Hydrogen sulfide: a novel gaseous signaling molecule and intracellular Ca$^{2+}$ regulator in rat parotid acinar cells

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Moustafa A, Habara Y. Hydrogen sulfide: a novel gaseous signaling molecule and intracellular Ca$^{2+}$ regulator in rat parotid acinar cells. Am J Physiol Cell Physiol 309: C480–C490, 2015. First published July 29, 2015; doi:10.1152/ajpcell.00147.2015.—In addition to nitric oxide (NO), hydrogen sulfide (H$_2$S) is recognized as a crucial gaseous messenger that exerts many biological actions in various tissues. An attempt was made to assess the roles and underlying mechanisms of both gases in isolated rat parotid acinar cells. Ductal cells and some acinar cells were found to express NO and H$_2$S synthases. Cevimeline, a muscarinic receptor agonist upregulated endothelial NO synthase in parotid tissue. NO and H$_2$S donors increased the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$). This was not affected by inhibitors of phospholipase C and inositol 1,4,5-trisphosphate receptors, but was decreased by blockers of ryanodine receptors (RyRs), soluble guanylyl cyclase, and protein kinase G. The H$_2$S donor evoked NO production, which was decreased by blockade of NO synthases or phosphoinositide 3-kinase or by hypotaurine, an H$_2$S scavenger. The H$_2$S donor-induced [Ca$^{2+}$]$_i$ increase was diminished by a NO scavenger or the NO synthases blocker. These results suggest that NO and H$_2$S play important roles in regulating [Ca$^{2+}$]$_i$, via soluble guanylyl cyclase-cGMP-protein kinase G-RyRs, but not via inositol 1,4,5-trisphosphate receptors. The effect of H$_2$S may be partially through NO produced via phosphoinositide 3-kinase-Akt-endothelial NO synthase. It was concluded that both gases regulate [Ca$^{2+}$]$_i$ in a synergistic way, mainly via RyRs in rat parotid acinar cells.

hydrogen sulfide; intracellular Ca$^{2+}$; ryanodine receptors; nitric oxide; parotid acinar cells


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Hydrogen sulfide (H$_2$S) is synthesized endogenously and modulates various physiological functions, including anti-inflammation (47), neuromodulation (1), vasoregulation (48), protection from myocardial ischemia-reperfusion injury (7), and reduction of oxidative stress (46), most of which overlap with the actions of NO. H$_2$S is generated mainly from cysteine and homocysteine by the enzymes cystathionine $\beta$-synthase (CBS) and cystathionine $\gamma$-lyase (CSE). The third synthase, 3-mercaptopyruvate sulfur transferase, also contributes to H$_2$S production in the presence of cysteine aminotransferase. In parotid acinar cells, neither the expression of H$_2$S-producing enzymes nor the roles of H$_2$S have been investigated.

In the last few years, interest has been directed toward a possible cross talk between H$_2$S and NO, because H$_2$S evokes NO production in some cells, as reported previously (3, 4, 10), and inhibits NO production in other cells (16, 32). In recent reports, we demonstrated that NO increases the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) via sGC-cGMP and inositol 1,4,5-trisphosphate (IP$_3$) in pancreatic acinar cells (30). We further examined potential intracellular cross reactions between H$_2$S and NO and proposed the presence of a novel cross talk in which H$_2$S stimulates NO production.

In the rat and ferret salivary glands, nNOS has been found to be expressed in nerve terminals located in the periphery of glandular tissues, both in acinar and ductal cells (2). This isoform was described to be predominant in rat parotid acinar cells and may be activated before amylase secretion in response to methacholine (14). The expression of nNOS was identified in the cytosol of rabbit parotid acinar cells but was barely expressed in ductal cells (38). In addition, nNOS-positive fibers were shown to distribute around acini in the submandibular gland but were scarce around the acini in the sublingual gland (40). Therefore, NOS expression in the salivary glands, including the parotid gland, varies depending on the isoforms, glands, cell types, and animals. NO exerts most of its effects by activating soluble guanylyl cyclase (sGC), resulting in the generation of guanosine 3',5'-cyclic monophosphate (cGMP). This cyclic nucleotide activates cGMP-dependent protein kinase G (PKG), which is assumed to evoke salivary secretion in the rat parotid gland (36). It has also been shown that NO promotes saliva secretion in a wide variety of species (21, 35).

The parotid gland releases both electrolytes and amylase in an intracellular Ca$^{2+}$-dependent manner in response to physiological secretagogues. Gaseous molecules that are produced endogenously as biological messengers have attracted great interest due to their diverse physiological and pathophysiological functions. We have previously demonstrated their roles in regulating intracellular Ca$^{2+}$ homeostasis in pancreatic acinar cells (28–30). Nitric oxide (NO) is a bioactive gas molecule and is synthesized mostly from L-arginine by NO synthases (NOSs). To date, three isoforms of NOS have been identified, including two isoforms that are constitutively expressed in various tissues and are Ca$^{2+}$-dependent neuronal NOS (nNOS) and endothelial NOS (eNOS) and a third isoform that is Ca$^{2+}$-independent inducible NOS (iNOS).

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glandular cells; second, to investigate the effects of both gases on intracellular Ca$^{2+}$ homeostasis; and third, to define the possible signaling pathways through which the two gaseous molecules may interact.

MATERIALS AND METHODS

Chemicals. Fluo 3-AM, EGTA, HEPES, $N^\omega$-monomethyl-l-arginine (l-NMMA) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) were obtained from Dojindo (Kumamoto, Japan). Diaminofluorescein-2 (DAF-2) diacetate was purchased from Daiichikagaku (Tokyo, Japan). Chromatographically purified collagenase was obtained from Worthington Biochemical (Lakewood, NJ). Cell-Tak was procured from BD Biosciences (San Jose, CA). LY-294002 was purchased from Calbiochem (La Jolla, CA). Eagle’s essential amino acid (MEM) was purchased from Invitrogen (Carlsbad, CA). U-73122, BSA, soybean trypsin inhibitor, hyaluronidase, ruthenium red (RR), KT5823, cevimeline hydrochloride, and hypotaurine (HT) were obtained from Sigma-Aldrich (St. Louis, MO). DETA NONOate was from Enzo Life Science. Sodium oxide

Fig. 1. Expression of nitric oxide (NO) and hydrogen sulfide (H$_2$S) producing synthases in parotid gland. A: immunohistochemically detected localization of neuronal NO synthase (nNOS), endothelial NO synthase (eNOS), cystathionine $\beta$-synthase (CBS), and cystathionine $\gamma$-lyase (CSE) in the parotid gland. Arrows represent ductal cells (intralobular and interlobular), and intercalated ductal cells are represented by arrowheads. B: immunohistochemical localization of eNOS at 0, 5, 10, and 30 min after the treatment of the parotid gland with cevimeline. Scale bar in negative control photographs: 50 $\mu$m. C: %area fraction of eNOS expression in parotid gland at 0, 5, 10, and 30 min after stimulation with cevimeline ($n$ = 12–22). ***$P < 0.001$, significant difference by unpaired Student’s t-test.
nitroprusside (SNP), sodium hydrosulfide (NaHS), 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ), xestospongin C, and other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan). Nembutal was purchased from Dainippon Sumitomo Pharma (Osaka, Japan).

Animals and solutions. Male SPF Wistar rats (70 rats, 8 wk old) were obtained from Clea Japan (Tokyo, Japan). They were maintained under a controlled environment at an ambient temperature of 22°C and a 12:12-h light-dark cycle. The animals were deprived of food overnight before the experiment, but were allowed free access to water. All experiments conformed to the guidelines on the ethical use of animals set by the US National Institutes of Health and were approved by the institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The HEPES-buffered standard solution used throughout the acinar isolation and experimentation contained the following (in mM): 138.0 NaCl, 4.7 KCl, 1.3 CaCl_2, 1.13 MgCl_2, 1.0 Na_2HPO_4, 5.5 d-glucose, and 10.0 HEPES, supplemented with MEM plus 2 mM L-glutamine and 1 mg/ml BSA. The pH was adjusted to 7.4 with NaOH. A nominally Ca^{2+}-free solution was prepared without the addition of CaCl_2 but with the addition of 1 mM EGTA.

Preparation of parotid acini. Isolated parotid acini were prepared according to a method described by Looms et al. (23) with some modifications originally adopted for isolation of pancreatic acinar cells (28, 29). Briefly, rats were anesthetized by CO_2 inhalation and euthanized by exsanguination. The parotid glands were excised and cut into small pieces and incubated with HEPES-buffered standard solution supplemented with 0.1 mg/ml soybean trypsin inhibitor, 75 U/ml collagenase, and 153 U/ml hyaluronidase for a total of 60 min at 37°C under vigorous shaking. The tissue was dispersed by gentle suction through pipettes with decreasing orificial size. The tissue suspension was then filtered through 150-µm nylon mesh, washed three times, pelleted (×60 g), and resuspended in a suitable amount of the standard solution.

Ca^{2+} and NO measurements. Intracellular changes in Ca^{2+} and NO were measured as described previously (28, 29). Briefly, isolated parotid acini were resuspended in the sonicated standard solution containing 10 µM fluo 3-AM or 10 µM DAF-2 diacetate and incubated for 60 min at 37°C with mild shaking under dark. Following incubation, the acini were washed with the standard solution, pelleted, resuspended, and transferred to a recording chamber, to the bottom of which a Cell-Tak-coated cover glass was attached. The chamber was placed on the stage of an inverted microscope (IX, Olympus, Tokyo, Japan) of a laser scanning confocal imaging system (LSM FV500, Olympus, Tokyo, Japan). The probe-loaded acini in the recording chamber were perfused with the standard solution at a flow rate of 1 ml/min before and throughout the experiments. The acini were illuminated at 488 nm with a krypton/argon laser, and the emission light (>505 nm) was guided through a ×40 water immersion objective to a pinhole diaphragm. Photodamage was minimized by attenuating the laser intensity with a neutral density filter interposed into the illumination path (1% transmission was sufficient to obtain fluorescence). Confocal images of fluo 3 or triazolofluorescein (DAF-2T) fluorescence were recorded at 10-s intervals. The time courses of changes in
fluorescence intensity (FI) at regions of interest set on several single cells within an acinus were analyzed using bundled software (FluoView 5.0 with Temo, Olympus, Tokyo, Japan). The change in FI was calculated as the percentage of basal FI obtained by setting the prestimulated fluorescence before the application of drugs at 100% (baseline) (FI/F0 X 100). The changes in [Ca2+]i, and NO production were estimated by calculating the summed area of fluorescence changes above the baseline. Summed area of fluorescence changes was calculated in multiple single cells within an acinus, and the mean (±SE) was obtained from the accumulated data obtained from multiple cells examined in several experiments for each specific experimental condition. All experiments for the measurement of [Ca2+]i, and NO were performed at room temperature, and isolated acini were used for experiments within 4 h after isolation.

Immunohistochemistry. Immunohistochemical detection of NOS isoforms (eNOS and nNOS), CBS, and CSE was conducted as reported previously (29). In brief, parotid glands were excised, fixed overnight in 4% paraformaldehyde in phosphate-buffered saline, dehydrated in an alcohol series, and embedded in paraffin. The paraffin block was cut into 3-μm sections and placed on poly-L-lysine-coated slides. The sections were deparaffinized, rehydrated, and washed twice with distilled water for 5 min each time. Antigen retrieval was conducted by heating in citrate buffer (pH 6.0) for 20 min. The endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 30 min at 4°C. Then the nonspecific antibody binding was blocked with 10% normal goat serum. Sections were incubated overnight with specific primary antibodies (polyclonal rabbit anti-eNOS, -nNOS, -CBS, or -CSE antibodies; 1:400; Santa Cruz Biotechnology, Santa Cruz, CA). The sections were subsequently incubated for 1 h with the biotinylated secondary antibody and then for 30 min with an avidin-biotin-peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA). 3,3′-Diaminobenzidine tetrahydrochloride was used as a chromogenic substance. Finally, the slides were washed and counterstained with hematoxylin. Negative control samples were treated in the same way, but the primary antibody was replaced with phosphate-buffered saline. Effects of cholinergic stimulation on eNOS expression in parotid gland were examined in vivo. Cevimeline (5.0 mg/kg) was intravenously injected in the tail vein of anesthetized rats with pentobarbital sodium (Nembutal, 40 mg/kg) and parotid glands were removed 0, 5, 10, and 30 min after the injection, fixed with 4% paraformaldehyde, and embedded in paraffin. Percentage of area fraction (percentage of immunopositive area against whole area) was estimated using ImageJ (version 1.46r).

Statistical analysis. The results are means ± SE. Statistical significance was determined using one-way ANOVA or unpaired Student’s t-tests. P < 0.05 was considered significant.

RESULTS

Expression and localization of NOSs, CBS, and CSE in rat parotid gland. First, we examined expression and localization of constitutive NOS isoforms and two H2S synthases in the rat parotid gland. Immunohistochemical analyses showed cytoplasmic expression of nNOS in intralobular and interlobular ducts, in addition to nuclear expression in intercalated ductal cells. This isofrom was also present in some nuclei of the acinar cells (Fig. 1A, top left). Expression of eNOS was found in the nuclei of all ductal cells and in the endothelial cells (Fig. 1A, top right). Second, we investigated the H2S synthases and found that both CBS and CSE were expressed in the nuclei of all ductal cells and in some nuclei of acinar cells, as shown in Fig. 1A, bottom. Therefore, in parotid acinar cells, all four synthases examined were found to be present with some variations in localization. Third, the expression and localization of NO- and H2S-producing synthases after the stimulation with 5 mg/kg cevimeline, a cholinomimetic muscarinic agonist, were examined. The restricted eNOS expression as described above was changed to scattered distribution at 10 min after cevimeline injection (Fig. 1B). This alteration was found at 5 and 30 min also, as indicated by percentage of area fraction (Fig. 1C). Cevimeline treatment had no appreciable effects on nNOS, CBS, and CSE expression in the parotid gland (data not shown).

Effect of NO and H2S on [Ca2+]i. To assess the roles of NO and H2S in [Ca2+]i regulation, SNP and NaHS were used as NO and H2S donors, respectively. Figure 2A shows typical pseudocolor images of [Ca2+]i changes recorded before and after treatment with 100 μM SNP (left) or 50 μM NaHS (right). Figure 2, B and C, depict the time courses of [Ca2+]i changes calculated in regions of interest, indicated with red ovals in the Fig. 2A, left and right, before and after treatment with 100 μM SNP and 50 μM NaHS, respectively. The [Ca2+]i increases were gradual for both donors, although some oscillatory changes were also found only in a small population of acinar cells treated with SNP (data not shown). With these concentrations, the donor-induced [Ca2+]i increase was much higher for NaHS than SNP. The donor-induced [Ca2+]i increases were dose dependent, as described in
Fig. 3, A and B (P < 0.001 for both donors by ANOVA). Like SNP, DETA NONOate, another NO donor (5–100 μM), induced [Ca^{2+}]_{i} increases as well (data not shown). Next, whether intracellular and/or extracellular Ca^{2+} is mandatory for the NO- and H_{2}S-induced [Ca^{2+}]_{i} increase was evaluated. Perfusion of isolated acini with EGTA-containing Ca^{2+}-free buffer before treatment with SNP (50–500 μM) or NaHS (5–300 μM) tended to decrease the donor-induced [Ca^{2+}]_{i} increases, but the effects were not significant (Fig. 3, A and B), suggesting that the source of Ca^{2+} for the gaseous molecule-induced [Ca^{2+}]_{i} increases is most likely to be intracellular stores, at least for the duration of treatment in this experiment.

Characterization of NO- and H_{2}S-induced Ca^{2+} release. Ca^{2+} release from intracellular stores can be initiated through either or both inositol trisphosphate receptors (InsP_{3}Rs) or ryanodine receptors (RyRs). We attempted to define internal stores that are involved in the SNP- and/or the NaHS-induced [Ca^{2+}]_{i} increase. First, possible involvement of PLC-IP_{3} pathway in the SNP- and the NaHS-induced [Ca^{2+}]_{i} increase was evaluated. Pretreatment of acini with a PLC inhibitor, U-73122 (2 μM), in the absence of extracellular Ca^{2+} had no effect on the [Ca^{2+}]_{i} increases elicited by SNP or NaHS (Fig. 4, A and C). Blockade of InsP_{3}Rs with its potent inhibitor xestospongin C (3 μM) in Ca^{2+}-free medium did not significantly decrease the SNP- or the NaHS-induced [Ca^{2+}]_{i} increase (Fig. 4, B and D). However, the SNP-induced [Ca^{2+}]_{i} was markedly elevated by xestospongin C (Fig. 4B, P < 0.001). These results indicate that the PLC-InsP_{3}Rs pathway is not significantly attributable to the SNP- or the NaHS-induced Ca^{2+} release. Second, we investigated whether RyRs are involved in the [Ca^{2+}]_{i} increase induced by both donors using RR as a RyRs blocker. Pretreatment of acini with RR at 30 μM in the absence of extracellular Ca^{2+} significantly attenuated the SNP- and the NaHS-induced [Ca^{2+}]_{i} increase (Fig. 5). The change from the unstimulated value was attenuated by 48 and 57%, respectively (Fig. 5, B, P < 0.05, and D, P < 0.001). These findings suggest that ryanodine-sensitive Ca^{2+} stores are responsible for the [Ca^{2+}]_{i} increase generated by SNP and NaHS in rat parotid acinar cells.

cGMP is an intracellular signal that mediates the actions of NO on various physiological functions. Therefore, whether the cGMP-linked pathway is involved in the NO- and H_{2}S-induced [Ca^{2+}]_{i} release was investigated. Pretreatment of acini with 100 or 300 μM ODQ, a potent and selective inhibitor of sGC, attenuated the [Ca^{2+}]_{i} increase elicited by 100 μM SNP (Fig. 6, A and B) by 54 and 59%, respectively (P < 0.01). However, 300 μM (P < 0.05) but not 100 μM ODQ significantly inhibited the 50 μM NaHS-induced [Ca^{2+}]_{i} increase by 50% (Fig. 6, C and D). Because one of the main cellular targets of cGMP is PKG, we next assessed its involvement in the [Ca^{2+}]_{i} increase. Pretreatment of acini with 2 μM KT5823, a highly cell-permeable and selective inhibitor of PKG, in Ca^{2+}-free buffer significantly reduced the [Ca^{2+}]_{i} increase elicited by 100 μM SNP or 50 μM NaHS (Fig. 7). The increase from the unstimulated value was attenuated by 53 and 46%, respectively (Fig. 7, B, P < 0.05 and D, P < 0.01). Together, these results indicate that SNP and NaHS induce the [Ca^{2+}]_{i} increase via a cGMP/PKG-dependent pathway.

H_{2}S-induced NO production in parotid acini. An interaction between H_{2}S and NO at different levels of the signaling cascade has been proposed. Here, we examined the effects of NaHS on NO production in parotid acinar cells using the...
Moreover, HT, an H2S scavenger, was used to further examine whether endogenously produced NO is H2S dependent or not. Expression of NOSs, CBS, and CSE in parotid gland. Immunohistochemical analyses in the present study showed that nNOS expression was localized in the cytoplasm of intralobular and interlobular ductal cells, the nuclei of intercalated ductal cells, and some nuclei of acinar cells. eNOS was localized in the nuclei of all parotid ductal cells in addition to endothelial cells. This feature was changed after in vivo stimulation of parotid gland with cevimeline. Expression of eNOS was thoroughly detected in acinar cell nuclei and cytoplasm after the stimulation of the gland. Reports of NOS expression in the salivary glands have varied according to isoforms and localization. NOS activity in the cytoplasmic fraction of the rat parotid gland was reported (27). β-Adrenergic receptor-acti-
vated NOS activity, as estimated by cGMP production, was detected in rat parotid acinar cells (42). Furthermore, nNOS-positive nerve fibers were identified around acini of rat parotid gland (2). The expression of nNOS in the glandular parenchyma and the excretory ducts of parotid and submandibular glands was reported (6). This isofor was also reported to be expressed in the cytosol of rabbit parotid acinar cells, but less in ductal cells (38). NOS activity, coupled with muscarinic receptor activation, was reported (35). Western blot analysis identified nNOS, but not iNOS or eNOS, in rat parotid acinar cells (14). However, parotid gland from duct-ligated rats presents an increase in iNOS activity (26). In the other two major salivary glands, expression of eNOS was detected exclusively in blood vessels and capillaries, whereas nNOS was found at high levels in the cell periphery, associated with the plasma membrane in all cell types of the submandibular gland (44). nNOS was not found in acinar cells of the submandibular gland, but was identified in neuroterminals, as well as excretory and striated ducts (21). In the submandibular gland, an nNOS-positive fiber was found around the acini, but expression was scarce in the sublingual gland (40). Reasons for these discrepancies are unclear, but, according to our present findings, it seems most likely that at least nNOS is expressed in rat parotid acinar cells. In major human salivary glands, most duct epithelial cells were found to be immunoreactive for nNOS, suggesting that NO might directly regulate saliva secretion, and it is a putative source of nitrates secreted into saliva (37).

However, increased iNOS expression was reported in salivary gland of Sjögren’s syndrome patients, suggesting that NO may contribute to inflammatory damage and acinar cell atrophy in the syndrome (18), and inhibition of NO synthesis was found to ameliorate the dysfunction of irradiated salivary glands (41). An alternative notable outcome found in the present study was nuclear localization of the H2S-producing enzymes, CBS and CSE, in all ductal cells, in addition to some nuclei of acinar cells. To the best of our knowledge, this is the first report showing the expression of CBS and CSE in the rat parotid gland, implicating a functional role(s) of H2S in parotid acinar cells. Together, it is conceivable that NO and H2S are endogenously produced in the rat parotid salivary gland.

Gaseous messenger-induced [Ca\textsuperscript{2+}], increase and underlying mechanisms. In our recent studies, carried out in rat pancreatic acinar cells, we found that both NO and H2S could increase the [Ca\textsuperscript{2+}], mainly through release from intracellular stores via InsP\textsubscript{3}R (28, 30). In the present study, we assessed whether a similar mechanism is operating in rat parotid acinar cells, which are exocrine cells functionally analogous to pancreatic acinar cells. In isolated parotid acinar cells, SNP elevated the [Ca\textsuperscript{2+}], in a dose-dependent manner, similar to our previous results in isolated pancreatic acinar cells. The characteristics of the [Ca\textsuperscript{2+}], increase induced by a NO donor were similar in pancreatic and parotid acinar cells. The NO-induced [Ca\textsuperscript{2+}], increase has already been reported in rat parotid acinar cells, and NO/cGMP signal transduction was reported to play...
a crucial role in the acetylcholine-stimulated Ca\(^{2+}\) homoeostasis in addition to the regulation of aquaporine-5 levels in the apical plasma membrane of rat parotid gland (13, 23). One novel finding obtained in the present study is that the H\(_2\)S donor could also increase the [Ca\(^{2+}\)]\(_i\), in a dose-dependent fashion in parotid acinar cells, which was clearly demonstrated in rat pancreatic acinar cells in our recent study (28). The presence of the H\(_2\)S-activated Ca\(^{2+}\) signaling pathway has been proposed in many types of cells, including astrocytes and cerebral arteriole smooth muscle cells (20, 31). In the present study, the H\(_2\)S-induced [Ca\(^{2+}\)]\(_i\) elevation was also presumed to be largely a result of intracellular Ca\(^{2+}\) release, at least for the duration of treatment with the donor. These findings obtained for NO and H\(_2\)S led us to define whether a very similar machinery is associated with the Ca\(^{2+}\) release initiated by NO and H\(_2\)S in parotid acinar cells.

In general, Ca\(^{2+}\) release is caused by InsP\(_3\)Rs and RyRs, which are embedded in the membranes of Ca\(^{2+}\) storing cellular organelles, such as sarcoplasmic and endoplasmic reticula. Our previous studies demonstrated that the IP\(_3\)-InsP\(_3\)Rs pathway is clearly associated with the NO- and the H\(_2\)S-induced [Ca\(^{2+}\)]\(_i\), increases in pancreatic acinar cells. In parotid acinar cells, receptors or channels involved in Ca\(^{2+}\) release have not been fully defined. Previous studies have proposed that RyRs, acting after secretagogue-induced Ca\(^{2+}\) release via InsP\(_3\)Rs, are involved in water secretion (12, 14). Both ryanodine-sensitive/ryanodine-insensitive stores contribute to the Ca\(^{2+}\) release (23, 42), and a Ca\(^{2+}\) release mechanism that is distinct from IP\(_3\)-sensitive Ca\(^{2+}\) channels is operating (33).

The present results that neither the PLC inhibitor nor the InsP\(_3\)Rs blocker was effective (Fig. 4) would rule out presumed involvement of the PLC-IP\(_3\)-InsP\(_3\)Rs pathway in the gaseous messenger-induced [Ca\(^{2+}\)]\(_i\) increases or suggest that this pathway, if available, plays only a minor role in the Ca\(^{2+}\) release by NO or H\(_2\)S, highlighting the second possibility that RyRs may be the major component that is related by the gaseous messenger-induced Ca\(^{2+}\) release in parotid acinar cells. The finding that RR, the RyRs blocker (8, 19, 24, 45), reduced the NO- and the H\(_2\)S-induced Ca\(^{2+}\) release in parotid acinar cells. However, we unexpectedly found that the NO- but not the H\(_2\)S-evoked [Ca\(^{2+}\)]\(_i\) increase was significantly potentiated in the presence of xestospongin C (Fig. 4B). Although the exact reasons are unclear, this phenomenon can be interpreted in light of the available evidence. In particular, two reports give a significant hint that may account for this result. 1) Whereas xestospongin C is a selective InsP\(_3\)Rs blocker extracted from a marine sponge, its hydroxylated derivatives, such as xestospongin B, showed a sensitizing effect on RyRs, inducing potentiation of Ca\(^{2+}\)-induced Ca\(^{2+}\) release by a mechanism mediated by RyR type 1 channels (39). In parotid acinar cells,
Fig. 8. NaHS elevates NO production. A: summarized dose-response relationship of NO production induced by different concentrations of NaHS (5–300 μM). Each bar represents mean SFC (±SE) of triazolofluorescein (DAF-2T) fluorescence (% of unstimulated value) of 50 μM NaHS alone (solid line), or in combination with 100 μM L-NMMA (dotted line) or 20 μM LY294002 (dashed line) or 100 μM HT (dotted line). Sodium hydrosulfide (NaHS)-loaded acini were perfused with the standard solution containing each inhibitor for 3 min before starting and throughout image acquisition. C: summarized SFC (±SE) obtained by treatment with 50 μM NaHS in the absence (n = 35) and presence of 100 μM L-NMMA (n = 28) or 20 μM LY294002 (n = 22) or 100 μM HT (n = 17). **P < 0.01, significant difference for NaHS alone vs. NaHS + L-NMMA and for NaHS alone vs. NaHS + HT. ***P < 0.001, significant difference for NaHS alone vs. NaHS + LY294002 and NaHS + HT by unpaired Student’s t-test.

Fig. 9. Effect of a NO scavenger and NOS inhibitor on the NaHS-induced [Ca2+]i increase. A: temporal changes in fluo 3 FI induced by 50 μM NaHS in the absence (solid line) and presence of 300 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; dotted line) or 100 μM L-NMMA (dashed line) with no added extracellular Ca2+. Acini were perfused with Ca2+-free buffer containing each inhibitor for 5 min before the application of 50 μM NaHS and throughout the experiments. B: summarized SFC (±SE) of 50 μM NaHS-induced [Ca2+]i changes in the absence (n = 37) and presence of 300 μM cPTIO (n = 33) or 100 μM l-NMMA (n = 28). *P < 0.05, significant difference for NaHS-induced SFC increase in the absence vs. presence of cPTIO by unpaired Student’s t-test. ***P < 0.001, significant difference for NaHS-induced SFC increase in the absence vs. presence of L-NMMA by unpaired Student’s t-test.

Fig. 10. Schematic diagram of putative cascade triggered by H2S and NO, and their possible cross talk in rat parotid acinar cells. Blockades by inhibitors used in the present study are described by dotted line. PI3K, phosphoinositide 3-kinase; ER, endoplasmic reticulum.
increase, a minor role for InsP3Rs in parotid acinar cells could not be ruled out (42, 22). If so, the potentiating effect of xestospongin C in the SNP-induced [Ca\textsuperscript{2+}] may be interpreted by putative interactions between InsP3Rs and RyRs, as proposed by McGeeown (25). Whatever the reasons, the present finding corroborates a major involvement of RyRs in the NO-induced [Ca\textsuperscript{2+}] increase in parotid acinar cells. However, the potentiating effect of xestospongin C was not observed in the case of H2S-induced [Ca\textsuperscript{2+}] increase. This may be due to a relatively strong effect of the H2S donor compared with the NO donor at the concentration used, which may mask the potentiating effect by xestospongin C.

Cyclic ADP-ribose (cADPR) has been first proposed as an endogenous activator of RyRs in the sea urchin egg (8) and in various tissues (49), including RyR type 1 in Purkinje cells (15). In addition, NO has been shown to induce Ca\textsuperscript{2+} mobilization via a signaling pathway involving cADPR in the sea urchin egg (43). The cADPR-RyRs-Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (33) and the NO-induced [Ca\textsuperscript{2+}] increase via cADPR-RyRs (23) are implicated in parotid acinar cells as well. H2S has been demonstrated to increase the [Ca\textsuperscript{2+}], due to the activation of RyRs in frog motor nerve endings (9). As mentioned above, our present findings suggested that the NO- and H2S-induced [Ca\textsuperscript{2+}] increase is through RyRs, but not InsP3Rs, raising the possibility that cADPR mediates RyRs activation. Together, it is likely that there is a prominent difference in the mechanism(s) of Ca\textsuperscript{2+} release between pancreatic acinar cells and parotid acinar cells; in the former, IP\textsubscript{3}-InsP3Rs-ind 

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