2 Experimental Observations on the Biological Significance of Hydrogen Sulfide in Carotid Body Chemoreception

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Abstract

The cascade of transduction of hypoxia and hypercapnia, the natural stimuli to chemoreceptor cells, is incompletely understood. A particular gap in that knowledge is the role played by second messengers, or in a most ample term, of modulators. A recently described modulator of chemoreceptor cell responses is the gaseous transmitter hydrogen sulfide, which has been proposed as a specific activator of the hypoxic responses in the carotid body, both at the level of the chemoreceptor cell response or at the level of the global output of the organ. Since sulfide behaves in this regard as cAMP, we explored the possibility that sulfide effects were mediated by the more classical messenger. Data indicate that exogenous and endogenous sulfide inhibits adenyl cyclase finding additionally that inhibition of adenylyl cyclase does not modify chemoreceptor cell responses elicited by sulfide. We have also observed that transient receptor potential cation channels A1 (TRPA1) are not regulated by sulfide in chemoreceptor cells.

Keywords

Carotid body • Sulfide • Catecholamine • cAMP • TRPA1

2.1 Introduction

Normal functioning of the CB consists of the detection of arterial blood gases levels by chemoreceptor cells, the transduction into a neurosecretory response and synaptic transmission to the CSN endings which generate action potentials that reach the nucleus of the tractus solitarius. This medullar nucleus is the first relay of the chemosensory activity and, from there on, information is channelled to different brain stem nuclei to

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generate integrated respiratory, cardiovascular and hormonal responses. If the central integration and mechanisms of reflex genesis of systemic responses are not well known, the initial steps of the whole chemoreceptor reflex, i.e., the detection-transduction of blood gases levels in chemoreceptor cells and synaptic transmission to CSN nerve endings is comparably poorly understood.

The transduction machinery is complex, and probably dual, that is, there is a machinery to detect arterial blood oxygen levels leading to chemoreceptor cell activation in hypoxic hypoxia and probably another to detect arterial hypercapnia and/or acidosis (Gonzalez et al. 1992, 2010). Likely, hypoxia and hypercapnia share some steps in the chain of events leading to cell activation (Buckler et al. 2000). Many primary molecular entities have been proposed as the primary O_2-sensor including a non-identified hemoprotein (Lopez-Lopez and Gonzalez 1992; Riesco-Fagundo et al. 2001; Park et al. 2009), mitochondrial cytochrome oxidase (Biscoe and Duchen 1990; Buckler and Turner 2013) NADPH-oxidase (Cross et al. 1990), hemoxoygenase-1 (Williams et al. 2004) adenosine-monophosphate activated protein kinase (Evans et al. 2005; Evans 2006), and potassium channels (McCartney et al. 2005). When PO_2 diminishes in the internal milieu bathing chemoreceptor cells, the molecular putative O_2-sensor(s), through ill-defined coupling mechanisms, diminish the opening probability of diverse O_2-sensitive K^+ channels leading to cell depolarization, activation of voltage operated Ca^{2+} channels, entry of Ca^{2+} and triggering of the release of several neurotransmitters which, in addition to activating the sensory nerve endings of the CSN, feedback control chemoreceptor cell activity via autoreceptors (Gonzalez et al. 1994; Conde et al. 2009; Nurse 2010). At every step from O_2-sensing to the release of neurotransmitters, there are second messengers which fine shape the exocytosis and other neurotransmitter releasing mechanisms, and therefore the level of activity generated in the CSN (e.g., Pérez-García et al. 1990, 1991; Gómez-Niño et al. 1994a, b; Rocher et al. 2009; He et al. 2007; Nunes et al. 2010; Kemp and Telezhkin 2014).

Among these second messengers there is one, namely hydrogen sulfide, whose potential capacity to modulate chemoreceptor activity, mechanisms of action, and physiological significance are not well defined (Anichkov and Belen'kii 1963; Telezhkin et al. 2009; Li et al. 2010; Peng et al. 2010; Fitzgerald et al. 2011; Olson 2011; Haouzi et al. 2011; Buckler 2012; Makarenko et al. 2012; Kemp and Telezhkin 2014). Main observations include that sulfide, applied in the form of NaSH, inhibits K^+ channels (Telezhkin et al. 2009; Buckler 2012), increases intracellular Ca^{2+} in a voltage dependent and dihydropyridine sensitive Ca^{2+}-dependent manner (Buckler 2012; Makarenko et al. 2012) and augments CSN activity and ventilation (Peng et al. 2010; Anichkov and Belen'kii 1963; Van de Louw and Haouzi 2012). Additional observations include that cystathionine-γ-lyase (CSE; one of the enzymes involved in sulfide synthesis) knockout mice exhibit a greatly diminished hypoxic ventilatory response, without alteration of the response to hypercapnia, and a diminished chemoreceptor cells Ca^{2+} response without alteration of the response to high extracellular K^+ (Peng et al. 2010; Makarenko et al. 2012). These data would indicate that in mouse the main sulfide synthesizing enzyme in the CB seems to be CSE. In rat, the enzyme responsible for sulfide generation in the CB seems to be cystathionine β-synthase (CBS) as its inhibition reproduces in their major the effects of CSE knockouts mice (Li et al. 2010).

In the present study we have explored the possible contribution of cAMP in sulfide signalling because, cAMP as sulfide production increases during hypoxia, and both messengers positively modulate the responses to hypoxia without affecting those elicited by high K^+ (Pérez-García et al. 1990, 1991); also, sulfide dose-dependently increases cAMP levels in rat retinal pigment epithelial cells (Njie-Mbye et al. 2012). In addition, the recent description that sulfide inhibits L-type voltage dependent Ca^{2+} channels in several tissues (Zhang et al. 2012; Streeter et al. 2012; Tang et al. 2013; Avanzato et al. 2014) does not support the notion that the positive modulation of the hypoxic response is linked to Ca^{2+} entry via L-type Ca^{2+} channels. Therefore we have

explored the possibility that sulfide activates alternative pathways for Ca^{2+} entry into chemoreceptor cells, namely transient receptor potential cation channel A1 (TRPA 1), a cationic channel with high Ca^{2+} permeability (Guimaraes and Jordt 2007) activated by many stimuli included sulfide and their derived polysulphates (see Kimura 2014). Additionally, TRPA1 can also be activated by hypoxia (Takahashi et al. 2012).

2.2 Material and Methods

2.2.1 Animals and Anaesthesia. Surgical Procedures

Experiments were performed with tissues obtained from male adult Wistar rats (280–350 g body weight). Animals were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) and euthanized by an intracardiac overdose of sodium-pentobarbital. We have followed the European Community Council directive of 24 November 1986 (86/609/EEC) for the Care and Use of Laboratory Animals with protocols for the experiments being approved by the Institutional Committee of the University of Valladolid for Animal Care and Use.

Anaesthetized animals were tracheotomised and bilateral blocks of tissue containing the carotid bifurcations were removed and placed in a dissecting chamber filled with ice-cold O_2-saturated Tyrode solution (in mM: NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1.1; HEPES, 10; glucose, 5; pH 7.40). The CBs were cleaned of surrounding tissues with the aid of a dissecting microscope. Cleaned CB, were collected and saved in glass vials containing O_2-saturated ice-cold Tyrode until use.

2.2.2 ^{3}H-Catecholamine (^{3}H-CA) Release Experiments Using Intact CBs

General procedures used to label chemoreceptor cells CA deposits and to later study their release have been described in previous publications (Vicario et al. 2000) and analytical methods have been described in detail in Conde et al. (2006). In brief, the CBs were incubated (2 h; 37 °C) in Tyrode solution containing ^{3}H-tyrosine (40–50 Ci/mmol; Perkin-Elmer España), 6-methyl-tetrahydropterine (100 μM) and ascorbic acid (1 mM). Following ^{3}H-CA labelling incubation, individual CBs were transferred to glass vials containing 2 ml of precursor-free Tyrode bicarbonate solution. Initial incubation in precursor-free normoxic (20 % O_2, 5 % CO_2, balance N_2) solution lasted 1 h, with solutions renewed every 20 min and discarded. Thereafter, incubating solutions were renewed every 10 min and collected for the analysis in their ^{3}H-CA content. Specific protocols for drug application are given in the Results sections. Drug solutions were freshly prepared as stock solutions and maintained at 0–4 °C in capped vials.

2.2.3 cAMP Measurement

Individual CBs were incubated (30 min) in Tyrode bicarbonate equilibrated with 95 % O_2/5 % CO_2. Thereafter the incubating solutions were renewed with an identical solution (control) or with test solutions as specified in the Results section; in every instance this second incubation lasted 20 min and contained IBMX 0.5 mM. At the end of the incubation, the organs were placed in homogenizers (0 °C) containing 150 μl of 6 % TCA and after 30 min were homogenized and centrifuged (2,000 × g; 15 min; 4 °C). The supernatant was extracted four times with 500 μl of diethyl ether saturated water. The upper diethyl ether layer from each extraction was discarded and the aqueous layer combined and lyophilized. Samples were stored at −80 °C until assay. Samples were reconstituted in a sodium acetate buffer (0.05 M; pH 5.8) containing BSA at 0.01 %. cAMP was measured using 96 wells commercial kit Healthcare Biosciences (EIA, RPN 2255, GE Healthcare Biosciences) following the instructions of the supplier. The standard curve (12.5–3,200 fmole), tissue extracts and blanks were assayed in duplicated. Tissue contents of cAMP, calculated by interpolation, are expressed as pmole/mg tissue.

2.2.4 Statistics

All data are expressed as the mean ± S.E.M. Statistical analysis were performed by two tails student t-test for unpaired data to compare two groups. Values of $p < 0.05$ indicate statistical significance.

2.3 Results

2.3.1 Sulfide and cAMP

Hydroxocobalamin is a vitamin B12 analogue that reacts with sulfide according to the reaction: $HS^- + H^+ + HO\text{-}Co\text{-}R \rightarrow HS\text{-}Co\text{-}R + H_2O$. As a result, it has been demonstrated both in vivo and in vitro models that hydroxocobalamin prevents the toxic effects of sulfide (Truong et al. 2007) and in our in vitro CB preparation fully prevents the release of CA elicited by NaSH. Hydroxocobalamin, at the concentration of 300 μM, augmented basal normoxic cAMP from 14.4 ± 1.8 (n = 10) to 27.0 ± 5.2 (n = 6) ($p < 0.01$) pmole/mg tissue and hypoxic cAMP from 47.4 ± 4.9 (n = 5) to 64.4 ± 5.2 (n = 5) ($p < 0.05$). These findings would imply that endogenous sulfide moderately inhibits adenylyl cyclase(s) (Fig. 2.1a). Consistent with these findings we also observed that NaSH (200 μM), diminished normoxic cAMP levels to 8.5 ± 0.8 pmole/mg tissue (n = 5; $p < 0.05$), with hydroxocobalamin fully reversing the effects of the sulfide donor, rising cAMP to 21.9 ± 2.8 pmole/mg tissue.

Figure 2.2 shows the effects of SQ22536, an inhibitor of adenylyl cyclase, on the release of ^{3}H-CA elicited by NaSH (200 μM). In panel A it can be seen that the inhibitor (80 μM) did not alter the time course of the NaSH elicited release response. Figure 2.2 panel B shows that the $^{3H\text{-}CA}$ evoked release, equivalent to the area under the curve in panel A, was nearly identical in CBs treated with NaSH and NaSH + SQ22536 and amounted to 8.0 ± 2.7 and 8.1 ± 2.7 (n = 6) percent of the total tissue ^{3}H-CA content.

2.3.2 Effects of TRPA1 Channel Inhibition of NaSH Elicited ^{3}H-CA Release

Using a slightly different protocol we tested the effect of the selective inhibitor of TRPA1 channel HC030031 at a concentration of 60 μM. Control CBs were stimulated twice with 200 μM NaSH and experimental CBs were similarly stimulated, but prior to and during the second stimulus the TRPA1 inhibitor was also present. Figure 2.3a shows the time course of the release of both control and experimental organs showing the apparent absence of effects of the inhibitor. Figure 2.3b shows the ratios of the evoked release in the second to the first stimulus (S2/S1) of both control and experimental CBs. The ratios were, respectively, 0.9 ± 0.1 (n = 4) and 1.1 ± 0.2 (n = 4) in control and HC030031 treated organs, confirming that TRPA1 does not participate in the NaSH response.

2.4 Discussion

Present experiments show that, contrary to the hypoxic natural stimulus, NaSH, the most commonly used sulfide donor, decreases the rate of cAMP accumulation in CBs incubated in the presence of the phosphodiesterase inhibitor IBMX. Therefore, our data indicate that sulfide inhibits adenylyl cyclase, the cAMP synthesizing enzyme (Pérez-García et al. 1990). Consistent with this effect of exogenous sulfide the hydroxyl form of vitamin B12 or hyroxocobalamin, which reacts with sulfide (Truong et al. 2007; as well as it reacts with HCN; Borron et al. 2007) reverses the effect produced by exogenous sulfide. Further, hydroxocobalamin which enters inside cells and mitochondria (Begley et al. 1993; Buccellato et al. 2004; see Depeint et al. 2006) and would purportedly react with endogenously generated sulfide, increases basal and hypoxic-stimulated accumulation of cAMP, implying that endogenous sulfide physiologically inhibits cAMP. It should be emphasized, however, that the inhibition

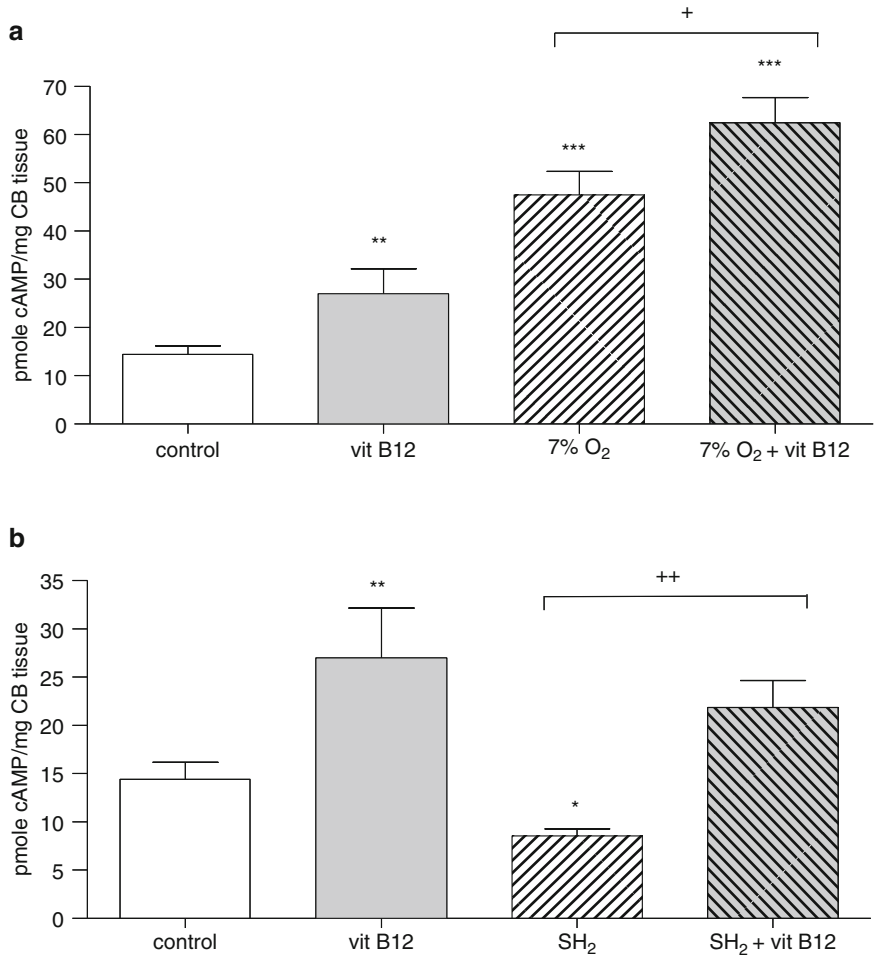

Fig. 2.1 Effects of different experimental manoeuvres on the rate of cAMP accumulation in the CB (See text for details)

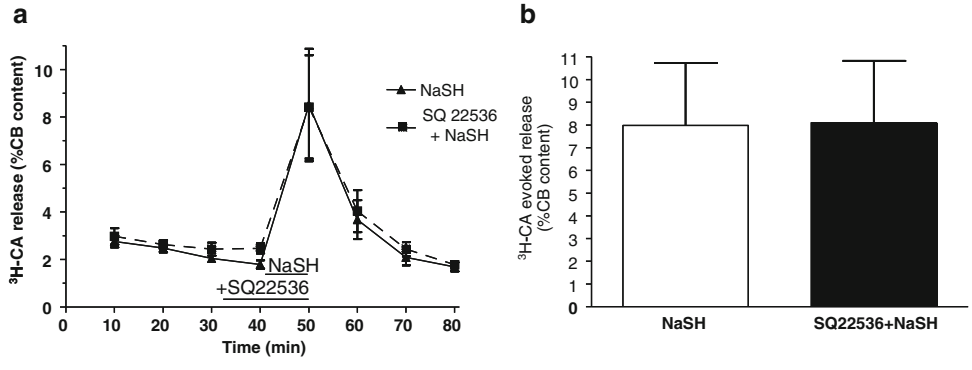

Fig. 2.2 Effects of the adenylyl cyclase inhibitor SQ22538 on the ^{3}H-CA release induced by sulphide

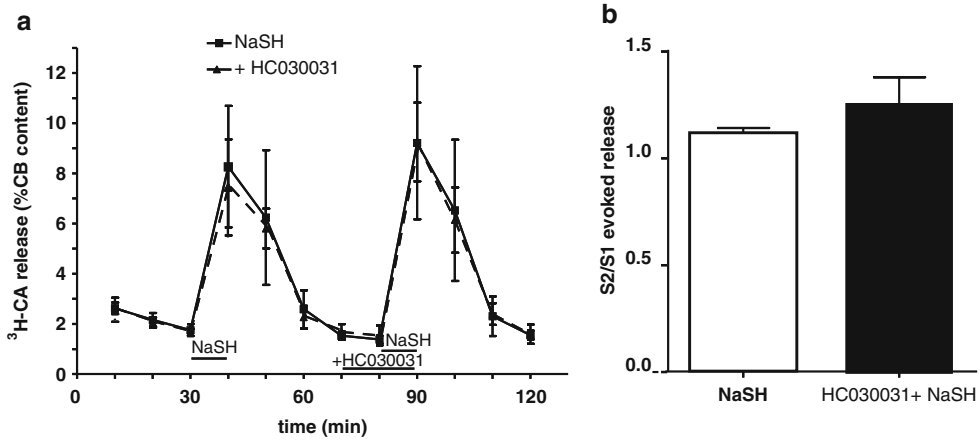

Fig. 2.3 Effects of the TRPA1 channel inhibitor HC030031 (See text)

exerted by endogenous sulfide, is quantitatively comparable in normoxia and hypoxia, implying that it does not interfere with the intimate mechanism used by hypoxia to activate the cycling enzyme.

The adenylyl cyclase inhibitor, SQ22536, which inhibits the release of ^{3}H-CA elicited by hypoxia by around 50 % (Rocher et al. 2009) does not alter the release response elicited by exogenous sulfide, behaving in this regard like the release response elicited by high external K^+ which is not affected by cAMP (Pérez-García et al. 1991). Thus, although hypoxia and high external K^+ share many steps in the cascade leading to activation of exocytosis (Gonzalez et al. 1994) they are fundamentally different stimulus. Similarly, sulfide and cyanide produce chemoreceptor cell depolarization, promote voltage dependent Ca^{2+} entry to chemoreceptor cells (Buckler 2012) and have a comparable IC_{50} for cytochrome oxidase (Wallace and Starkov 2000; Cooper and Brown 2008), yet cyanide-induced release response is modulated by cAMP while sulfide response is not. These considerations lead us to conclude that even if chemoreceptor cell depolarization and triggering of voltage dependent Ca^{2+} entry shared by many stimuli, each one seems to activate a specific second messenger system, shaping specificity to the responses (Pérez-García et al. 1991; Gómez-Niño et al. 1994a, b).

We also have explored the possibility that TRPA1 represented an alternative or additional pathway to voltage dependent L-type Ca^{2+} channel (Buckler 2012; Makarenko et al. 2012) for Ca^{2+} entry in chemoreceptor cells. The rationale for this search rests on two different observations: one, sulfide activates TRPA1, a Ca^{2+} selective cationic channel, in many structures (Guimaraes and Jordt 2007; Kimura 2014); and, two, sulfide inhibits L-type channels in many preparations (Zhang et al. 2012; Streeter et al. 2012; Tang et al. 2013; Avanzato et al. 2014) making possible that sulphide activates chemoreceptor cells by pathways alternative to L-type channels. Our findings did not support our working hypothesis: either chemoreceptor cells do not express TRPA1 or it is not amenable to sulfide regulation.

In conclusion, exogenous and endogenously produced sulfide inhibits adenylyl cyclase in the carotid body, behaving in this regard in an opposite manner to hypoxia. Similarly, inhibition of adenylyl cyclase does not affect the release response elicited by exogenous sulfide, while that elicited by hypoxia is greatly diminished.

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