphorylation and activation of p38 kinase. NaHS increases **glucagon-like peptide-1** (GLP-1) secretion in mice via activation of p38 (Pichette *et al.*, 2017). Activation of p38 in response to NaHS was detected in human monocytes (Sulen *et al.*, 2016). Garlic-derived DATS ameliorates cisplatin-induced oxidative injury in NCI-H460 human lung carcinoma cells in a similar manner (Jiang *et al.*, 2017).

Inhibitory effects of sulfide on p38 kinase is more widely accepted. Inhibition of p38 by sulfide was detected in human, murine, rat neuronal tissues and cell lines (Li *et al.*, 2016; Lee *et al.*, 2016b). Inhibitory action of sulfide on p38 MAPK was reported in non-neuronal cells, (Wu *et al.*, 2017). The organic compound DADS inhibited p38 in C28/I2 human chondrocyte cell line (Hosseinzadeh *et al.*, 2017).

Casein kinase 2 (CK2)

CK2 is a highly conserved and ubiquitous serine/threonine protein kinase. CK2 is currently gaining significance in the regulation of Th17 immune response and inflammatory processes involving glomerulonephritis, intestinal inflammation, pulmonary disease, multiple sclerosis and contact dermatitis (Gibson and Benveniste, 2018). One suggested phosphorylation site of TRPA1 channels by CK2 is threonine number 1078 in the C-terminal cytoplasmic part of the ion channel. Replacement of threonine 1078 with aspartate,

mimicking phosphorylation, strongly influences electrophysiological properties of the channel in response to Ca^{2+} (Kádková *et al.*, 2017). Protection of rat myocardial cells from ischaemia-reperfusion injury and amelioration of apoptosis by NaHS involved increased CK2 activity (Yao *et al.*, 2012). The activity of CK2 is increased when intramolecular disulfides are reduced to the corresponding thiols (Zhang *et al.*, 1998). Recently, sulfide has been demonstrated to participate in the reduction of disulfides under physiological conditions, offering another possible mechanism for the interaction with CK2 (Vasas *et al.*, 2015).

AMP-activated protein kinase (AMPK)

AMPK functions as an energetic sensor of cells being activated by increased **AMP** (and therefore decreased **ATP**) concentrations. AMPK is either activated by phosphorylation or a Ca^{2+} signal via **Ca²⁺/calmodulin-activated protein kinase β** . Recently, the role of AMPK has been demonstrated in the regulation of cell autophagy, atherosclerosis, inflammatory disease and cancer. AMPK down-regulated TRPA1 channels in DRG neurons, as shown by a lowered amount of membrane-bound TRPA1 protein in response to metformin and elevated level of TRPA1 protein when AMPK activity was decreased (Wang *et al.*, 2018). Altogether the link between TRPA1 channels and AMPK is rather weak and is included to provide a full view of the area. Wang *et al.* (2017) provide an excellent review on the modulation of AMPK by sulfide. No evidence on the interaction of sulfide with AMPK is available in neurons. However, microglia-mediated neuroinflammation was ameliorated by various donors of sulfide via activation of AMPK (Wang *et al.*, 2017). Na_2S increased AMPK activity and attenuated myocardial damage due to smoking, high fat diet, ischaemia or cardiac arrest (Minamishima *et al.*, 2009; Wang *et al.*, 2017). Similar positive effects were recorded in rat aortic endothelial cells following challenge by high glucose and **palmitic acid**. Sulfide elicited this action by activating **nuclear factor erythroid 2-related factor 2** (Nrf2), a transcription factor involved in the response to oxidants and electrophiles (Wang *et al.*, 2017). Activation of Nrf2 involves thiol modification and inhibition of a regulatory protein – **Kelch-like ECH-associated protein 1** (Keap1) – that normally promotes ubiquitination and degradation of Nrf2 (Taguchi *et al.*, 2011). Nrf2-mediated protection against oxidative stress was detected in mouse neuroblastoma cells and glomerular endothelial cells in response to NaHS and polysulfide (Koike *et al.*, 2013; Wang *et al.*, 2017). Viability of rat embryonic cardiac cells in a high glucose environment was enhanced by the slow-release sulfide donor GYY4137 due to AMPK phosphorylation (Wang *et al.*, 2017). Elevated phosphorylation of AMPK in response to sulfide was detected in hepatic cells, colonic epithelial cells, osteoblasts and human monocyte cell line (Wang *et al.*, 2017). Garlic-derived organic disulfide ajoene initiated similar signalling mechanisms in hepatocytes (Wang *et al.*, 2017).

I κ B kinase complex (IKK)

Activation of the pivotal and ubiquitous transcription factor NF- κ B might occur via phosphorylation and consequent degradation of its inhibitory protein I κ B- α by the **IKK**. Functional data on nociceptor neurons genetically lacking

IKK suggest that the kinase complex suppresses expression of TRPA1 channels (Bockhart *et al.*, 2009). There is evidence that both inorganic sulfide and organic polysulfides modulate IKK and in this way could affect the expression of TRPA1 channels. Regulation of the IKK complex by sulfide was only investigated in non-neuronal tissues. Garlic-derived DATS promoted apoptosis in primary effusion lymphoma cells by inhibiting IKK activity, preventing proteasome-driven degradation of I κ B- α and consequently suppressing NF- κ B (Shigemi *et al.*, 2016). Similar effect of DADS was detected in carbon tetrachloride-exposed rat hepatocytes (Lee *et al.*, 2014). Inhibition of NF- κ B signalling by modulation of IKK by inorganic sulfide was described in rat cardiomyocytes exposed to haemorrhagic shock (Gao *et al.*, 2012). Sulfide-releasing diclofenac could prevent breast cancer-induced osteoclast formation via a comparable mechanism (Frantziadis *et al.*, 2012).

Oestrogen

The mechanism of modulating TRPA1 channels by oestrogen shown below is highly debatable and rests upon relatively limited evidence, but the authors feel obliged to include it in order to provide a full picture. There is a single report of ovariectomy-related apoptosis of rat hippocampal and DRG neurons partly relies on Ca^{2+} influx mediated by TRPA1 channels (Yazgan and Naziroglu, 2017). It is well known that oestrogen increases the expression of sulfide-synthesizing enzymes (Li *et al.*, 2017) and some data suggest that sulfide might reciprocally affect oestrogen synthesis or metabolism. Treatment with the sulfide precursor cysteine for 8 weeks elevated the serum concentration of **17 β -oestradiol** in rats (Han *et al.*, 2016). A garlic-derived organic sulfide, DAS, was reported to interact with intracellular **constitutive androstane receptor** and induce oestrogen metabolizing sulfotransferases. However, this did not affect the serum oestrogen concentration but only that of exogenously applied hormone (Green *et al.*, 2007; Sueyoshi *et al.*, 2011).

Hypoxia inducible factor 1 α (HIF-1 α)

Cigarette smoke extract induces expression of TRPA1 channels via **HIF-1 α** activation in A549 human alveolar adenocarcinoma cells (Nie *et al.*, 2016). The influence of sulfide on HIF-1 α activity and expression is still debatable. Some authors have reported destabilization and lowered expression of the transcription factor in various cell lines (Wu *et al.*, 2012). Others found the opposite, with activation and stabilization of HIF-1 α by sulfide in human THP-1 macrophages and rat endothelial cells (Liu *et al.*, 2010; Flannigan *et al.*, 2015; Lohninger *et al.*, 2015).

Mitochondrial dysfunction and oxygen radicals

Inhibition of complex III of the mitochondrial electron transport chain by antimycin A leads to the activation of TRPA1 channels in murine bronchopulmonary C fibres. The effect was mediated by ROS resulting from mitochondrial dysfunction (Nesuashvili *et al.*, 2013). Large amounts of sulfide are well known to inhibit complex IV (cytochrome c oxidase) and open the way for the production of ROS and depletion of GSH. The effect of sulfide on oxidative phosphorylation offers an indirect way of activating TRPA1 channels (Figure 3).

Concluding remarks

The biological importance of H₂S and polysulfides has been extensively investigated *in vitro* and *in vivo*. The role of TRPA1 channels and the signalling effects of sulfide compounds have been demonstrated separately in different organs either in the CNS or on the periphery in numerous physiological and pathological processes, including neuroimmune modulation, metabolic pathways, cardiovascular system, tumour growth, inflammation and pain. The present review provides a comprehensive overview about potential interactions between TRPA1 channel- and sulfide-activated signal transduction pathways. There are several sets of experimental data demonstrating direct binding and agonist effect of sulfide containing ligands on TRPA1 channels. They open the ion channel presumably by covalent modification of cysteine and lysine residues within the N-terminus and transmembrane domain. Analysing the list of recently published data, it is very likely that, apart from direct activation, sulfides modulate TRPA1 channels indirectly, via signalling kinases, as well. The key link to reveal the molecular mechanism is that sensitization or activation of TRPA1 channels has followed the phosphorylation of the protein. There is substantial evidence that sulfides can activate several kinases (PKA, PKC, Src, p38 MAPK, CK2, AMPK and IκB) in the signal transduction pathways. Modulation of TRPA1 channels by transcription factors, hormones and reactive species is also documented in the literature. TRPA1-independent and TRPA1-dependent effects – resulting from either by direct or indirect mechanisms – of sulfides play pivotal roles in regulation of cellular functions. Understanding interactions between TRPA1 channels and sulfides may contribute to the discovery of novel drug targets for the treatment of a range of diseases.

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 (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c,d,e,f).

Acknowledgements

This study was funded by the following grants of the National Research, Development and Innovation Office – NKFIH, Hungary: OTKA PD 112171, OTKA NN 114458. GINOP-2.3.2.-15-2016-00050 'PEPSYS', EFOP-3.6.3-VEKOP-16-2017-00009 and EFOP-3.6.2-16-2017-00009 'HECRIN' from the European Regional Development Fund. This project was supported by the János Bolyai Research Scholarship (G.P.) and by János Szentágothai Fellowship A2-SZJÖ-TOK-13-0149 (E.P.) of the Hungarian Academy of Sciences (Magyar Tudományos Akadémia) and by the ÚNKP-18-4 New National Excellence Program of the Ministry of Human Capacities.

Conflict of interest

The authors declare no conflicts of interest.

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