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Hydrogen sulfide induces hyperpolarization and decreases the exocytosis of secretory granules of rat GH3 pituitary tumor cells

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ABSTRACT

The aim of the present study was to evaluate the effects of hydrogen sulfide (H_2S) on the membrane potential, action potential discharge and exocytosis of secretory granules in neurosecretory pituitary tumor cells (GH3). The H_2S donor – sodium hydrosulfide (NaHS) induced membrane hyperpolarization, followed by truncation of spontaneous electrical activity and decrease of the membrane resistance. The NaHS effect was dose-dependent with an EC_{50} of 152 μM (equals effective H_2S of 16–19 μM). NaHS effects were not altered after inhibition of maxi conductance calcium-activated potassium (BK) channels by tetraethylammonium or paxilline, but were significantly reduced after inhibition or activation of ATP-dependent potassium channels (K_{ATP}) by glibenclamide or by diazoxide, respectively. In whole-cell recordings NaHS increased the amplitude of K_{ATP} currents, induced by hyperpolarizing pulses and subsequent application of glibenclamide decreased currents to control levels. Using the fluorescent dye FM 1–43 exocytosis of secretory granules was analyzed in basal and stimulated conditions (high K^+ external solution). Prior application of NaHS decreased the fluorescence of the cell membrane in both conditions which links with activation of K_{ATP} currents (basal secretion) and activation of K_{ATP} currents and BK-currents (stimulated exocytosis). We suggest that H_2S induces hyperpolarization of GH3 cells by activation of K_{ATP} channels which results in a truncation of spontaneous action potentials and a decrease of hormone release.

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1. Introduction

Hydrogen sulfide (H_2S), a member of the gasotransmitter family, is endogenously synthesized and participates in the regulation of a great variety of physiological and pathophysiological processes [1–8]. In the central nervous system H_2S induces long-term potentiation in the hippocampus [9], and modulates neuronal excitability and transmitter release in the central and peripheral nervous system [10–15].

Ion channels are a main target of H_2S action in excitable cells [3], among which are NMDA-receptors [9], K^+ and Ca^{2+} channels [1,5,7,8,16–19]. Endocrine pituitary cells are neurosecretory cells, expressing Na^+ Ca^{2+}, K^+ and Cl^- channels which are involved to establish the membrane resting potential, modulate the electrical discharge activity and generate spontaneous activity, which is observed not only in cell lines *in vitro* but also in rat pituitary slices *in situ* [20].

The fluorescent dye FM1-43 has been used extensively to study secretory activity [21] and provides the ability to label selectively those structures that are undergoing exocytosis and endocytosis in living cells in real time [22].

The aim of this study was to evaluate the effects of H_2S on membrane potential and exocytosis of secretory granules in neurosecretory pituitary tumor GH3 cells using electrophysiological and fluorescent techniques.

Abbreviations: H_2S, hydrogen sulfide; TEA, tetraethylammonium; BAPTA AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; DMSO, dimethyl sulfoxide; NaHS, sodium hydrosulfide; BK-channels, maxi conductance calcium-activated potassium channels; K_{ATP}, ATP-dependent potassium channels.

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2. Materials and methods

2.1. Cell culture

GH3 pituitary cells were obtained from the European Collection of Cell cultures (ECACC). Cells were cultured at 37 °C and 90% humidity in MEM (minimal essential medium) supplemented with 7% fetal calf serum and 3% horse serum [7]. Cells were split, grown on poly-D-lysine coated coverslips and used for recordings from 3 to 5 days after seeding. Culture media were from Sigma (St. Louis MO, USA) and sera from Invitrogen (Carlsbad CA, USA).

2.2. Electrophysiological recording and analysis

Patch pipettes were pulled from borosilicate glass capillaries (BF 150-86-10, Sutter Instrument, Novato, CA, USA) with resistances of 2–4 MΩ which gave access resistance of 3–10 MΩ [7]. Recordings in whole cell mode were made with an Axopatch-200B amplifier connected to a Digidata 1440A interface, using pClamp 10 software. Data were analyzed using Clampfit software (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA). Membrane potential was recorded in current clamp mode. For analysis of the membrane resistance current pulses were injected into the cell with an amplitude of 0.02 nA. To study ATP-dependent potassium (K_{ATP}) currents a voltage clamp protocol was used with hyperpolarizing test pulses of 200 ms which were applied at 10 mV increments from −20 to −140 mV, the holding potential was clamped at −20 mV [6]. Current–voltage (I–V) relationships were constructed from measurements of current amplitudes at the end of the 200 ms test pulses.

2.3. Solutions and chemicals

The standard extracellular bath solution contained in mM – 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES, pH 7.2, recording temperature was 20–22 °C. The pipette solution contained in mM – 145 KCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA, 3.63 $CaCl_2$ – resulting in 0.5 μM free Ca^{2+} as calculated with the Webmaxc extended calculator (http://web.stanford.edu/~cpatton/webmaxcE.htm).

High K^+ extracellular solution containing 100 mM KCl was used to trigger exocytosis. The concentration of NaCl was correspondingly decreased to 50 mM to maintain isotonicity in high K^+ solutions. Sodium hydrosulfide (NaHS) was used as a H_2S donor. Our previous experiments indicate that from a concentration of 300 μM NaHS, only 11–13% is effective as H_2S in solution when taking pH, temperature, salinity of the perfusate and evaporation of H_2S into account [8]. Tetraethylammonium (TEA) and EGTA was solved in extracellular solution while glibenclamide, diazoxide, paxilline and BAPTA AM (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid) were dissolved in dimethyl sulfoxide (DMSO). For fluorescence staining FM1-43 was used. All chemical unless noted otherwise were from Sigma–Aldrich, St Louis, MO, USA.

2.4. Fluorescence imaging and analysis

Staining of cells with FM 1–43 (4 μM) was performed using the following protocol: cells were perfused with dye-containing solution for 5 min, then a high K^+ dye-containing solution was applied for 5 min, afterwards cells were washed with normal dye-free solution for 10 min. Fluorescence imaging of FM1-43 stained cells was performed using an AxioScope A1 microscope (Carl Zeiss, Germany) equipped with a water immersion objective PlanNeofluar 63 × /0.9NA (Carl Zeiss, Germany), an excitation filter (BP450-490 nm), a beam splitter filter (FT510) and an emission filter (LP555 nm). Fluorescence patterns were recorded with a high-speed camera AxioCam MRm (Carl Zeiss, Germany). The image acquisition time was 300–500 ms per frame. The total cellular fluorescence was measured from the region containing the cell. For background subtraction, fluorescence was measured in the same way from regions containing no cells. The fluorescence intensity of labeled membranes was analyzed with ImageJ software (NIH, USA). The fluorescence intensity profiles were drawn in perpendicular directions for the association of the plasma membrane and nucleus. The peak fluorescence was expressed as relative change in fluorescence $(F-F_0)/F$ for each frame of each measurement, where F is the peak fluorescence of stained cells and F_0 is the background fluorescence closely to a given cell. Quantitative analyses of fluorescence images were performed using OriginPro 8.5 software (OriginLab, USA).

To reveal a possible effect of NaHS on the dye properties the fluorescence emission and absorbance spectra in the 350–700 nm range was measured using spectrofluorometry (LAMBDA 35 UV/Vis) (PerkinElmer, USA) from the same samples. Excitation and emission slit widths were both set to 5 nm. All measurements were carried out at room temperature in quartz cuvettes having 1 cm optical path-length (QS-115, Hellma, Müllheim, Germany). NaHS (300 μM) did not affect the spectral properties of FM 1–43.

2.5. Statistical analysis

All data are presented as the mean value ± standard error; n is the number of independent experiments. Significance levels were determined by using the paired Student's t test, Kolmogorov–Smirnov test or a one way analysis of variance (ANOVA) test followed by Bonferroni's post test.

3. Results

3.1. NaHS effects on BK-, K_{ATP} channels, membrane potential and spontaneous action potentials

Employing our experimental conditions about 45% of cells generated spontaneous action potentials. NaHS in a concentration of 300 μM hyperpolarized the membrane of the cells from −44.31 ± 1.98 mV to −56.72 ± 2.78 mV ($n = 16$, $p < 0.05$) (Fig. 1A). In 50% of cells the effect of NaHS was reversible and after washout the membrane potential returned to its initial level −45.27 ± 2.83 mV ($n = 8$). Other cells displayed different responses like further hyperpolarization, no changes or partial repolarization, which may be explained by different electrical properties of cells in a different stage of the cell cycle [23]. As indicated by the brief downward deflections caused by negative current injections of 0.02 nA NaHS induced a decrease of the membrane resistance from 0.94 ± 0.12 to 0.71 ± 0.10 GΩ ($n = 7$, $p < 0.05$), indicating an increase of ion conductance during NaHS application. The H_2S effect on membrane potentials was dose-dependent. NaHS in concentrations from 10 to 500 μM induced a dose-dependent hyperpolarization with an EC_{50} of 151.56 ± 9.88 μM (effective H_2S about 16–19 μM [8]) (Fig. 1B). Activation of maxi conductance calcium-activated potassium (BK) channels may underlie the hyperpolarization caused by NaHS. To test this hypothesis we used TEA which in low concentrations (1 mM) inhibits BK-channels [24,25] and selectively blocks BK channels in GH3 cells [7,26]. TEA by itself was unable to significantly change the membrane resting potential or membrane resistance. Subsequent application of NaHS hyperpolarized cells to the same extend as under control conditions – the membrane potential hyperpolarized by 9.50 ± 1.11 mV ($n = 13$, $p < 0.05$) and the membrane resistance decreased from 2.47 ± 0.35 to 1.54 ± 0.36 GΩ ($n = 6$, $p < 0.05$) (Fig. 1C, D). Similar effects were obtained when NaHS was applied after inhibition of BK channels with specific

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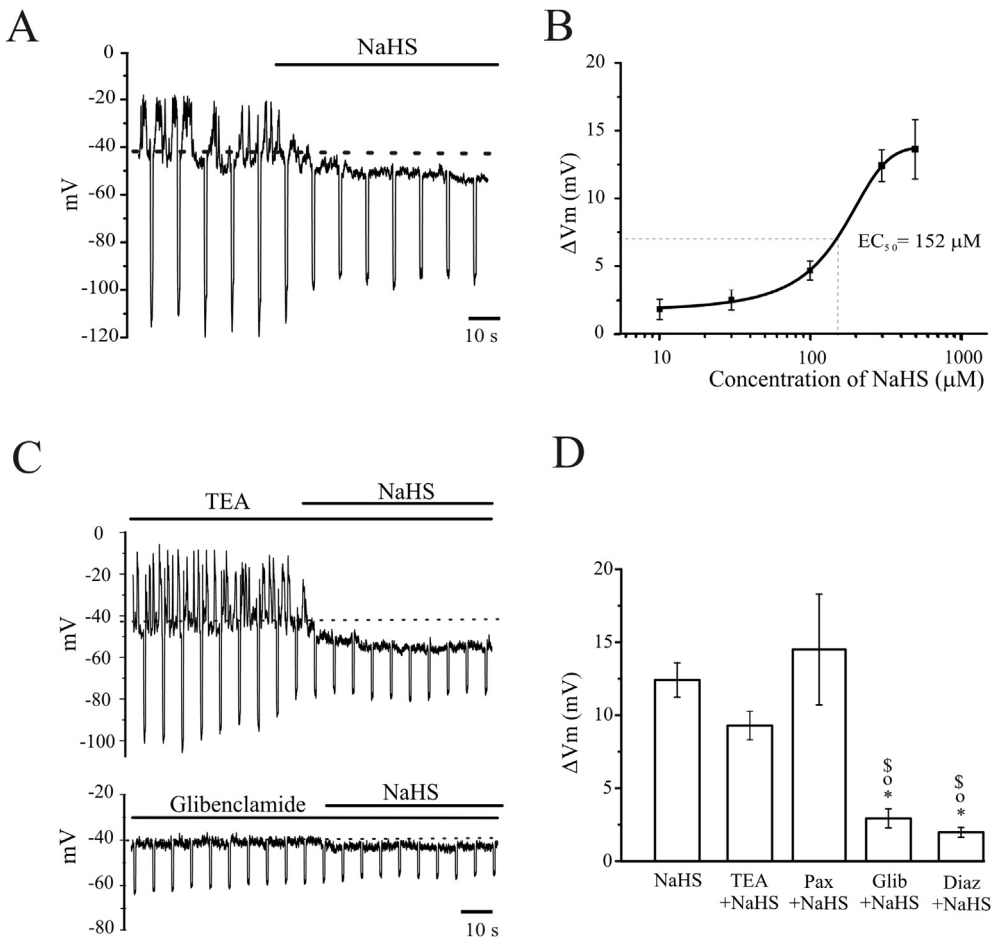

Fig. 1. The effect of the H_2S donor NaHS on the membrane potential and spontaneous action potentials of rat pituitary GH3 cells. A – Application of NaHS (300 μM) induced membrane hyperpolarization, a truncation of spontaneous action potentials and finally a cessation of discharge activity. Brief downward voltage deflections indicate changes of membrane conductance. B – Dose-response of NaHS (μM) effects on the membrane potentials (ΔVm). C – Examples of membrane potential recordings showing the effect of NaHS in the presence of TEA (1 mM) and glibenclamide (10 μM). D – Hyperpolarizing effect of NaHS during control and after application of TEA (1 mM), paxilline (pax) (1 μM), glibenclamide (glib) (10 μM), diazoxide (diaz) (100 μM). * indicates a significant difference at $p < 0.05$ compared to the NaHS effect in control conditions, o indicates a significant difference at $p < 0.05$ when compared to TEA + NaHS, $ indicates a significant difference at $p < 0.05$ compared to pax + NaHS (analyzed by one-way ANOVA).

blocker paxilline (1 μM) - the membrane potential hyperpolarized by 14.50 ± 3.77 mV ($n = 7$, $p < 0.05$) (Fig. 1D) and the membrane resistance decreased by 1.20 ± 0.58 GΩ ($n = 4$, $p < 0.05$).

As suggested by previous studies the activation of K_{ATP} channels may underlie the hyperpolarization caused by H_2S [17,19]. In our experiments application of the K_{ATP} channels inhibitor glibenclamide (10 μM) induced a depolarization of the membrane by 3.10 ± 0.80 mV ($n = 7$, $p < 0.05$). After application of glibenclamide the effect of NaHS was significantly smaller compared to the NaHS effect in control. The membrane hyperpolarized by 2.93 ± 0.66 mV ($n = 7$, $p < 0.05$) and the membrane resistance decreased from 0.93 ± 0.19 to 0.71 ± 0.19 GΩ ($n = 6$, $p < 0.05$) (Fig. 1C, D). Diazoxide (100 μM), a K_{ATP} channels opener, induced hyperpolarization of the membrane by 2.12 ± 0.79 mV ($n = 13$, $p < 0.05$). NaHS induced a further hyperpolarization by 1.99 ± 0.34 mV ($n = 10$, $p < 0.05$) (Fig. 1D). The membrane resistance decreased from 1.76 ± 0.54 to 1.55 ± 0.56 GΩ ($n = 5$, $p < 0.05$) under this experimental condition. After preliminary application of TEA and glibenclamide NaHS significantly hyperpolarized the membrane potential from −38.27 ± 1.59 to −41.10 ± 1.38 mV ($n = 9$, $p < 0.05$) and Rm decreased non significantly from 1.64 ± 0.25 to 1.56 ± 0.24 GΩ ($n = 8$, $p > 0.05$).

We conclude that activation of K_{ATP} channels is the main mechanism underlying the hyperpolarization during H_2S application.

3.2. NaHS increases the amplitude of K_{ATP} currents

To analyze the effects of NaHS on K_{ATP} currents using voltage-clamp experiments hyperpolarizing steps were applied. 300 μM NaHS significantly increased the amplitude of inward currents at voltage steps from −90 to −140 mV and at −120 mV an enhancement from −97.12 ± 15.75 pA to −139.48 ± 16.00 pA ($n = 15$, $p < 0.05$) (Fig. 2A, B) was observed. Subsequent application of glibenclamide (10 μM) reversed currents to the control level ($n = 15$, $p < 0.05$) (Fig. 2A, B). As H_2S may inhibit cytochrome *c* oxidase, a depletion of intracellular ATP levels ($[ATP]_i$) [27] may activate K_{ATP} channels. To avoid the possible decrease of $[ATP]_i$ levels we conducted experiments with ATP 0.5 mM in the pipette solution. Under these conditions NaHS still induced an increase of inward currents and at −120 mV an enhancement from −49.95 ± 8.94 pA to −69.68 ± 9.92 pA ($n = 6$, $p < 0.05$) (Fig. 2C, D) was observed. Thus, activation of K_{ATP} channels by H_2S and subsequent hyperpolarization of GH3 cells are unlikely to result from the reduction of $[ATP]_i$.

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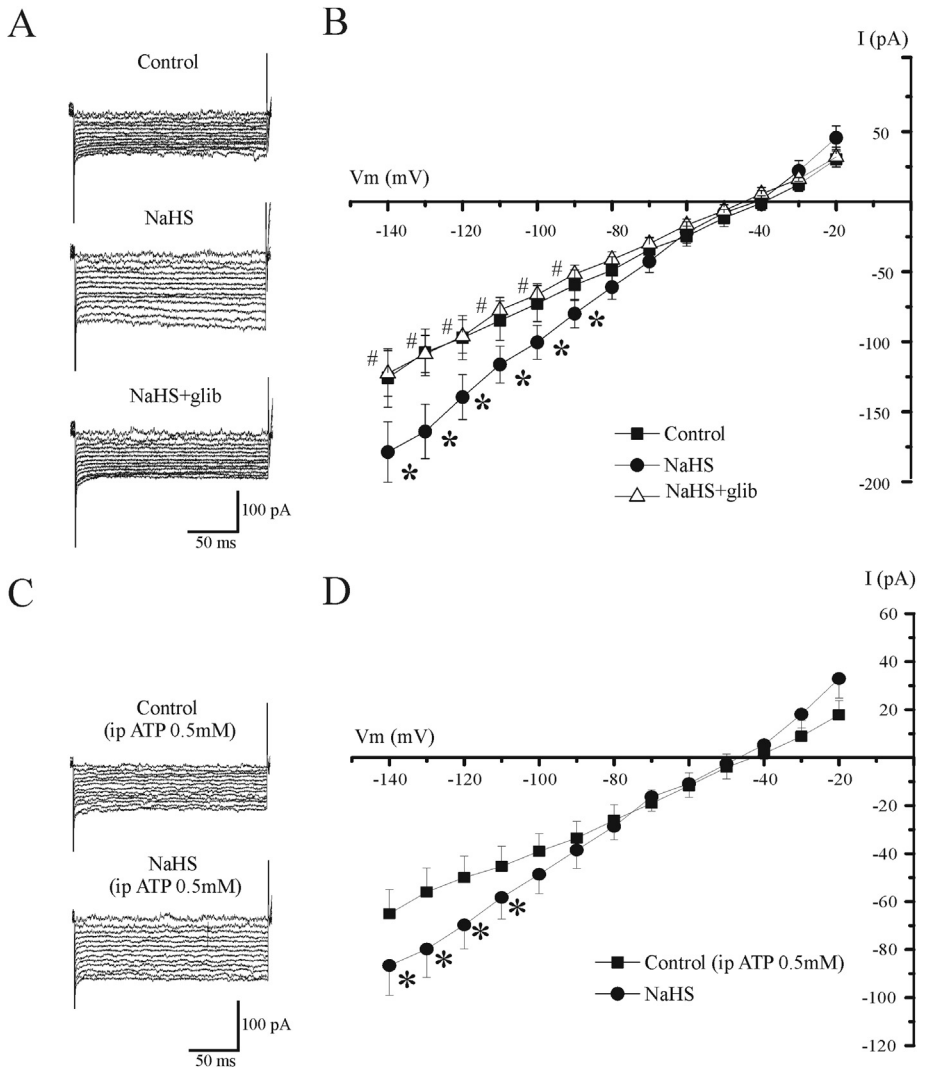

Fig. 2. Effect of NaHS on whole-cell K_{ATP} currents. A – Representative K_{ATP} currents under control conditions, after application of NaHS (300 μM) and glibenclamide (glib) (10 μM). B – The mean I–V relationships of whole-cell K_{ATP} currents (n = 15) under control conditions (filled squares), after the application of NaHS (filled circles) or glib (open triangles). * indicates a statistical significant difference at $p < 0.05$ compared to controls. # indicates a statistical significant difference at $p < 0.05$ of the glibenclamide effect compared to the effect of NaHS. C – Representative K_{ATP} currents during control and 3 min after NaHS application (the intrapipette solution (ip) contained 0.5 mM ATP). B – The mean I–V relationship of whole-cell K_{ATP} currents (n = 6) under control conditions (filled squares) and after NaHS application (filled circles). * – indicates a statistical significance at $p < 0.05$ compared to controls.

3.3. *NaHS decreases the exocytosis of secretory granules both during resting conditions and in response to depolarization*

To reveal the effect of NaHS on the exocytosis of secretory granules the fluorescent dye FM 1–43 was used, which labels membranes undergoing exocytosis. In our experiments FM 1–43 was present in the perfusate before and during KCl stimulation. In control experiments we observed an increase of basal membrane fluorescence during 5 min of dye perfusion by 0.34 ± 0.06 ΔF/F (n = 5, 12 cells (Fig. 3Aa,B)). In order to stimulate cells by prolonged depolarization an extracellular high K^+-containing solution (100 mM) was applied for 5 min. High extracellular K^+ induced a large increase of fluorescence up to 0.72 ± 0.09 ΔF/F (n = 5, 12 cells, $p < 0.05$) (Fig. 3Aa, B). Washout of cells with a dye-free solution decreased the fluorescence intensity within 10 min to 0.11 ± 0.07 ΔF/F (Fig. 3B).

To evaluate the effect of NaHS on exocytosis of secretory granules, cells were incubated in a solution containing NaHS (300 μM) for 10 min. Under these conditions the basal and K^+ stimulated fluorescence was significantly lower compared to control levels (Fig. 3A–D). The NaHS effect on basal exocytosis was prevented by glibenclamide (10 μM) (n = 5, 23 cells) but remained unchanged by paxilline (1 μM) (n = 5, 25 cells) (Fig. 3C). In glibenclamide or paxilline containing solution NaHS decreased the stimulated fluorescence to a lesser degree compared to control (Fig. 3D).

It is known that in neuroendocrine cells Ca^{2+}-dependent exocytosis occurs through fusion of intracellular membrane compartments with the plasma membrane [28]. To investigate a Ca^{2+}-dependent mechanism in GH3 cells we pre-incubated cells with 2 μM of the cell-permeable Ca^{2+}-chelator – BAPTA-AM for 30–60 min at 37 °C. Subsequently cells were washed with a Ca^{2+}-free solution containing 10 mM EGTA, which remained present in the course of the experiment. Application of FM1-43 in these experiments did not significantly change the fluorescence intensity of cells, neither in control nor in the high K^+ stimulated environment (n = 3; 6 cells, Fig. 3B) indicating that exocytosis in these cells is Ca^{2+} dependent.

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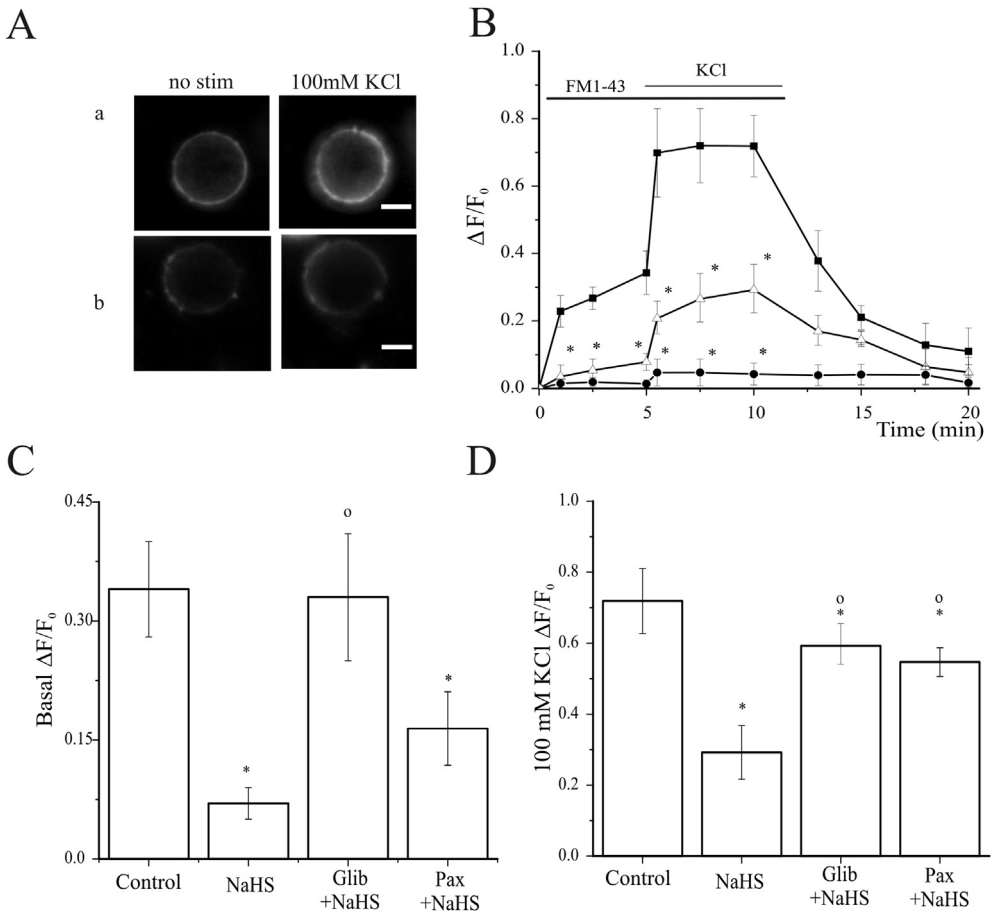

Fig. 3. NaHS decreases FM 1–43 fluorescence in GH3 cells. A – Fluorescence images of GH3 cells incubated in FM 1–43 (4 μM) in resting conditions (5 min) (no stim) and after stimulation with a high K^+-solution (100 mM) (5 min) a) in control, (b) after previous application of NaHS (300 μM) for 10 min. Note that both basal and evoked fluorescence decreased in NaHS. The scale bar equals 5 μm. B – The mean of fluorescence intensity of cell membranes during resting conditions (the first 5 min), after subsequent stimulation with 100 mM KCl and washout in control solution (filled squares), after previous incubation in NaHS (open triangles) Filled circles indicate an experiment where cells were pre-incubated in a Ca^{2+}-free solution containing EGTA and BAPTA-AM. Note that both basal and stimulated fluorescence are significantly lower in cells pre-incubated in NaHS and almost negligible in Ca^{2+}-free conditions. * indicates a statistical significance at $p < 0.05$ compared to controls. C and D – The mean of fluorescence intensity in basal (C) or KCl–evoked (D) conditions in control, after previous incubation in NaHS (300 μM), NaHS + glibenclamide (10 μM) and NaHS + paxilline (1 μM), * indicates a significant difference at $p < 0.05$ compared to control, o indicates a significant difference at $p < 0.05$ compared to NaHS (analyzed by one-way ANOVA). Note: NaHS and NaHS + paxilline in C are not significantly different.

4. Discussion

The main finding of our study is that the H_2S donor NaHS induces a dose-dependent hyperpolarization and truncation of spontaneous action potentials in rat pituitary GH3 cells as a result of activation of K_{ATP} channels. This results in a decrease of secretion as indicated in the fluorescence experiments both in resting and stimulated conditions.

Electrophysiological experiments show that an onset of NaHS effects on the membrane potential can be already seen at a concentration of 30 μM NaHS which relates to an effective H_2S concentration of 3.4–4.1 μM [8]. These concentrations are close to the range of endogenous H_2S concentrations from 10 nM to 3 μM as reported for mammal tissues [29]. We reported previously that NaHS increased the open probability of BK-channels in different types of GH cells [7,8], which may underlie hyperpolarizing effects of NaHS. However, in experiments using TEA (1 mM) or paxilline (1 μM), selective BK channel inhibitors, it was shown that the hyperpolarizing effect of NaHS was not mediated by the activation of BK channels.

In GH3 cells inward rectifier K-channels (K_{ir}), including K_{ATP} channels, have been shown to participate in the maintenance of the resting potential [30,31]. We suggested that H_2S increases the activity of K_{ATP} channels as it was shown in other preparations like smooth muscle cells, where the activation of K_{ATP} by H_2S mediates H_2S-induced vasorelaxation [5,17]. Indeed in our experiments glibenclamide or diazoxide decreased NaHS-induced hyperpolarization significantly. A slight hyperpolarizing effect of NaHS was maintained after inhibition of both K_{ATP} and BK-channels simultaneously. Our results indicate that the hyperpolarization of GH3 cells by NaHS is mediated mainly by an activation of K_{ATP} channels, but a small fraction (about 16%) has to be attributed to a non-identified current component, like other members of K_{ir} channels which are known to be constitutively active in GH3 cells [31].

The NaHS effect on K_{ATP} currents was supported by voltage clamp experiments where NaHS increased the amplitude of inward currents at hyperpolarizing steps and a subsequent application of glibenclamide returned currents back to the initial level. It may be argued that the increase in K_{ATP} currents in the presence of H_2S results from the interfered ATP metabolism by H_2S [27]. However, when cells were dialyzed with a pipette solution contained 0.5 mM ATP NaHS still induced an increase of inward currents, which indicates a direct effect of NaHS on K_{ATP}-channels. The direct effect of H_2S on K_{ATP} channels was shown previously in excised membrane

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patches where NaHS increased the open probability of K_{ATP} channels independent of ATP levels [6]. The analysis of molecular mechanisms of H_2S action revealed a direct modification of SUR1 subunits [18] or Kir 6.1 subunits [32].

The level of the membrane potential is a key factor for the determination of excitability of somatotrophs as well as for their ability to generate action potentials, calcium oscillations and hormone release [31]. Hyperpolarization of GH3 cells by H_2S may in consequence reduce the hormone release in these cells. Indeed, using the fluorescence marker FM1-43 we found that NaHS decreased the fluorescence intensity of the cell membrane during basal conditions as well as in response to a KCl-induced depolarization which reflects the amount of vesicular membrane added during exocytosis [28]. It is known that basal or spontaneous prolactin and growth hormone secretion in GH3 cells is high and occurs as a result of spontaneous action potentials which in turn induce transients of intracellular free Ca^{2+} concentration and lead consequently to basal growth hormone/prolactin secretion [31,33]. Indeed we observed almost negligible fluorescence after cells were pre-incubated in zero Ca^{2+} solutions. Our experiments using glibenclamide and paxilline suggested that only activation of K_{ATP} currents by NaHS underlies the decrease in basal exocytosis. In addition to K_{ATP} BK currents activated by depolarization (as it is the case under high external K^+) are further increased by NaHS [7] in the evoked secretion experiments. This extra BK channel activation may in addition favor shortening of action potentials and a subsequent decrease in Ca^{2+}-influx.

Inhibition of hormone release by H_2S was shown for other endocrine cells. In rat hypothalamus explants H_2S and S-adenosyl methionine (an activator of H_2S synthesis) reduced the release of corticotropin-releasing hormone [34]. Activation of K_{ATP} channels by H_2S also underlies the inhibitory effect of H_2S on the on insulin secretion from INS-1E cells [6]. These inhibitory H_2S actions are opposite to the effect of NaHS in the peripheral nervous system where it was shown that NaHS increases the exocytosis of synaptic vesicles in motor nerve ending of frog and mice as measured by electrophysiological and fluorescence methods [14,15,35]. Such differences may be a consequence of peculiar properties of processes of exo- and endocytosis between synaptic vesicles and secretory granules and different targets of H_2S in excitable cells.

To conclude H_2S induces a dose-dependent hyperpolarization of GH3 cells which results in the cessation of spontaneous action potentials and a decrease of secretion.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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