Hydrogen Sulfide and T-Type Ca\textsuperscript{2+} Channels in Pain Processing, Neuronal Differentiation and Neuroendocrine Secretion

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Hydrogen sulfide · Ca\textsubscript{v}3.2 T-type calcium channel · Prostate · Pain

Abstract

Background: Hydrogen sulfide (H\textsubscript{2}S), a gasotransmitter, is generated from L-cysteine by mainly 3 enzymes, cystathionine-γ-lyase (CSE), cystathionine-β-synthase, and 3-mercaptoppyruvate sulfurtransferase in cooperation with cysteine aminotransferase. The H\textsubscript{2}S-forming enzymes, particularly CSE, are overexpressed under the pathological conditions such as inflammation, neuronal or neuroendocrine differentiation and cancer development. Given that Ca\textsubscript{v}3.2 T-type Ca\textsuperscript{2+} channels mediate some of the biological activity of H\textsubscript{2}S, we focus on the role of the H\textsubscript{2}S/Ca\textsubscript{v}3.2 pathway in regulating the neuronal and neuroendocrine function. Summary: In the neuronal system, H\textsubscript{2}S regulates the activity of various ion channels including Ca\textsubscript{v}3.2. Exogenous and endogenous H\textsubscript{2}S enhances the Ca\textsubscript{v}3.2 channel activity, promoting somatic and visceral pain signaling. The H\textsubscript{2}S/Ca\textsubscript{v}3.2 pathway also facilitates neuritogenesis or neuronal differentiation. Interestingly, endogenous H\textsubscript{2}S formed by CSE regulates secretory function by enhancing Ca\textsubscript{v}3.2 channel activity in neuroendocrine-differentiated prostate cancer cells or carotid glomus cells. Key Messages: The H\textsubscript{2}S/Ca\textsubscript{v}3.2 pathway may serve as therapeutic targets for treatment of intractable pain, neuronal injury, androgen-independent prostate cancer, cardiovascular diseases, etc.

Introduction

Like nitric oxide and carbon monoxide, hydrogen sulfide (H\textsubscript{2}S) is now considered a gasotransmitter in the mammalian body. H\textsubscript{2}S is generated endogenously from L-cysteine (L-Cys) mainly by 3 enzymes, cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptoppyruvate sulfurtransferase (3-MST) in cooperation with cysteine aminotransferase (CAT) [1–3]. Numerous physiological and pathophysiological roles of endogenous H\textsubscript{2}S have been reported in the cardiovascular, neuronal, gastrointestinal, urinary, and endocrine systems [1, 3, 4]. There is also evidence that H\textsubscript{2}S has the paradoxical actions on cancer cells, whereby lower (endogenous) production of H\textsubscript{2}S tends to promote, while its higher production (from exogenously added H\textsubscript{2}S donors) tends to inhibit cancer cell proliferation [5]. H\textsubscript{2}S appears to target a variety of voltage-dependent and ligand-gated ion channels, such as ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channels, voltage-gated T-type and L-type Ca\textsuperscript{2+} channels, transient...
receptor potential ankyrin-1 (TRPA1) channels, large conductance Ca^{2+}-activated K^+ (BK) channels, cystic fibrosis transmembrane conductance regulator Cl^- channels, NMDA receptors, in addition to transcription factors and protein kinases [2, 3, 6, 7]. In the nociceptor neurons, H_{2}S enhances the function of Ca_{3.2} T-type Ca^{2+} channels [8–12] and TRPA1 channels [13, 14], leading to the neuronal excitation and then pain sensation or hyperalgesia/allodynia [3, 7, 15]. We have also shown that the acceleration of Ca_{3.2} channel activity by exogenous H_{2}S causes neurtogenesis and neuronal differentiation in NG108-15 cells [9, 10, 16], and that the endogenous H_{2}S/Ca_{3.2} pathway regulates secretory function in the neuroendocrine-like differentiated human prostate cancer LNCaP cells [17]. Here, we thus focus on the roles of Ca_{3.2} T-type Ca^{2+} channels and H_{2}S in pain processing, neuronal differentiation and neuroendocrine secretion.

**H_{2}S-Mediated Functional Acceleration of T-Type Calcium Channels**

Low-voltage-activated Ca^{2+} channels, known as T-type Ca^{2+} channels, are classified into 3 different types, Ca_{3.1}, Ca_{3.2} and Ca_{3.3} [18]. Unlike high-voltage-activated (HVA: L-, N-, P/Q-, and R-types) Ca^{2+} channels, T-type Ca^{2+} channels appear to consist of only α1 subunit and do not require coassembly with auxiliary subunits such as α2δ, β and γ that are essential for HVA Ca^{2+} channels. Under the physiological conditions, T-type Ca^{2+} channels are activated by depolarization near a resting membrane potential, thereby regulating neuronal excitability. There is evidence for the involvement of T-type Ca^{2+} channels in secretion of hormones or neurotransmitters, muscle contraction and cell differentiation or proliferation [19, 20]. Among 3 isoforms of T-type Ca^{2+} channels, Ca_{3.2} appears to mediate some of biological actions of H_{2}S. H_{2}S donors, NaHS and L-Cys, facilitate T-type Ca^{2+} channel currents (T-currents) in NG108-15 (mouse neuroblastoma × rat glioma) cells, isolated dorsal root ganglion (DRG) neurons and neuroendocrine-like differentiated human prostate cancer LNCaP cells that naturally express Ca_{3.2} [9–11, 17], and also in Ca_{3.2}-transfected HEK293 cells [21]. The T-currents are suppressed by DL-propargylglycine (PPG), a CSE inhibitor, in neuroendocrine-differentiated LNCaP cells and Ca_{3.2}-transfected HEK293 cells that abundantly express CSE, an H_{2}S-forming enzyme, suggesting that the function of Ca_{3.2} is positively regulated by endogenous H_{2}S in those cells [17, 21]. These findings are in agreement with recent evidence from an independent group that endogenous H_{2}S formed by CSE following hypoxia and H_{2}S donor-derived exogenous H_{2}S cause Ca^{2+} influx in glomus cells and sensory excitation of the carotid body in wild-type mice, and these effects of H_{2}S are greatly attenuated in Ca_{3.2}-knockout mice and by selective T-type Ca^{2+} channel blockers [22]. In physiological conditions, Ca_{3.2}, but not Ca_{3.1} or Ca_{3.3}, is considered to be tonically attenuated by zinc binding to the histidine residue at position 191 (His^{191}) in the second extracellular loop of domain I of the channel protein [23]. The His^{191} of Ca_{3.2} is conserved through different species including human, mouse and rat, and contributes to much higher sensitivity of Ca_{3.2} to inhibition with metals such as zinc, copper and nickel, compared to Ca_{3.1} or Ca_{3.3} [15, 20]. It is considered that typical zinc chelators and also some substances capable of interacting zinc, such as H_{2}S and L-Cys, cancel inhibition of channel activity by zinc binding to His^{191} of Ca_{3.2}. In addition, Ca_{3.2}, but not Ca_{3.1} or Ca_{3.3}, is suppressed by ascorbic acid (vitamin C) via the metal-catalyzed oxidation at His^{191} [24]. The NaHS-induced facilitation of T-currents in NG108-15 cells is also attenuated by ascorbic acid [10]. There is a conflicting report showing that NaHS at low and high concentrations suppresses and enhances T-currents, respectively, in Ca_{3.2}-transfected HEK293 cells [12], although the physiological significance of the H_{2}S-induced T-current attenuation, if any, remains unclear. Nevertheless, we have never detected such inhibitory effect of H_{2}S donors in an extensive concentration range on T-currents in HEK293 cells transfected with Ca_{3.2} or other cells that naturally express Ca_{3.2} [7, 9–11, 16, 17, 21].

**Roles of Endogenous H_{2}S and T-Type Calcium Channels in Pain Processing**

The concentrations of endogenous H_{2}S in the mammalian tissues are regulated by 3 principal H_{2}S-producing enzymes, CSE, CBS, and 3-MST/CAT. It has been reported that CSE and CBS are upregulated in inflammatory conditions such as sepsis, pancreatitis and cystitis in rats or mice [25–28]. On the other hand, Ca_{3.2} targeted by H_{2}S is upregulated de novo or functionally in the DRG and/or spinal cord in laboratory animals with surgically induced neuropathic pain [15, 29–33]. In mice or rats, intraplantar and intracolonic administration of NaHS, an H_{2}S donor, evokes mechanical hyperalgesia/allodynia and visceral nociceptive behavior accompanied by re-
ferred hyperalgesia, respectively, which are blocked by T-type Ca\(^{2+}\) blockers or gene silencing of Ca\(_v\)3.2 [8, 11, 29, 34, 35]. In the mice with cerulein-induced pancreatitis and with cyclophosphamide-induced cystitis, CSE protein is markedly upregulated in the pancreas and bladder, respectively, and the concomitant pancreatic and bladder pain are abolished by a CSE inhibitor or distinct T-type Ca\(^{2+}\) channel blockers, and by knockdown of Ca\(_v\)3.2 in DRG by intrathecal administration of the Ca\(_v\)3.2-targeting antisense oligodeoxynucleotide [26, 27, 36]. The CSE inhibitor, T-type Ca\(^{2+}\) channel blockers and knockdown of Ca\(_v\)3.2 also suppress the neuropathic hyperalgesia induced by L5 spinal nerve injury in rats, and upregulation of Ca\(_v\),3.2 channels, but not CSE, at protein levels are detectable in the DRG following the development of neuropathy [31]. Paclitaxel-evoked painful neuropathy in rats is also suppressed by the inhibitors of CSE and T-type Ca\(^{2+}\) channels, while the expression levels of CSE and Ca\(_v\)3.2 proteins in the DRG and hindpaws do not change after paclitaxel treatment [37]. In both type I (streptozotocin (STZ) injection-induced diabetes) and type II (leptin-deficient (ob/ob) mice) diabetic mouse models, the diabetic neuropathy is considered to involve the upregulation of Ca\(_v\)3.2 T-type Ca\(^{2+}\) channels in small-medium size DRG neurons [38, 39]. There is also evidence that hyperalgesia/allodynia accompanying STZ-induced diabetic neuropathy in rats is suppressed by systemic administration of inhibitors of CSE (PPG) or CBS (hydroxylamine) [40]. The increased production of endogenous H\(_2\)S by CSE or CBS and possibly concomitant enhancement of Ca\(_v\)3.2 channel activity would thus appear to be involved in diabetic neuropathy. Together, studies on the roles of the endogenous H\(_2\)S/Ca\(_v\)3.2 pathway in pain processing might unveil novel therapeutic avenues for treatment of intractable pain including neuropathic and visceral pain (fig. 1). TRPA1 in addition to Ca\(_v\)3.2 mediates the pronociceptive effect of H\(_2\)S [15, 41] (fig. 1). We have shown that the somatic hyperalgesia following intraplantar injection of NaHS and prompt phosphorylation of ERK in the spinal dorsal horn following NaHS injection into the pancreatic duct are reduced by pharmacological inhibition or knockdown of not only Ca\(_v\)3.2 but also TRPA1 [34, 36]. It has been reported that H\(_2\)S activates TRPA1 through sulfhydration of the intracellular cysteine residue of this channel and that polysulfides are more potent in activating TRPA1 than H\(_2\)S [42]. The effect of polysulfides on Ca\(_v\)3.2 channels is still open to question, and needs to be examined in future.

**Fig. 1.** A scheme for the pain processing by H\(_2\)S via Ca\(_v\)3.2 and TRPA1 in the sensory neurons.
Role of Endogenous H$_2$S and T-Type Calcium Channels in Neuronal Differentiation

H$_2$S may regulate differentiation of various cells [16, 43, 44]. In neuronal progenitor-like NG108-15 cells that abundantly express Ca$_v$3.2 channels, dibutyryl cyclic AMP (db-cAMP), a membrane-permeable analogue of cAMP, causes neuronal differentiation characterized by neurite outgrowth and functional upregulation of HVA Ca$^{2+}$ channels [45, 46], being dependent on Ca$_v$3.2 channel activity [45]. Interestingly, NaHS and Na$_2$S, H$_2$S donors, also cause similar neurite outgrowth and functional upregulation of HVA channels in NG108-15 cells [9, 10, 16]. The NaHS-evoked neuritogenesis is inhibited by BAPTA-AM, an intracellular Ca$^{2+}$ chelator, or mibefradil, a T-type Ca$^{2+}$ channel blocker, and by zinc chloride and ascorbic acid that inhibit Ca$_v$3.2, but not Ca$_v$3.1 or Ca$_v$3.3, as mentioned above, suggesting the involvement of Ca$^{2+}$ influx through Ca$_v$3.2 channels in the H$_2$S-dependent neuronal differentiation [9, 10]. The NaHS-induced neuritogenesis is not affected by inhibition of other ion channels known as possible targets for H$_2$S, K$_{ATP}$, TRPA1 and HVA Ca$^{2+}$ channels [10]. The activity of Src kinase appears to be required for the NaHS-evoked neuronal differentiation of NG108-15 cells [10].

Roles of Endogenous H$_2$S and T-Type Calcium Channels in Neuronal and Neuroendocrine Secretion

H$_2$S appears to regulate secretory function in the pancreas, hypothalamus and pituitary gland; for example, H$_2$S decreases insulin secretion via activation of K$_{ATP}$ channels in rat insulinoma INS-1E cells or inhibition of L-type Ca$^{2+}$ channels in mouse pancreatic $\beta$ cells [47, 48], enhances 5-hydroxytryptamine release via activation of TRPA1 channels in clonal rat pancreatic delta RIN14B cells [49] and reduces the exocytosis of secretory granules via activation of K$_{ATP}$ channels in rat pituitary tumor GH3 cells [50]. Interestingly, T-type Ca$^{2+}$ channels trigger Ca$^{2+}$ entry during small depolarization around the resting potential and concomitant vesicular exocytosis in the neuronal system, which is essential for synaptic neurotransmitter release at rest [51]. Thus, we next focus on the roles of the endogenous H$_2$S/Ca$_v$3.2 pathway in neuronal or neuroendocrine secretion in neuroendocrine-differentiated prostate cancer cells, glomus cells in the carotid body, and chromaffin cells in the adrenal medulla [17, 52, 53].

H$_2$S donors, NaHS and Na$_2$S, further enhance the increased T-currents dependent on the upregulated Ca$_v$3.2 in the neuroendocrine-like differentiated LNCaP cells. Interestingly, the increased T-currents in the neuroendocrine-like LNCaP cells are partially attenuated by the treatment with the CSE inhibitors, PPG and $\beta$-cyano-L-alanine, but not a CBS inhibitor aminooxy acetic acid (AOAA), suggesting that endogenous H$_2$S produced by the upregulated CSE, but not CBS, enhances the activity of the overexpressed Ca$_v$3.2 channels in neuroendocrine-like LNCaP cells. In parallel with the upregulation of Ca$_v$3.2 channels, spontaneous Ca$^{2+}$-dependent secretion increases following neuroendocrine-like differentiation with db-cAMP plus IBMX in LNCaP cells [60]. The increased spontaneous secretory function in the neuroendocrine-like LNCaP cells is abolished by mibefradil, a T-type Ca$^{2+}$ channel blocker, or PPG, but not AOAA,
whereas mibefradil does not affect the secretory response in undifferentiated LNCAp cells that do not abundantly express CSE and CaV3.2 [17]. Together, it is considered that, in neuroendocrine-differentiated prostate cancer LNCAp cells, H2S generated by the upregulated CSE further enhances the channel activity of the upregulated CaV3.2 and causes the augmentation of the secretory function (fig. 2). Since the neuroendocrine-like differentiation of LNCAp cells by db-cAMP plus IBMX results in the upregulation of neurotensin and PTHrP, mitogenic neuropeptides [17], the enhanced secretory function brought about by the CSE/H2S/CaV3.2 pathway may contribute to the increased secretion of those mitogenic factors in the differentiated cells, leading to androgen-independent proliferation in the surrounding undifferentiated cells (fig. 2). This hypothesis is in agreement with the evidence that the conditioned culture medium from the neuroendocrine-like differentiated LNCAp cells and neurotensin promote mitogenesis of androgen-independent prostate cancer PC3 cells [65]. Therefore, pharmacological inhibition of the CSE/H2S/CaV3.2 pathway might be beneficial to prevent the acquisition of androgen-independent property of prostate cancer. However, the long-term inhibition of CSE might cause unexpected serious biological events, because endogenous H2S plays important physiological roles throughout the mammalian body including the cardiovascular, gastrointestinal and neuronal systems [1, 4]. In addition, CSE contributes to not only L-Cys metabolism to H2S and pyruvate, but also L-cystathionine metabolism to L-Cys and α-ketobutyrate [3], so that the abnormal metabolism of L-Cys or L-cystathionine caused by CSE inhibitors might also limit their therapeutic use. In contrast, the pharmacological blockade of CaV3.2 T-type Ca2+ channels might be suitable for the treatment of prostate cancer. Actually, several blockers of T-type Ca2+ channel appear to inhibit the proliferation and migration of androgen-independent prostate cancer DU-145 cells [66]. Although there is no report concerning the development of selective blockers of CaV3.2 channels, ascorbic acid (vitamin C) and zinc block CaV3.2, but not CaV3.1 or CaV3.3 [23, 24]. Strikingly, it has been reported that the pharmacological dose of ascorbic acid suppresses tumor growth and metastases of implanted hormone-resistant prostate adenocarcinoma cells in rats [67].

Involvement of the H2S/CaV3.2 Pathway in the Hypoxia-Induced Catecholamine Secretion in the Carotid Body and Adrenal Medulla

Hypoxia induces Ca2+-dependent release of catecholamines (CAs) in glomus cells of the carotid body, an organ sensing arterial blood O2 concentration, which is essential for controlling the function of respiratory and cardiovascular systems [22]. The hypoxia-induced secretion of CAs is observed also in the adrenal medullary chromaffin cells [52]. It has been reported that CSE, an H2S-forming enzyme, is expressed in glomus cells in the carotid body and adrenal medullary chromaffin cells, and that the hypoxia-evoked CA secretion is attenuated in CSE knockout mice or by the treatment with CSE inhibitors, indicating the critical role of endogenous H2S formed by CSE in the respiratory and cardiovascular homeostatic control in response to hypoxia [22, 52]. Voltage-gated HVA and T-type Ca2+ channels are also expressed in glomus cells of the carotid body [68, 69], and the hypoxia-evoked elevation of intracellular Ca2+ concentration and CA secretion in the glomus cells are attenuated by inhibitors of L-type (nifedipine) or T-type (mibefradil and TTA-A2) Ca2+ channels, or in CaV3.2 knockout mice [22, 70]. Therefore, endogenous H2S produced by CSE is considered to cause Ca2+ influx through both CaV3.2 T-type channels and L-type HVA channels in the carotid body, regulating the hypoxia-evoked CA secretion. Recently, it has also been reported that H2S inhibits background K+ currents.

Fig. 2. Involvement of the H2S/CaV3.2 T-type Ca2+ channel pathway in secretory function in neuroendocrine-like differentiated prostate cancer LNCAp cells.
through TWIK-related acid-sensitive potassium (TASK) channels in carotid body type 1 (glomus) cells, leading to membrane depolarization and elevation of the intracellular Ca\(^{2+}\) concentration via voltage-dependent Ca\(^{2+}\) channels [71]. Thus, in addition to the direct enhancement of Ca\(^{3.2}\) activity by H\(_2\)S, indirect activation of Ca\(^{3.2}\) following membrane depolarization by the H\(_2\)S inhibition of TASK channels might also contribute to H\(_2\)S-enhanced Ca\(^{2+}\) influx and CA secretion in response to hypoxia. The adrenal chromaffin cells of mammalian species express all types of voltage-gated Ca\(^{2+}\) channels, whereas CA secretion is mostly mediated by Ca\(^{2+}\) influx via HVA Ca\(^{2+}\) channels (L-, N-, P/Q- and R-types) [72]. On the other hand, T-type Ca\(^{2+}\) channels are poorly expressed or absent in adult chromaffin cells, but are functionally expressed in the embryonic and neonatal chromaffin cells [51, 72]. In addition, Ca\(^{3.2}\) T-type Ca\(^{2+}\) channels in adult chromaffin cells are upregulated in response to various long-term stressful stimuli, such as chronic hypoxia, β-adrenergic and high-frequency sympathetic stimulation and exposure to cAMP, and contribute to low-threshold CA secretion [19, 51]. On the other hand, NaHS, an H\(_2\)S donor, induces CA secretion in rat adrenal chromaffin cells [53]. CSE is detected in the chromaffin cells, and upregulated and involved in the hypoxia-evoked CA secretion in response to hypoxia [52]. Collectively, the CSE/H\(_2\)S/Ca\(^{3.2}\) pathway may play a role in the hypoxia-evoked CA secretion in adrenal chromaffin cells, as in glomus cells of the carotid body. This hypothesis has yet to be demonstrated by future more in-depth studies.

**Conclusion**

As described so far, the CSE/H\(_2\)S/Ca\(^{3.2}\) T-type Ca\(^{2+}\) channel pathway plays roles in the pain processing, neuronal differentiation and neuronal or neuroendocrine secretion. Thus, pharmacological inhibition or acceleration of this pathway may be beneficial for treatment of intractable pain, neuronal or axonal injury, prostate cancer and impaired homeostatic regulation of the respiratory and cardiovascular systems.

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**Disclosure Statement**

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**References**

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