ANTIOXIDANTS & REDOX SIGNALING
Volume 17, Number 1, 2012

DOI: 10.1089/ars.2011.4359

FORUM REVIEW ARTICLE

Modulation of Ion Channels by Hydrogen Sulfide

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Abstract

Significance: Evidence of the ability of the gasotransmitter hydrogen sulfide (H_2S) to serve as a regulator of many physiological functions, including control of blood pressure, regulation of cardiac function, protection of neurons, and cardiomyocytes against apoptosis, and in pain sensation is accumulating. However, the mechanisms accounting for its many actions are not yet well understood. ***Recent Advances:*** Following the pioneering studies of the regulation of *N*-methyl-D-aspartate receptors and ATP-sensitive K^+ channels by H_2S, data continue to emerge indicating that H_2S modulates other ion channel types. This article reviews the numerous, yet diverse, types of ion channels now reported to be regulated by H_2S. ***Critical Issues:*** Currently, a critical issue within this field is to determine the mechanisms by which H_2S regulates ion channels, as well as other target proteins. Mechanisms to account for regulation include direct channel protein sulfhydration, channel redox modulation, effects mediated by interactions with other gasotransmitters (carbon monoxide and nitric oxide), and indirect effects, such as modulation of channel-regulating kinases. Through such modulation of ion channels, novel roles for H_2S are emerging as important factors in both physiological and pathological processes. ***Future Directions:*** Increasing current awareness and understanding of the roles and mechanisms of action of ion channel regulation by H_2S will open opportunities for therapeutic intervention with clear clinical benefits, and inform future therapies. In addition, more sensitive methods for detecting relevant physiological concentrations of H_2S will allow for clarification of specific ion channel regulation with reference to physiological or pathophysiological settings. *Antioxid. Redox Signal*. 17, 95–105.

Introduction

Hydrogen sulfide (H_2S) is an endogenous, biologically active gas of both physiological and pathophysiological importance in the cardiovascular and nervous systems, and elsewhere (29). It is regarded as the third gasotransmitter, receiving increasing interest over the past decade or so, much as nitric oxide (NO) and carbon monoxide (CO) have previously (28, 36, 60). H_2S is generated from cysteine primarily by two widely distributed enzymes, cystathionine γ lyase (CSE) and cystathionine β synthetase (CBS). More recently, 3-mercaptopyruvate sulfurtransferase (3MST; interestingly found in mitochondria) has also been demonstrated to generate H_2S in the brain and vasculature. 3MST generates H_2S from 3-mercaptopyruvate, which is itself generated from cysteine aminotransferase [CAT; (23)]. Other sources of H_2S include red blood cells, which can generate H_2S nonenzymically from inorganic polysulfides. This has led to the suggestion that H_2S may mediate the beneficial vascular effects of dietary garlic (3). In addition, H_2S can be liberated from sulfur stores (sulfur bound to proteins in mitochondria or the cytosol) in a redox- or pH-sensitive manner (23). CBS predominates in the central nervous system, along with 3MST (28), whereas CSE is believed to generate the majority of H_2S in the periphery, including the vasculature. The involvement of H_2S in various fundamental biological processes is increasingly being reported, ranging from nociception and neuroprotection, through modulation of insulin secretion, to inflammation and apoptosis (29, 67). Indeed, the anti-inflammatory and cardioprotective properties of H_2S are being exploited therapeutically through the development of H_2S-releasing compounds (27, 29).

When the widespread biological actions of H_2S were first being realized, initial estimates of dissolved H_2S levels ranged from 20 to 100 μM, but it should be noted that H_2S detection is problematic, and these values are likely to be overestimates. Indeed, more recent measurements have downsized these initial values by orders of magnitude [reviewed by Li *et al.* (29)]. Nevertheless, its biological importance, along with that of NO and CO, has continued to grow apace, and in time all of these gasotransmitters could well be regarded as ubiquitously influential. This is highlighted by the growing number of pathological conditions under which H_2S metabolism is altered. Certainly, the list of signaling pathways and effectors

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regulated by H_2S continues to grow, and one major group of target proteins regulated by H_2S appears to be ion channels (29). This in itself implies that H_2S is influential in membrane excitability as well as any other transmembrane process that might depend on electrochemical gradients.

In this article, we discuss the regulation of known ion channel targets of H_2S. While the first ion channel identified as being regulated by H_2S was the *N*-methyl-D-aspartate (NMDA) receptor (1), and the list of subsequent channels has grown considerably (63) in the intervening years, we focus primarily on K^+ and Ca^{2+} channels, and emphasize the widespread physiological influence modulation of such ion channels may have. We also describe candidate mechanisms that might account for the actions of H_2S, and detail current shortcomings in our knowledge, which require resolution before we can fully exploit the therapeutic potential of this gasotransmitter.

ATP-Sensitive K^+ Channels

One of the best characterized ion channels identified as a target for regulation by H_2S is the vascular ATP-sensitive K^+ (K_{ATP}) channel (79). This channel, the pore of which is composed of four inwardly rectifying (K_{IR} 6.X) channel subunits, associated with four sulfonylurea receptors (SURs) (Fig. 1), which confer unique pharmacological as well as other properties on the channel, is widely distributed (12). Its activation by H_2S, shown schematically in Figure 1, was an exciting breakthrough, since it could account simply for the vasodilatory actions of H_2S: K_{ATP} activation leads to hyperpolarization of vascular smooth muscle cells, causing vasodilation by reducing voltage-gated Ca^{2+} influx. Subsequent studies indicated that in addition to this action on K_{ATP} channels in vascular smooth muscle, there was a significant endothelial component to the vasodilation caused by H_2S; this arose from the ability of endothelium-derived NO to stimulate both the activity and expression of CSE (Fig. 1), enhancing H_2S production (78). Since this observation, evidence has accumulated that H_2S activation of K_{ATP} channels (which are found in the mitochondrial as well as the plasmalemmal membrane) contributes to myocardial protection against ischemia/reperfusion (I/R) injury, control of insulin secretion from pancreatic β cell-derived cell lines, and regulation of inflammation and nociception [reviewed in Ref. (68)]. In addition, activation of neuronal K_{ATP} channels by H_2S has been suggested to contribute to the protective actions of H_2S against glutamate-induced toxicity (24, 25), as discussed in the subsequent section concerning K^+ channels and apoptosis.

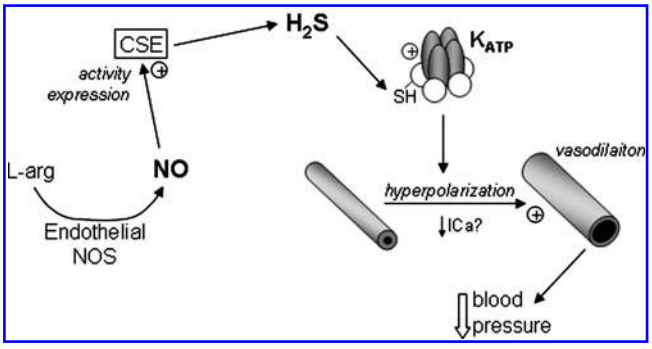

FIG. 1. Schematic representation of hydrogen sulfide (H_2S)-mediated regulation of vascular ATP-sensitive K^+ (K_{ATP}) channels by H_2S. The K_{ATP} channel, comprising four inwardly rectifying channel subunits (*shaded*), associated with four sulfonylurea receptors (SUR) (*white circles*), is activated by H_2S *via* SUR cysteine modification. Activation leads to hyperpolarization and relaxation of vascular smooth muscle, which in turn contributes to a lowering of blood pressure. These actions of H_2S are regulated in turn by the endothelium, since endothelial nitric oxide (NO) stimulates both expression and activity of cystathionine γ lyase (CSE), the main source of vascular H_2S.

The observation that K^+ currents generated by heterologous expression of K_{IR} subunits (in the absence of SUR receptors) were insensitive to H_2S strongly suggested that SUR receptors were the sites of modulation by H_2S (19). Such a proposal is supported by earlier data indicating that H_2S increased the open probability of K_{ATP} channels in excised membrane patches independently of ATP levels (73), which argues strongly for a direct mechanism of H_2S action. Further insight was gained when it was found that either alkylation or oxidation of key cysteine residues located on the extracellular face of the SUR1 protein prevented regulation by H_2S (19). Most recently, Mustafa *et al.* (38) demonstrated that Kir6.1 is directly sulfydrated by H_2S, leading to a reduction of ATP binding (which normally leads to channel closure) and promoting channel binding to phospholipid phosphatidylinositol (4,5)-bisphosphate, which enhances channel activity. This study represents the most detailed investigation published to date of the mechanism by which H_2S regulates ion channels; other candidate mechanisms are discussed in detail below.

$Ca2^+$-Activated K^+ Channels

Telezhkin and colleagues have demonstrated that H_2S (applied as NaHS) inhibited both human recombinant Ca^{2+}-activated K^+ (BKCa) channel α subunits (KCNMA1) expressed in HEK293 cells and native BKCa channels expressed in type I cells from the carotid body of the rat (65) (Fig. 2). This channel is widely distributed in most, if not all, tissues, and has well-described roles in the central nervous system and vasculature [reviewed in Ref. (13)]. It is of particular importance to the process of oxygen-sensing by the carotid body, being inhibited by hypoxia and so contributing to type I cell depolarization (47). This in turn leads to voltage-gated Ca^{2+} entry and transmitter release, which excites afferent sensory nerves and so, ultimately, increases our ventilation in response to acute hypoxia [reviewed in Ref. (26)]. This channel is also sensitive to the gasotransmitter CO, which augments its activity (see Fig. 2) and has been postulated as a mechanism by which the channel may sense hypoxia: in the rat type I cell, the CO-generating enzyme heme oxygenase-2 associates with BKCa channels and regulates their activity through the tonic generation of CO (*via* degradation of heme, which is an O_2-dependent process) (70). It is of interest that both H_2S and CO regulate this channel since both gases—and the channel itself—are of influence in the O_2-sensing mechanism, which is fundamental to the chemosensory ability of the carotid body. Telezhkin *et al.* (65) demonstrated that H_2S inhibited the channel *via* a mechanism distinct from its regulation by CO: thus, CO activation was blocked by cyanide, but this had no

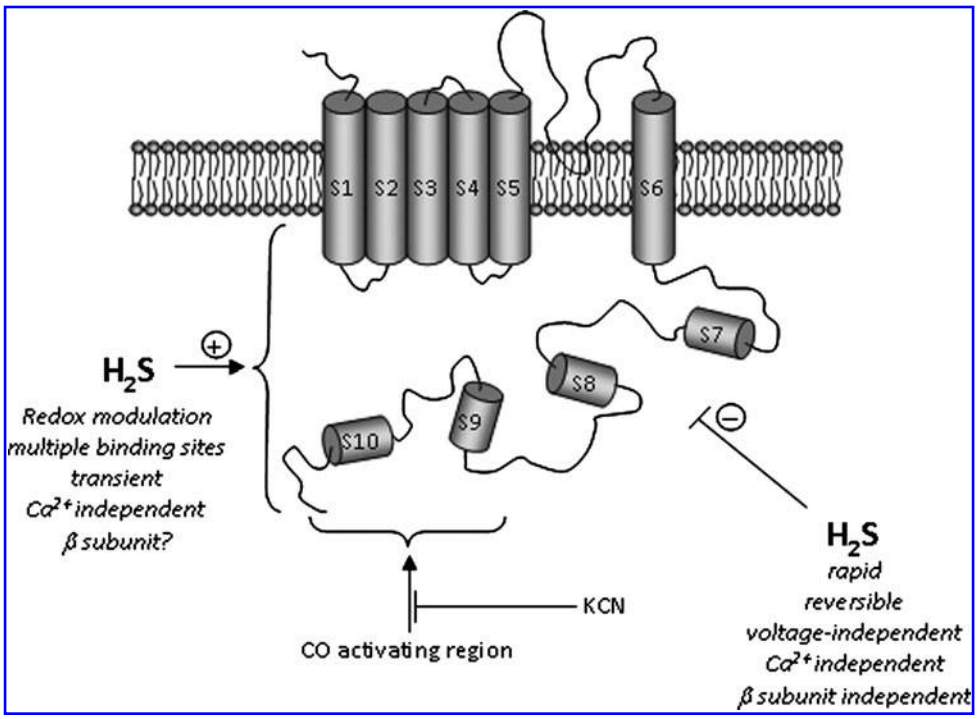

FIG. 2. Conflicting reports on the regulation of Ca^{2+}-activated K^+ channel (BKCa) channels by H_2S. One study (53) indicates that H_2S augments channels activity *via* redox modulation of low- and high-affinity intracellular sites. Whether β subunits were involved was not determined. By contrast, inhibition of BKCa channels has been shown to be independent of β subunits, and discounted the involvement of the region of the C-terminal domain responsible for activation *via* CO (ref 63). In addition, this study indicated that KCN, which prevented CO activation, did not alter H_2S inhibition.

effect on channel inhibition by H_2S. Furthermore, the authors generated a BKCa chimera that lacked both Ca^{2+} and CO sensitivity, yet remained sensitive to inhibition by H_2S.

This study—and its importance for carotid body function—was supported by experiments in which mouse carotid body excitation was observed in response to H_2S application. Thus, Li *et al.* (30) demonstrated the excitatory effects of H_2S and, crucially, found that hypoxic excitation of the carotid body was inhibited by suppression of endogenous H_2S using two distinct CBS inhibitors (surprisingly, given that type I cells also express CSE, inhibition of this enzyme was found to be without effect). Patch-clamp recordings indicated that H_2S mimicked the ability of hypoxia to inhibit BKCa channels in isolated type I cells, and in fact the ability of hypoxia to reduce channel activity was suppressed by a CSE inhibitor, suggesting involvement of H_2S in this effect also. An additional study has confirmed the central importance of H_2S in carotid body O_2 sensing by demonstrating a markedly weakened response to hypoxia in $CSE^{-/-}$ mice (48), although this study did not include direct analysis of ion channel modulation.

Collectively, the above-described studies strongly suggest that inhibition of BKCa channels in type I glomus cells by H_2S is of fundamental physiological importance to carotid body function. However, another report has indicated that H_2S increases the activity of BKCa channels expressed in the rat pituitary cell line, GH3 (55). The authors proposed that H_2S acted in a Ca^{2+}-independent manner to reduce cysteine residues accessible from the cytosolic face of the channel protein. Presently, there is no explanation to account for these opposing reports on BKCa modulation. Possibilities, some offered by the authors (55), include differential effects on splice variants, differing phosphorylation states of the channel protein, involvement of auxiliary (β4) subunits, or the differential presence of unidentified, associated proteins which may regulate channel activity in a H_2S-sensitive manner. Interestingly, a most recent report has suggested that H_2S can also activate BKCa channels in smooth muscle of small mesenteric arteries (15). Based in part on the ability of the BKCa inhibitor iberiotoxin to occlude the effects of H_2S, this study concluded that H_2S generated by endothelial CSE caused vasorelaxation not *via* activation of K_{ATP} channels (as described above) but instead by activating BKCa channels, thereby hyperpolarizing and relaxing smooth muscle cells. Although no direct patch-clamp electrophysiological evidence for BKCa activation by H_2S was provided, this study supported the idea that in tissues other than the clonal GH3 cell line H_2S can activate BKCa channels.

Clearly, further work is required to resolve these opposing effects of H_2S on BKCa channels, and indeed to fully characterize the underlying mechanisms in each case. However, it already appears that BKCa represents an influential physiological target for regulation by H_2S.

K^+ channels and apoptosis

In the central nervous system (CNS), H_2S is clearly neuroprotective. There are a number of mechanisms that have been proposed to account for its actions in this regard. It has, for example, been shown to increase neuronal levels of reduced glutathione (GSH) in part through increasing cysteine uptake (23). Interestingly, expression of 3MST and CAT in neuronal mitochondria increases resistance to oxidative stresses, implying that mitochondria may be an important source of deleterious reactive oxygen species (ROS) that could lead to neurodegeneration.

Numerous studies have implicated the delayed rectifier channel Kv2.1 as being important in oxidative stress-induced neuronal apoptosis (Fig. 3). Since K^+ acts to regulate caspase activation, mitochondrial membrane potential and volume, and (through its abundance) osmolarity and hence cell volume, cellular apoptosis is strongly influenced by intracellular K^+ levels (75). K^+ loss is a key early stage in apoptosis, and efflux

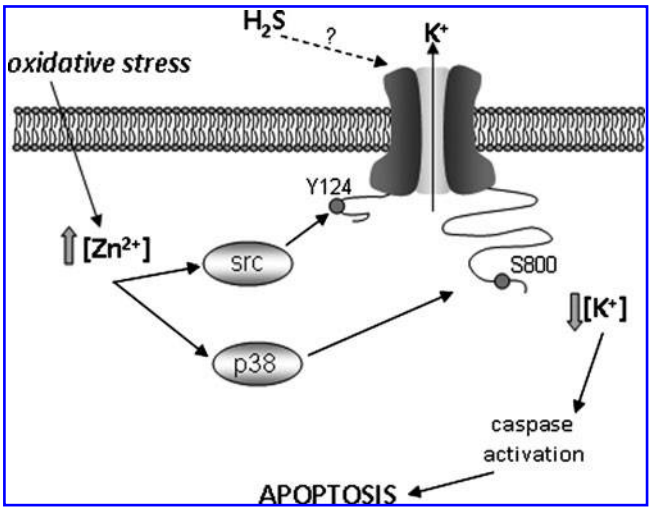

FIG. 3. The role of Kv2.1 in apoptosis. Schematic indicating oxidant-induced apoptosis: oxidative stress, by mobilizing free intracellular Zn^{2+}, activates p38 MAP kinase, which in turn phosphorylates Kv2.1 at serine 800. In addition, Zn^{2+} increases src kinase activity to phosphorylate tyrosine residue 124. This increases insertion of functional channels into the plasma membrane, and the resultant loss of intracellular K^+ triggers caspase activation and hence apoptosis. The potential effects of H_2S on Kv2.1 channel function remain undetermined.

occurs through K^+ channels to trigger the apoptotic cascade: cell shrinkage, mitochondrial disruption, cytochrome c release, and caspase activation (75). K^+ loss occurs *via* K^+ channels: thus, K^+ channel inhibitors can protect against apoptosis triggered by a variety of insults, including oxidative stress (2, 4) and exogenously applied amyloid β peptide of Alzheimer's disease (76). Theoretically, any class of K^+ channel could serve to permit K^+ loss, but evidence suggests a particularly important role for the delayed rectifier channel Kv2.1 in mediating K^+ efflux that leads to neuronal apoptosis: neurons expressing dominant negative Kv2.1 constructs (therefore lacking functional Kv2.1 channels) were protected against experimentally induced apoptosis, and expression of Kv2.1 in Chinese hamster ovary (CHO) cells increased their susceptibility to apoptosis (44). Pro-apoptotic agents cause a rapid increase in the surface expression of Kv2.1 channels (Fig. 3), a process thought to require p38 MAP kinase phosphorylation at Ser-800 in the intracellular C-terminal region of the channel protein and additional phosphorylation of an *N*-terminal tyrosine (Y124) regulated *via* Src kinase (51).

An additional feature of neuronal oxidative stress is the upregulation of the inducible form of the CO-generating enzyme heme oxygenase (HO-1). Since CO provides neuronal protection against stresses such as stroke and excitotoxicity (77), we recently investigated whether CO might regulate Kv2.1 (6). We found that CO specifically protected HEK293 cells overexpressing Kv2.1 against oxidative stress, while the vulnerability of untransfected cells to oxidative stress was unaffected by CO. Furthermore, CO reversibly inhibited the Kv2.1 currents in these Kv2.1-expressing cells. Importantly, in hippocampal neurons, CO selectively inhibited Kv2.1 and reversed the dramatic oxidant-induced increase in K^+ current density as well as providing protection against oxidant-induced apoptosis (6). It will be of importance to determine in future experiments whether some of the neuroprotective effects of H_2S may involve interruption of Kv2.1 trafficking or activity and hence may be antiapoptotic *via* this pathway.

Although a role for Kv2.1 in neuroprotection by H_2S remains speculative at present, there is much evidence that K_{ATP} channels are involved in this process. For example, Tay *et al.* (64) demonstrated that prior exposure of neuroblastoma cells to hypoxic/ischemic conditions *in vitro* provided striking protection (*via* application of NaHS at 10–100 μM). Protection was prevented by the plasmalemmal K_{ATP} channel blocker glibenclamide (a mitochondrial K_{ATP} channel blocker was ineffective), and was also dependent on the involvement of protein kinase C/extracellular receptor kinase (ERK1/2) activation, as well as activation of HSP90, which interacts with and prevents activity of various pro-apoptotic proteins (see Fig. 4 for schematic representation). Such an action of H_2S is in addition to its known effect to increase reduced GSH levels and so afford protection against oxidative stress (24, 25). This effect may also be of importance in protection against hypoxic insults, since some studies indicate that hypoxia can increase ROS production from mitochondria, thereby causing a seemingly paradoxical oxidative stress (11). It is important to note, however, that convincing evidence for a protective role of H_2S in the central nervous system in response to hypoxic/ischemic challenges requires *in vivo* evidence. Indeed, we need to understand more completely the relationship between oxygen levels and levels of H_2S: while some studies suggest that hypoxic/ischemic conditions dramatically reduce endogenous H_2S levels (64) (which would remove or reduce protective effects of H_2S), other studies argue that tonic production of H_2S leads to increased net levels in hypoxia, *via* H_2S

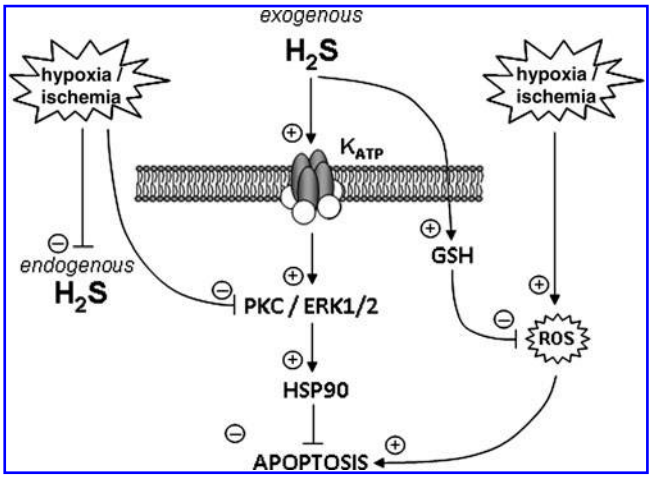

FIG. 4. Pathways involved in the neuroprotective effects of H_2S. Hypoxic/ischemic conditions can inhibit endogenous H_2S production and inhibit protein kinase C (PKC), leading to apoptosis through the inhibition of HSP90. Stimulation of K_{ATP} channels, *via* H_2S, can increase PKC and extracellular receptor kinase (ERK)1/2 activity to stimulate HSP90 and so afford protection against apoptosis. The possible effects of hypoxia to increase pro-apoptotic intracellular reactive oxygen species (ROS) is also countered by H_2S *via* its stimulation of intracellular glutathione, the reduced form of which glutathione (GSH) acts to buffer ROS levels.

accumulation, since its oxidation is limited by available oxygen (43).

T-Type Ca^{2+} Channels

T-type Ca^{2+} channels are a unique class of voltage-gated Ca^{2+} channel (VGCC), distinguished functionally from other VGCCs by their rapid activation and inactivation properties, slow deactivation, smaller single-channel conductances, and their low membrane potential threshold for activation, which is more negative than the threshold for other VGCCs [they were originally termed low-voltage-activated currents (49)]. T-type Ca^{2+} channels are encoded by three genes (*CACNA1G*, *CACNA1H*, and *CACNA1I*) giving rise to voltage-sensing, pore-forming α_{1G}, α_{1H}, and α_{1I} subunits, now termed Cav3.1, Cav3.2, and Cav3.3, respectively. Also distinguishing them from other VGCCs is their pharmacology and the fact that heterologous expression of these channels gives rise to currents which are remarkably similar to native currents, suggesting that their function is primarily determined by the pore-forming α subunits alone, without major regulation by auxiliary subunits [although their trafficking may be altered (8)]. T-type Ca^{2+} channels are widely expressed in both central and peripheral nervous systems and serve a wide variety of functions (62). In central neurons T-type Ca^{2+} channels are responsible for pacemaker activity and low threshold spikes, which in turn give rise to Na^+-dependent action potential bursts, and also contribute to rebound bursts of spiking activity following a hyperpolarizing inhibitory postsynaptic potential. This latter property contributes to oscillatory activity, and thus T-type Ca^{2+} channels have become a focus of study in the treatment of epilepsy [reviewed in Ref. (40)]. They also display a small, but significant, window current (*i.e.*, are tonically active) at membrane potentials close to resting potential, so can contribute to tonic Ca^{2+} influx (49).

In the periphery T-type Ca^{2+} channels play an important role in nociception: they are prominently expressed in small nociceptive dorsal root ganglion (DRG) neurons. Cav3.2 (α_{1H}) is the dominant form of T-type Ca^{2+} channel in these neurons, and although their role in controlling DRG neurons is less well defined than in central neurons, they control burst firing and so influence excitability (40). This implies that they are of central importance to nociception since stimulus intensity correlates with burst frequency, and much information supports this. For example, native DRG T-type currents (and recombinant Cav3.2) are enhanced or inhibited by reducing and oxidizing agents, respectively. Accordingly, hindpaw injections of reducing agents induced thermal and mechanical hyperalgesia, an effect prevented with the T-type inhibitor mibefradil and absent in Cav3.2$^{-/-}$ mice (40, 41). These data clearly suggest that augmentation of T-type Ca^{2+} currents can be hyperalgesic, while their inhibition may lead to analgesia. Altered function or expression of T-type Ca^{2+} channels has also been implicated in chronic pain, as studied experimentally in models such as diabetic neuropathy and constriction-induced chronic pain (16, 59). Thus, for example, T-type Ca^{2+} current amplitudes are enhanced in DRG neurones in experimental diabetic neuropathy (17), and *in vivo* antisense knockdown of Cav3.2 suppresses hyperalgesia and DRG T-type Ca^{2+} currents in this model (35).

The above-described studies shed important light on the mechanism that can account for the hyperalgesic effects of H_2S. Thus, for example, the effects of intraplantar injection of NaHS can be abolished by both the oxidizing agent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and by known T-type Ca^{2+} channel inhibitors. Hyperalgesia could also be suppressed by blockade of endogenous H_2S production (22). Subsequent studies concluded that H_2S could activate or sensitize Cav3.2 channels either in primary afferent or spinal sensory neurons to account for hyperalgesia and in models of chronic pain the associated hyperalgesia and allodynia could be prevented by inhibitors of CSE as well as the T-type channel inhibitor, mibefradil or by antisense knock-down of T-type Ca^{2+} channels (61). Finally, in a model of H_2S-induced colonic pain, the actions of H_2S could be mimicked by chelation of Zn^{2+} (34), which normally tonically blocks Cav3.2 by binding to an extracellular histidine residue (see below) (41).

These behavioral studies strongly indicate that H_2S regulation of T-type Ca^{2+} channels is an important feature of both acute and chronic pain sensation. It is surprising, therefore, that there is no detailed electrophysiological investigation of the modulation of T-type Ca^{2+} channels by H_2S: Kawabata *et al.* (22) indicated that T-type Ca^{2+} currents in the NG108-15 hybridoma line were enhanced after 2–3-min exposure to NaHS, and such effects were abolished by either DTNB or the reducing agent dithiothreitol (DTT), but this was only effective at a high concentration of 1.5 m*M*. There was no evidence presented to indicate that H_2S was effective through direct channel modulation (redox or otherwise) or, for example, by altering channel trafficking, which could conceivably be

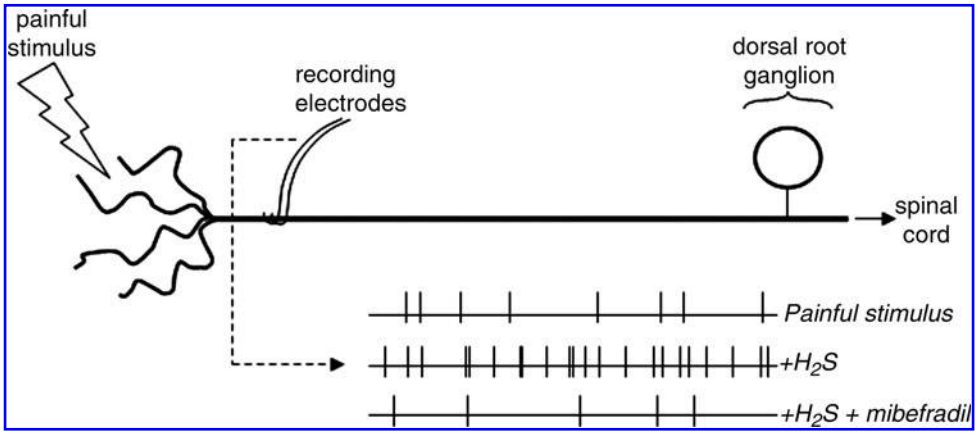

FIG. 5. Schematic illustrating the putative effects of H_2S on nociception. In response to a painful stimulus, afferent sensory nerve discharge increases. In the presence of H_2S, this increase in discharge frequency is enhanced, potentially due to augmentation of T-type Ca^{2+} channel activity. In the presence of H_2S and also mibefradil, a T-type Ca^{2+} channel blocker, no pro-nociceptive augmentation of nerve discharge is observed.

modified within such a timeframe. A schematic summarizing the possible role of H_2S in regulating nociception *via* modulation of T-type channels is shown in Figure 5.

L-type Ca^{2+} channels

The voltage-gated L-type Ca^{2+} channel (Cav1.x) has also been shown to be targeted by H_2S, but published reports of resulting effects are mixed. In neuronal tissue [both the human neuroblastoma SH-SY5Y (74) and primary cultures of cerebellar granule neurons (10)] evidence has been presented that H_2S raises intracellular calcium concentration ($[Ca^{2+}]_i$) *via* activation of L-type Ca^{2+} channels. In SH-SY5Y cells, this effect was acute, and rises of $[Ca^{2+}]_i$ were observed in response to submillimolar levels of NaHS, peaking after ~15 min. These rises were inhibited by L-type Ca^{2+} channel blockers [and also by mibefradil, which preferentially inhibits T-type Ca^{2+} channels, although SH-SY5Y cells do not express these channels (52)] and also inhibitors of protein kinases A and C. In cerebellar granule neurons, H_2S (50–300 μM) also caused rises of $[Ca^{2+}]_i$, which could be inhibited by known L-type Ca^{2+} channel blockers, but these responses were only reported after much longer periods of exposure (~1–2 h). Importantly, these rises of $[Ca^{2+}]_i$ led to glutamate release and subsequent neurotoxicity, which could be prevented with NMDA receptor antagonists (Fig. 6). This finding contrasts with the previously reported neuroprotective effects of H_2S (25), and may also involve the known enhancing effects of H_2S on NMDA receptors (1). Despite the general agreement that H_2S activates these neuronal L-type Ca^{2+} channels, conclusions were based on indirect measurements of channel activity (*i.e.*, fluorometric detection of $[Ca^{2+}]_i$) and more direct patch-clamp recordings were lacking.

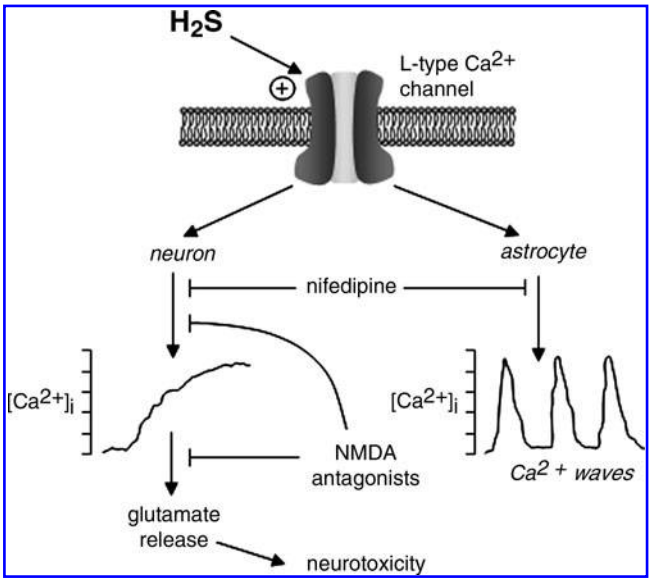

FIG. 6. Schematic illustrating the consequences of activation of L-type Ca^{2+} channels by H_2S in the central nervous system. Activation of L-type Ca^{2+} channels in neurons can lead to elevation of intracellular Ca^{2+} levels sufficient to trigger glutamate release and so initiate excitotoxicity. Influx and toxicity can be prevented by the L-type Ca^{2+} channel inhibitor nifedipine or by *N*-methyl-D-aspartate (NMDA) receptor antagonists. In astrocytes, activation of L-type Ca^{2+} channels can trigger Ca^{2+} waves that can propagate between adjacent astrocytes.

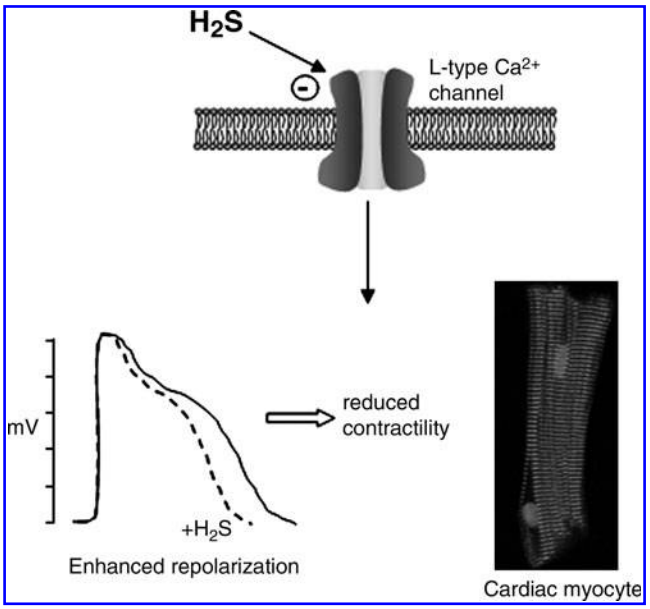

FIG. 7. Schematic illustrating the consequences for myocardial excitability and contractility of reducing Ca^{2+} influx *via* L-type Ca^{2+} channels by H_2S. Inhibition of the L-type Ca^{2+} current will reduce the depolarizing influence of Ca^{2+} influx, thereby allowing more rapid myocyte repolarization, which will limit contractility.

As was the case in neurons, exposure of hippocampal astrocytes to H_2S also raised $[Ca^{2+}]_i$ and in some cases induced Ca^{2+} waves (39), as illustrated in Figure 6. This stimulatory effect of H_2S was attributed in part to activation of L-type Ca^{2+} channels, since effects were reduced in the presence of nifedipine. However, induction and propagation of these Ca^{2+} waves also involved intracellular Ca^{2+} stores and alternative (*i.e.*, non-L-type Ca^{2+} channel dependent) Ca^{2+} entry pathways, so the relative importance of astrocytic L-type Ca^{2+} channels—and their regulation by H_2S—is not fully understood.

In stark contrast to the effects of H_2S on neuronal L-type Ca^{2+} channels, those recorded in cardiac myocytes were inhibited by H_2S (58). This study used whole-cell patch-clamp recordings to measure Ca^{2+} currents directly, and inhibition by H_2S or NaHS was observed at concentrations lower than those required to activate K_{ATP} channels. H_2S inhibition of the L-type Ca^{2+} channel current was voltage independent, without effect on channel gating kinetics and independent of activation status (Fig. 7). As might be predicted, these inhibitory effects were associated with a shortening of the cardiac action potential duration and a negative inotropic effect (58). Finally, caffeine-induced Ca^{2+} transients (arising from mobilization of Ca^{2+} from intracellular stores *via* ryanodine receptor activation) were unaffected by H_2S, as was also the case also in cultured neurons (10). The authors reported a similar inhibitory effect of H_2S on L-type Ca^{2+} currents in normotensive and spontaneously hypertensive rat strains, and speculated that this important modulatory effect of H_2S may contribute not only to a reduction in blood pressure, but also to longer-term protective effects, being potentially

antiapoptotic and antihypertrophic. Further studies will resolve the significance of this effect. However, at present it is important to understand why such opposing effects are found on L-type Ca^{2+} channels in neurons as compared with cardiac myocytes. One suspects that this will only be resolved when we have a more complete understanding of the mechanisms by which H_2S regulates ion channels.

Other Ion Channels

A recent study has demonstrated that native (jejunum smooth muscle) and recombinant (Nav1.5) Na^+ channels are augmented by H_2S (applied as NaHS) with an associated positive shift in steady-state activation and inactivation kinetics (56). These effects were partially reproduced by the reducing agent DTT and prevented by either an oxidant or the alkylating agent N-ethylmaleimide. Although the high (m*M*) concentrations of NaHS employed in this study suggest that caution should be applied as to the physiological significance of such an effect, the potential influence extends beyond the jejunum, since Nav1.5 is expressed in other tissues, notably the heart, where it gives rise to the upstroke of the cardiac action potential.

Chloride channels represent a widespread, diverse, and relatively understudied group of ion channels that serve numerous roles *via* permitting Cl^- movement not only across the plasma membrane but also across intracellular membranes (42). Indirect evidence, based on the responses to known Cl^- channel blockers, suggested that H_2S inhibition of Cl^- channels in the hippocampal-derived neuronal cell line HT22 contributes to its protective effects against glutamate-induced oxidative stress (24). Similarly, Cl^- channels are important in cardiac I/R injury and a recent study demonstrated that Cl^- channels isolated from cardiac myocyte mitochondria and inserted into artificial lipid bilayers could be inhibited by H_2S and NaHS directly (32). Reduced channel activity was observed when H_2S was applied to either face of the channel protein, and was poorly reversible, although no mechanism was proposed for this action. Nevertheless, it could account at least in part for the cardioprotective effects of H_2S (27).

Evidence is emerging that H_2S can also modulate specific members of the family of cation channels known as transient receptor potential (TRP) channels (45). This channel family is widely distributed and influential in physiological processes as diverse as pain sensation, cardiovascular and renal function, inflammation, and secretion, and as such is currently the focus of intense research. To date, limited studies have suggested that H_2S can activate TRPV1 in sensory nerves innervating the airways and so stimulate neurokinin-mediated neurogenic airway inflammation (66). Similarly, TRPA1 in neurons innervating the urinary bladder can be activated by H_2S in order to regulate micturition (57). In addition, H_2S evoked rises of $[Ca^{2+}]_i$ in CHO cells heterologously expressing TRPA1, supporting the notion that H_2S may directly activate this channel. Given the diverse and widespread roles of TRP channels throughout the body, their regulation by H_2S is likely to account for multiple effects of this gasotransmitter.

Potential Mechanisms

While our appreciation of the widespread and diverse nature of ion channel regulation by H_2S is expanding, our insight into possible mechanisms by which such regulation may occur is currently lacking. The most striking candidate mechanisms are illustrated in the schematic of Figure 8 and include the following processes:

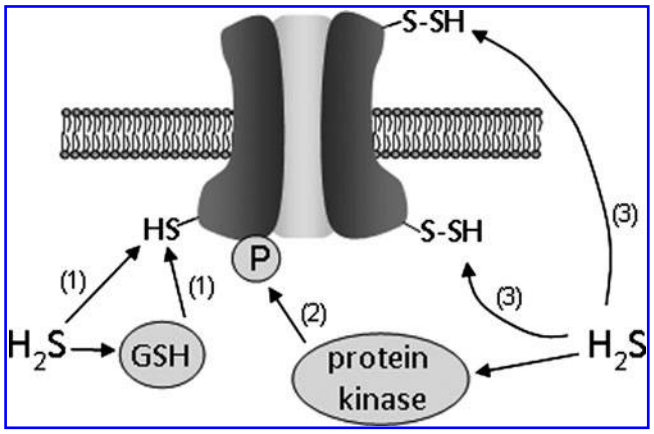

FIG. 8. Schematic indicating possible mechanisms by which H_2S might modulate ion channel activity. H_2S may act through reduction of sulfhydryl groups (1) either directly or *via* increased levels of reduced GSH, through altered phosphorylation status (2), or *via* sulfhydration of cysteines (formation of–S-SH groups from–SH groups (3).

Sulfhydration

Snyder and colleagues have recently shown that H_2S exerts many of its biological effects through sulfhydration of cysteine thiol groups in target proteins. In this process, –SH groups of cysteine residues are converted to –S-SH groups *via* addition of sulfur from H_2S (37). This post-translational modification appears widespread and analogous to nitrosylation of cysteines (forming –SNO groups) by NO. Indeed, by modifying the biotin switch assay (originally developed to detect NO-mediated nitrosylation of cysteine thiol groups) to permit detection of sulfhydrated proteins, these authors showed a striking loss of physiological protein sulfhydration by comparing proteins from $CSE^{-/-}$ mice with controls, and indicated that it is a remarkably widespread form of protein modification. It remains to be determined whether sulfhydration is a primary mechanism by which H_2S regulates ion channels as very little direct information is available. However, likely candidates include the SUR receptor of the K_{ATP} channel; these channels are regulated in excised membrane patches (which discounts effects mediated by soluble second messengers) and activation by H_2S could be prevented by sulfhydryl alkylation or oxidation (19). Sulfhydration would also seem to be a possible mechanism of regulation of mitochondrial Cl^- channels, since these channels were sensitive to H_2S when incorporated into lipid bilayers and so H_2S could presumably modulate the channels only through a direct effect rather than *via* involvement of other mediators as discussed below (32). However, sulfhydration is a nonspecific covalent modification with no characterized reversal process (31), so the extent to which it influences specific roles of H_2S require further investigation.

Redox modulation

Much evidence indicates that H_2S can act as an antioxidant, because it has reducing properties (it may break disulfide bonds) and also increases intracellular GSH levels (29). It

should also be noted that CSE deficiency might be expected to lead to reduced cysteine and hence reduced GSH levels; this would in theory compromise a cell's antioxidant capabilities. However, while one study does indeed report reduced GSH levels in $CSE^{-/-}$ (associated with marked vulnerability to oxidative injury and myopathy when dietary cysteine was reduced) (14), another, while reporting a similar hyperhomocysteinemia in $CSE^{-/-}$ mice, indicates that they display only slightly lower levels of intracellular GSH, probably due to the minor reduction of circulating L-cysteine (71); hence, reduced GSH levels may in fact not play a significant role in the effects of H_2S. Clearly, this issue requires resolution.

The above-described enhancing effect of H_2S on neuronal T-type channels has been attributed to a channel-reducing effect, being inhibited by the oxidant, DTNB (22). Certainly, T-type Ca^{2+} current amplitudes can be enhanced in nociceptive DRG neurons (which express primarily Cav3.2) by reducing agents (DTT or L-cysteine) and inhibited by DTNB. Recombinant Cav3.2 is similarly modulated (41). However, the acute vascular effects of H_2S, which could involve T-type Ca^{2+} channels, given their emerging role in the control of vascular tone, are not believed to be mediated by altered redox status (68). Interestingly, redox modulation of neuronal T-type Ca^{2+} channels has been attributed to the relief of tonic block by Zn^{2+}, which is normally bound to the channel causing tonic inhibition at an extracellular histidine (H191). Thus, the Cav3.2 mutant H191Q was relatively insensitive to reducing agents, and sensitivity of Cav3.1 to reducing agents was conferred by the analogous reverse mutation [Q172H; (41)]. However, it is important to note that these effects are specific to Cav3.2, and the crucial Zn^{2+} binding residue is not present in Cav3.1 or 3.3. Crucially, others have described a number of cysteine residues conserved among T-type Ca^{2+} channels, the redox status of which can have marked effects on channel function (21). It is therefore apparent that all T-type Ca^{2+} channels can be subject to redox modulation.

Whether or not redox modulation accounts for the actions of H_2S on T-type Ca^{2+} channels (and hence nociception) remains to be determined. However, some interesting and potentially conflicting issues are emerging concerning gasotransmitter regulation of other channel functions and the involvement of ROS. For example, it has been suggested that H_2S enhances BKCa activity *via* cysteine residue reduction (55), yet previous reports indicate that BKCa is enhanced by oxidation (53). Furthermore, it is noteworthy that CO inhibits the cardiac L-type Ca^{2+} channel, and that this inhibition arises from increased mitochondrial ROS, which regulate the channel *via* redox control of key cysteine residues (54). It seems unlikely, therefore, that H_2S (which exerts a similar inhibitory effect on the channel) acts through channel protein reduction. However, it should be noted that there are numerous conflicting reports of the effects of reducing and oxidizing agents on the cardiac L-type channel [reviewed in Ref. (80)]. An intriguing possible interaction of gasotransmitters arises from these observations, in that CO is able to stimulate ROS production from mitochondria, yet in some cells 3MST is localized to mitochondria where it can scavenge ROS and provide protection against oxidative stress (23).

Involvement of CO/NO production

Interestingly, H_2S has been shown to increase nuclear localization of the transcription factor Nrf2, thereby increasing expression of HO-1. Furthermore, exogenous H_2S has been shown to increase levels of HO-1 protein and mRNA in pulmonary arteries (50). This enzyme generates CO, which itself is known to modulate ion channels *via* a number of signaling pathways (46, 69). This raises the possibility that H_2S may modulate ion channels (at least chronically, if not acutely) *via* the generation of CO, adding further complexity to the biological interaction of these gases. Additionally H_2S regulates the vascular bioavailability of the third gasotransmitter NO and therefore may mediate some of the reported effects on ion channels through NO-mediated mechanisms (20).

Involvement of kinases

Ion channels are recognized target proteins for phosphorylation, which can have profound effects not only on their activity but also on their localization, since trafficking of ion channels can be directed by phosphorylation. The two kinases most notably associated with H_2S are ERK1/2 and p38 MAP kinase. Much evidence indicates that H_2S activates ERK1/2 in vascular smooth muscle and other cells (18, 72), and this represents an attractive alternative mechanism by which H_2S might regulate T-type Ca^{2+} channels; in fact, there is a complex association between ERK activation and T-type Ca^{2+} channel activity: Chen *et al.* (5) have shown that Ca^{2+} influx *via* T-type Ca^{2+} channels in sensory neurons leads to ERK activation, which may reflect a positive feedback mechanism since ERK1/2 activation is required for neurotrophic factor-mediated up-regulation of neuronal T-type Ca^{2+} channels (5) and up-regulation in a recombinant expression system (7). However, in cardiac myocytes ERK1/2 appears to mediate down-regulation of T-type Ca^{2+} channels by estrogen (33). It remains to be determined whether ERK1/2 inhibitors (*e.g.*, U0126) can affect the ability of H_2S to modulate T-type Ca^{2+} channels either acutely or, following chronic exposure, *via* altered trafficking. Since ERK1/2 can regulate numerous other ion channels [*e.g.*, Kv2.1 (6)] its involvement in their regulation by H_2S is worthy of further study.

Reports also indicate that H_2S modulates the activity of p38 mitogen-activated protein kinase (MAPK), but there are some conflicts as to its effects and consequences. In vascular smooth muscle H_2S activates p38 MAPK, leading to apoptosis (72). Interestingly, p38 MAPK phosphorylation of Kv2.1 is required for its insertion into the plasma membrane as an initial step in the apoptotic response to oxidative stress (51). However, another report suggests that H_2S suppresses agonist-induced MAPK activity (9). These issues and further accounts of the regulation of kinases and their downstream targets (including ion channels) will continue to emerge in due course. It is evident from the widespread regulation of ion channels by phosphorylation and the increasing awareness of H_2S as a modulator of kinase activity/expression that kinases will be seen to account for many of the signaling effects of H_2S on ion channels, as well as other target proteins.

Concluding Remarks

It is clear from the growing number of reports that ion channels are an important and diverse family of target proteins for regulation by H_2S. Further research will expand this family, and will hopefully provide greater insight into mechanisms of channel modulation—it will be particularly interesting to determine whether the novel, post-translational

modification by sulfhydration can account for any of the effects described herein. Future studies must also take advantage of transgenic models in order to elucidate the tonic, physiological regulation of channel expression and activity by endogenous H_2S. The field of H_2S biology in general would also benefit enormously from a means by which to detect H_2S levels dynamically at the sub-cellular level. Such developments are hopefully near at hand to allow us to explore the regulation of ion channels by H_2S more fully, and so provide new opportunities for therapeutic intervention in a wide variety of clinical applications.

Acknowledgments

We are grateful to colleagues for discussions and permitting us to illustrate this review with schematic diagrams based on their data, and apologize to those contributors to the field whose work we could not cite due to space limitations. The authors' own contributions to the field are supported by the Wellcome Trust, the Alzheimer's Society, and the British Heart Foundation.

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Date of first submission to ARS Central, October 24, 2011; date of final revised submission, October 27, 2011; date of acceptance, November 11, 2011.

Abbreviations Used

BKCa = Ca^{2+}-activated K^+ channel
CHO = Chinese hamster ovary
CO = carbon monoxide
CAT = cysteine aminotransferase
CBS = cystathionine β synthetase
CSE = cystathionine γ lyase
DRG = dorsal root ganglion
DTNB = 5,5′-dithio-bis(2-nitrobenzoic acid)
DTT = dithiothreitol
ERK = extracellular receptor kinase
GSH = glutathione
HO-1 = heme oxygenase-1
H_2S = hydrogen sulfide
I/R = ischemia/reperfusion
K_{ATP} channel = ATP-sensitive K^+ channel
K_{IR} = inwardly rectifying K^+ channel
MAPK = mitogen activated protein kinase
3MST = 3-mercaptopyruvate sulfurtransferase
NMDA = *N*-methyl-D-aspartate
NO = nitric oxide
SUR = sulfonylurea receptor
TRP = transient receptor potential
VGCC = voltage-gated Ca^{2+} channel

This article has been cited by:

1. Luca Munaron, Daniele Avanzato, Francesco Moccia, Daniele Mancardi. 2012. Hydrogen sulfide as a regulator of calcium channels. *Cell Calcium* . [CrossRef]
2. Ferdinando Fusco, Roberta d'Emmanuele di Villa Bianca, Emma Mitidieri, Giuseppe Cirino, Raffaella Sorrentino, Vincenzo Mirone. 2012. Sildenafil Effect on the Human Bladder Involves the L-cysteine/Hydrogen Sulfide Pathway: A Novel Mechanism of Action of Phosphodiesterase Type 5 Inhibitors. *European Urology* . [CrossRef]