RESEARCH PAPER

The gasotransmitter hydrogen sulphide decreases Na⁺ transport across pulmonary epithelial cells

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BACKGROUND AND PURPOSE

The transepithelial absorption of Na⁺ in the lungs is crucial for the maintenance of the volume and composition of epithelial lining fluid. The regulation of Na⁺ transport is essential, because hypo- or hyperabsorption of Na⁺ is associated with lung diseases such as pulmonary oedema or cystic fibrosis. This study investigated the effects of the gaseous signalling molecule hydrogen sulphide (H₂S) on Na⁺ absorption across pulmonary epithelial cells.

EXPERIMENTAL APPROACH

Ion transport processes were electrophysiologically assessed in Ussing chambers on H441 cells grown on permeable supports at air/liquid interface and on native tracheal preparations of pigs and mice. The effects of H₂S were further investigated on Na⁺ channels expressed in *Xenopus* oocytes and Na⁺/K⁺-ATPase activity *in vitro*. Membrane abundance of Na⁺/K⁺-ATPase was determined by surface biotinylation and Western blot. Cellular ATP concentrations were measured colorimetrically, and cytosolic Ca²⁺ concentrations were measured with Fura-2.

KEY RESULTS

H₂S rapidly and reversibly inhibited Na⁺ transport in all the models employed. H₂S had no effect on Na⁺ channels, whereas it decreased Na⁺/K⁺-ATPase currents. H₂S did not affect the membrane abundance of Na⁺/K⁺-ATPase, its metabolic or calcium-dependent regulation, or its direct activity. However, H₂S inhibited basolateral calcium-dependent K⁺ channels, which consequently decreased Na⁺ absorption by H441 monolayers.

CONCLUSIONS AND IMPLICATIONS

H₂S impairs pulmonary transepithelial Na⁺ absorption, mainly by inhibiting basolateral Ca²⁺-dependent K⁺ channels. These data suggest that the H₂S signalling system might represent a novel pharmacological target for modifying pulmonary transepithelial Na⁺ transport.

Abbreviations

ALI, acute lung injury; AMPK, adenosine monophosphate-activated protein kinase; ARDS, acute respiratory distress syndrome; BK_{Ca}, large conductance K_{Ca} channel; CBS, cystathionine-β-synthase; CO, carbon monoxide; CPA, cyclopiazonic acid; CSE, cystathionine-γ-lyase; DMSO, dimethyl sulfoxide; DNP, 2,4-dinitrophenol; ENaC, epithelial Na⁺ channel; FCS, fetal calf serum; Glib., glibenclamide; Go6983, 3-[1-[3-(dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione; H₂S, hydrogen sulfide; H-7, (+/-)-1-(5-isoquinolinesulfonyl)-2-methylpiperazine; I_{ami}, amiloride-sensitive current; IK_{Ca}, intermediate K_{Ca} channel; I_{lido}, lidocaine-sensitive current; I_{NaHS}, NaHS-sensitive current; I_{ouab}, ouabaine-sensitive current; I_{SC}, short-circuit current; K_{ATP}, ATP-dependent K⁺ channel; K_{Ca}, calcium-dependent K⁺ channel; K_V, voltage-dependent K⁺ channel; R_T, transepithelial resistance; SK_{Ca}, short conductance K_{Ca} channel; TBS, tris-buffered saline; TP_{EA}, tetrapentylammonium; V_T, transepithelial potential; XE991, 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracene

Introduction

Na⁺ absorption by pulmonary epithelia is crucial for the maintenance of lung fluid homeostasis. Na⁺ ions apically enter the pulmonary epithelial cells through amiloride-sensitive Na⁺ channels, such as the epithelial Na⁺ channel (ENaC) (Matalon *et al.*, 2002), or amiloride-insensitive Na⁺ channels, such as the cyclic nucleotide-gated channel A1 (CNGA1) (Wilkinson *et al.*, 2011). The Na⁺ ions are extruded at the basolateral membrane of the epithelium by the activity of the Na⁺/K⁺-ATPase, consequently leading to the net movement of Na⁺ ions from the apical to the basolateral side of the epithelium. This transepithelial Na⁺ transport results in an osmotic gradient across the epithelium and is thus the main driving force for transepithelial fluid absorption.

Disregulated Na⁺ transport across pulmonary epithelia is associated with lung diseases that are characterized by disturbed fluid homeostasis. Decreased Na⁺ transport, especially across the alveolar epithelium, can, due to impaired alveolar fluid clearance, promote the development of pulmonary oedema (Althaus *et al.*, 2011). In the airways, ENaC hypoactivity, as in type 1 pseudohypoaldosteronism, will lead to increased volume of the airway lining fluid (Kerem *et al.*, 1999). By contrast, hyperactive Na⁺ transport, especially in the airway epithelia, produces mucus thickening and cystic fibrosis-like lung disease (Mall *et al.*, 2004). Consequently, novel therapeutic strategies for the treatment of those diseases currently focus on a pharmacological intervention of Na⁺ transport, and its regulation in the respiratory system (Althaus *et al.*, 2011; Becq *et al.*, 2011). Therefore, it is important to explore novel regulators of pulmonary transepithelial Na⁺ transport, and thus identify novel putative pharmacological targets.

Recently, there is a growing body of evidence that small gas molecules, so called gasotransmitters, may regulate transepithelial ion transport processes. It was recently shown that the classical gasotransmitters NO and CO both decrease pulmonary transepithelial Na⁺ transport: Whereas NO decreases the activity of amiloride-sensitive Na⁺ channels as well as of the Na⁺/K⁺-ATPase (Guo *et al.*, 1998; Helms *et al.*, 2008; Althaus *et al.*, 2010), CO solely decreases the activity of amiloride-sensitive Na⁺ channels in the lung (Althaus *et al.*, 2009). Besides NO and CO, hydrogen sulphide (H₂S), a gas with the characteristic odour of rotten eggs, has been suggested to be another gasotransmitter (Wang, 2002), that influences a variety of cellular and organ functions (for detailed review, see Olson, 2011). H₂S is endogenously generated in humans from cysteine, mainly by the enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) (Stipanuk and Beck, 1982; Wang, 2002). H₂S production has also been demonstrated in lung tissue homogenates (Olson *et al.*, 2010).

In the respiratory system, H₂S has profound vasoactive and anti-inflammatory effects (Olson, 2011). It mimics the vasoactive effects of hypoxia (Olson *et al.*, 2010), and administration of the H₂S-donating drug NaHS causes relaxation of mouse bronchial smooth muscle cells (Kubo *et al.*, 2007). Na₂S, another H₂S-donating molecule, reduces mortality and lung damage in a burn- and smoke-induced acute lung injury (ALI) model (Esechie *et al.*, 2009). NaHS also reduces inflammation in a rat model of ventilator-induced lung injury (VILI)

(Aslami *et al.*, 2010). Inhaled H₂S gas has beneficial effects in a rat model of VILI, which is due to inhibition of pro-inflammatory cytokine release and apoptosis (Faller *et al.*, 2010). Similar to the gasotransmitter CO (Ryter *et al.*, 2007), H₂S is therefore suggested as a potential therapeutic option for the treatment of inflammatory lung diseases, including ALI (Esechie *et al.*, 2009; Otulakowski and Kavanagh, 2010).

Although there are currently no studies that have investigated the effect of H₂S on pulmonary epithelia, evidence for regulation of transepithelial ion transport processes by H₂S comes from studies on rat distal colonic preparations, where an increased Cl⁻ secretion is observed after administration of NaHS (Hennig and Diener, 2009; Pouokam and Diener, 2011). However, whether or not H₂S affects transepithelial Na⁺ transport processes, is currently unknown.

The present study investigated the effect of H₂S on Na⁺ transport by a human airway line (H441), native tracheal preparations of pigs and mice and on Na⁺ transporting molecules *in vitro* and in an heterologous expression system. These studies revealed that H₂S decreases transepithelial Na⁺ transport indirectly by inhibiting basolateral calcium-dependent K⁺ channels. These data suggest that the H₂S signalling system might represent a novel pharmacological target for the treatment of lung diseases that are associated with deregulated Na⁺ and water homeostasis.

Methods

Cell culture

The H441 human bronchiolar epithelial cell line was purchased from the American Type Culture Collection (distributed by LGC Standards, Wesel, Germany) in the 65th passage. Cells were cultured in flasks with standard Roswell Park Memorial Institute culture medium containing 1640/L-glutamine medium (PAA, Cölbe, Germany), which was supplemented with 10% FCS (PAA), 1 mM Na⁺ pyruvate, 1% insuline-transferrin-sodiumselenite medium supplement (Sigma, Taufkirchen, Germany) at 37°C in humidified atmosphere containing 5% CO₂. For Ussing chamber recordings, cells were removed from culture flasks using trypsin/EDTA (Sigma), resuspended in medium and seeded in confluent densities on permeable membrane supports (type 3801, Snapwell, Corning, Amsterdam, The Netherlands) under liquid/liquid conditions in standard medium supplemented with 1% antibiotic/antimycotic mix (Sigma). One day after seeding the medium on the apical side was aspirated, and the basolateral medium was supplemented with 200 nM dexamethasone (Sigma). Medium was changed every 48 h. The cells were cultured for an additional 7 days under air/liquid conditions in the presence of dexamethasone. The monolayers were used for Ussing chamber studies from day 8 onwards.

Native tracheal tissue preparations

Native tracheal epithelial preparations were freshly prepared from pigs and mice. Tracheae of pigs were obtained from a local butcher and kept at 4°C until the experiments were performed. The tracheae were incised longitudinally and the tissue containing the epithelium was dissected from the cartilage. The preparations were placed between two plastic rings

and mounted into Ussing chambers. Ussing chamber experiments were performed exactly as described later.

Adult mice (C57/Bl/6) were obtained from the local animal breeding facility (Justus-Liebig University of Giessen). Animals were killed by isoflurane overdose followed by aortic exsanguinations. The preparation of the tracheae and Ussing chamber recordings were performed exactly as described in Hollenhorst *et al.* (2012). The results of all studies involving animals are reported in accordance with the ARRIVE guidelines reporting experiments involving animals (McGrath *et al.*, 2010). Experiments were performed according to the current law on the protection of animals in Germany and the National Institutes of Health guidelines for the care and use of laboratory animals.

Ussing chamber studies

Confluent monolayers, grown on membrane supports, or tracheal preparations were mounted in perfusion Ussing chambers and bathed from both sides with physiological saline containing 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 1 mM CaCl_2 , 0.5 mM MgCl_2 , 10 mM HEPES and 10 mM glucose (37°C, pH 7.35, NaOH). For the detection of electrical parameters, custom-made Ag/AgCl electrodes with KCl/agar bridges were used to connect the chamber bath with the voltage-clamp amplifier (custom made). Monolayers were maintained under open-circuit conditions and the spontaneous transepithelial potential (V_T) was monitored until a stable value was reached (~15 min). The V_T was then clamped to 0 mV by the voltage-clamp amplifier, and short-circuit current (I_{sc}) was monitored (strip-chart recorder, Kipp&Zonen, Delft, The Netherlands) and recorded online using an analogue/digital interface (National Instruments, München, Germany). In order to estimate transepithelial resistance (R_T), voltage pulses of 2.5 mV were applied to the monolayers. The resulting current deflections were used to calculate R_T using Ohm's law. All experiments were performed at 37°C.

Measurement of basolateral K^+ channels. In order to measure current fluxes through basolateral K^+ channels, H441 monolayers in Ussing chambers were apically permeabilized with nystatin (75 μM) in the presence of solutions, which generated a K^+ gradient from the apical to the basolateral compartment. The apical solution contained 30 mM KCl, 100 mM K^+ gluconate, 1.5 mM KH_2PO_4 , 11 mM calcium gluconate, 0.5 mM MgSO_4 , 10 mM HEPES and 10 mM glucose (pH 7.35/KOH). In order to block epithelial sodium channels, the solution also contained amiloride (10 μM). The basolateral solution consisted of 30 mM NaCl, 100 mM Na^+ gluconate, 1.5 mM KH_2PO_4 , 11 mM calcium gluconate, 0.5 mM MgSO_4 , 10 mM HEPES and 10 mM glucose (pH 7.35/NaOH). The solution also contained ouabain (1 mM) in order to block the Na^+/K^+ -ATPase.

Patch-clamp studies

For Patch-clamp studies, H441 cells were seeded in culture dishes (Thermo Scientific, Langenselbold, Germany) in standard culture medium. One day after seeding, 200 nM dexamethasone was added to the medium. After 48–72 h of dexamethasone exposure, whole-cell patch-clamp experiments were performed on single cells, which were located

within small groups of cells. For experiments, culture dishes were washed with bath solution containing 140 mM NaCl, 4.5 mM KCl, 2.5 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES and 5 mM D-glucose (pH 7.4). Patch-pipettes were pulled from borosilicate capillaries and filled with a solution containing 10 mM NaCl, 18 mM KCl, 92 K^+ gluconate, 0.5 mM MgCl_2 , 1 mM EDTA and 10 mM HEPES (pH 7.2). Whole-cell recordings were performed with a HEKA EPC 9 patch-clamp amplifier (HEKA Electronics, Lambrecht, Germany) at a holding potential of –48 mV. Together with the liquid junction potential of the bath and pipette solutions employed this resulted in a membrane potential of –60 mV during experiments. Electrical signals were acquired by an ITC-16 interface with the HEKA Pulse software (version 8.77). Chemicals were directly applied to cells by a pressure-driven perfusion system (custom made with a compressor, valves and microtubings). Experiments were performed at room temperature.

Heterologous expression of ENaC and two-electrode voltage clamp (TEVC) recordings

The effect of H_2S on ENaC was investigated by the TEVC technique in the *Xenopus* oocyte expression system. The α , β , γ and δ ENaC subunits were previously cloned from human lung mRNA (Fronius *et al.*, 2010; Wesch *et al.*, 2010). Defolliculated *Xenopus* oocytes were injected with cRNA encoding the human $\alpha\beta\gamma$ ENaC or $\delta\beta\gamma$ ENaC subunits (for oocyte preparation and cRNA injection see Fronius *et al.*, 2010). The RNA concentration was 10 ng- μL^{-1} per subunit and a volume of 23 nL was injected per oocyte. Experiments were performed 24–48 h after injection. For this purpose, oocytes were placed in a lucite chamber and perfused by a gravity driven system with oocyte Ringer's solution, containing 90 mM NaCl, 1 mM KCl, 2 mM CaCl_2 and 10 mM HEPES (pH 7.4). Glass capillaries with an outer diameter of 1.2 mm were pulled to microelectrodes and filled with 1 M KCl. The membrane voltage was clamped to –60 mV using a TEVC amplifier (Warner Instruments, Hamden, CT, USA) and transmembrane currents (I_m) were continuously recorded with a strip chart recorder (Kipp&Zonen).

H_2S administration and chemicals used

Due to the high toxicity of H_2S gas, the H_2S -donating drug NaHS (Sigma) was employed. In aqueous solution, NaHS dissociates into Na^+ , $\text{H}_2\text{S}/\text{HS}^-$ and OH^- . Due to the formation of OH^- , buffers were adjusted to keep the pH stable over the time course of experiments. Stock solutions of NaHS were freshly prepared in water before each experiment. The final concentration of NaHS in Ussing chamber recordings was 300 μM , of which approximately one-third (100 μM) represents free H_2S (Reiffenstein *et al.*, 1992). For experiments that were performed at room temperature (patch clamp, voltage clamp and calcium imaging), a higher concentration of NaHS was employed (1 mM).

Unless indicated otherwise, all chemicals were obtained from Sigma. All inhibitors employed, their targets and concentrations are listed in Table 1. The nomenclature of drugs and targets conforms to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2011). Where necessary, drugs were dissolved in dimethyl sulfoxide (DMSO, Sigma) and control

Table 1

Purpose and concentrations of the inhibitors employed

Substance	Purpose	C (M)	Company	References
Amiloride	ENaC inhibitor	10 ⁻⁵	Sigma	Canessa <i>et al.</i> , 1994
Ouabain	Na ⁺ /K ⁺ -ATPase inhibitor	10 ⁻³	Sigma	Ramminger <i>et al.</i> , 2004; Woollhead <i>et al.</i> , 2007
Lidocaine	Non-specific K ⁺ channel inhibitor	3 × 10 ⁻³	Sigma	Inglis <i>et al.</i> , 2007
Glibenclamide	K _{ATP} channel inhibitor	10 ⁻⁴	Sigma	Barrett-Jolley and Davies, 1997; Hennig and Diener, 2009
TPeA	K _{Ca} channel inhibitor	3 × 10 ⁻⁴	Sigma	Hennig and Diener, 2009; Maguire <i>et al.</i> , 1999
XE991	K _V channel inhibitor	10 ⁻⁴	Biozol	Greenwood <i>et al.</i> , 2009; Wang <i>et al.</i> , 1998; 2000
Dynasore	Dynamin-inhibitor	7 × 10 ⁻⁵	Biozol	Macia <i>et al.</i> , 2006
H-7	Non-selective PKC inhibitor	2 × 10 ⁻⁵	Biozol	Hidaka <i>et al.</i> , 1984
Staurosporine	Non-selective PKC inhibitor	2 × 10 ⁻⁷	Biozol	Rüegg and Burgess, 1989; Tamaoki <i>et al.</i> , 1986
Go6983	Broad spectrum PKC inhibitor	5 × 10 ⁻⁶	Biozol	Gschwendt <i>et al.</i> , 1996
PKC peptide inhibitor	PKC inhibitor	6 × 10 ⁻⁵	Sigma	Eichholtz <i>et al.</i> , 1993

C, concentration; TPeA, tetrapentylammonium.

experiments were performed with analogous concentrations of DMSO. For permeabilization experiments, nystatin (Sigma) was used. This drug generates ion selective pores, which are permeable for monovalent ions such as Na⁺, K⁺ and Cl⁻ (Russell *et al.*, 1977).

Surface biotinylation and Western blotting for the detection of membrane proteins

Plasma membrane proteins were isolated and purified by the method described previously (Woollhead *et al.*, 2007; Tan *et al.*, 2011). Briefly, monolayers were transferred to ice, and both filter compartments rinsed with ice-cold PSS (pH 7.4). Biotinylation solution [1.5 mL of 0.5 mg·mL⁻¹ sulfo-N-hydroxysuccinimide-biotin (sulfo-NHS-biotin) in borate buffer pH 8.2] was added to the basolateral compartment and the monolayers incubated with gentle rocking for 30 min. Apical biotinylation was prevented by adding 500 µL PSS + 10% FBS to the apical chamber. The biotinylation solution was removed and PSS + 10% FBS was added to quench all unreacted sulfo-NHS-biotin groups, followed by three washes with PSS. Cells were then lysed, proteins solubilized, and the protein concentration was then determined by Bradford assay. Similar amounts of total protein were incubated overnight with streptavidin agarose beads at 4°C. The following day, biotinylated proteins bound to beads were separated from non-biotinylated proteins by centrifugation. Bound biotinylated proteins were eluted into 30 µL sample loading buffer by heating at 95°C for 5 min. Bound and unbound (30 µg) proteins were separated by NuPAGE (Invitrogen, Paisley, UK) on 4–12% Bis-Tris gels, and transferred to low fluorescence PVDF membrane. Membranes were blocked for 1 h at room temperature in Odyssey blocking buffer (LI-COR, Lincoln, NE, USA), then incubated with primary antibodies overnight at 4°C. Membranes were washed in Tris buffered saline + 0.01% TWEEN, and incubated with a fluorescent secondary antibody diluted in Odyssey buffer for 1 h at room temperature. Proteins were visualized and band density analysed using the Odyssey system (LI-COR). Anti α1-Na⁺/

K⁺ATPase monoclonal antibody was developed by D Fambrough and was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development maintained by the University of Iowa, USA. Anti beta-actin was obtained from Ab-cam (Cambridge, UK). Fluorescent secondary antisera were obtained from Odyssey (LI-COR).

Calcium imaging experiments

Changes in cytosolic Ca²⁺ concentrations were measured using Fura-2. For the experiments, H441 cells were seeded on glass cover slips and cultured in the presence of dexamethasone for 48–72 h. Cells were washed with PSS, and incubated for at least 45 min at 37°C with 2.5 µM Fura-2 AM (Invitrogen, Darmstadt, Germany) and 0.05% pluronic (20% solution in DMSO, Invitrogen). After incubation, cells were washed twice and perfused with PSS (5 mL·min⁻¹). NaHS (1 mM) and cyclopiazonic acid (CPA; 10 µM; Alexis, Grünberg, Germany or Biozol, Eching, Germany) were then applied via the perfusion system. Changes in cytosolic Ca²⁺ concentrations were measured at room temperature as changes in the Fura-2 ratio (emission at an extinction of 340 nm divided by emission at an extinction of 380 nm) exactly as previously described (Pouokam and Diener, 2011).

Colometric determination of catalytic Na⁺/K⁺-ATPase activity

The catalytic activity of the Na⁺/K⁺-ATPase was determined by a colometric assay (EC 3.6.1.3, Sigma). This *in vitro* assay is based on the measurement of the amount of inorganic phosphorous (P_i) that is liberated upon ATP hydrolysis by a purified Na⁺/K⁺-ATPase (Sigma). The reaction, with and without 1 mM NaHS, was performed exactly according to manufacturer's instructions. The supernatant of the enzyme reaction was used to determine the amount of inorganic phosphorus according to the method described by Taussky and Shorr (1953), by quantification at 660 nm with a spectrophotom-

eter (Krüss Optronic, Hamburg, Germany). The activity of the Na^+/K^+ -ATPase was calculated by the subtraction of background activity, which was determined with the Na^+/K^+ -ATPase inhibitor ouabain (1 mM). The total amount of P_i was determined with a standard curve made from phosphorus standard solution (Sigma).

Colorimetric determination of cellular ATP levels

For the determination of cellular ATP levels a colorimetric ATP assay kit (Abnova, Heidelberg, Germany) was used. H441 cells were cultured on permeable supports, as described for Ussing chamber experiments. Cells were apically exposed to 300 μL PSS, with or without NaHS (300 μM or 1 mM) for 15 min at 37°C and 5% CO_2 in humidified atmosphere. 2,4-Dinitrophenol (DNP; 500 μM ; 30 min exposure) was used as a positive control. Because DNP was dissolved in DMSO, all solutions contained 0.1% DMSO. After incubation of the monolayer with the drugs described, cells were cooled to 4°C and all media were aspirated. Afterwards, cells were frozen at -80°C. Cells were then thawed and lysed in 100 μL of ATP assay buffer as supplied by the manufacturer. Further sample preparation and colorimetric determination of ATP was performed exactly according to manufacturer's instructions by measuring the absorbance at 570 nm with a spectrophotometer (Krüss Optronic). ATP concentrations were estimated with an ATP standard curve.

Statistics

Values are presented as mean $\pm$ SEM. For statistical analysis, mean values of unpaired experiments (e.g. parallel conducted controls and drug exposed monolayers from identical passages) were compared by Student's unpaired *t*-test. For the comparison of dependent means (e.g. before and after exposure to reagents), Student's paired *t*-test was used. For the statistical comparison of more than two groups, one-way ANOVA followed by Bonferroni's multiple comparison test was used. Statistical tests were performed with Microsoft Excel 2003 or GraphPad Prism versions 4 and 5. The number of experiments is indicated by *n*, which represents single H441 monolayers on permeable supports for Ussing chamber experiments; single cells from separate culture dishes for patch-clamp recordings; single oocytes injected with ENaC-encoding RNA; and single animals from which tracheae were obtained. In experiments with pig tracheal preparations, in total seven tissue preparations were made from three pigs and Ussing chamber studies were performed independently. All control experiments were performed from the same cell or oocyte preparations, for example, cells were derived for Ussing chamber cultures from the same culture flask, or oocytes were harvested from the same donor frog. *P*-values ≤ 0.05 were regarded to be significant, and marked in the figures by an asterisk (*).

Results

Effects of H_2S on Na^+ absorption by H441 cells and native tracheal preparations

The effect of H_2S on Na^+ absorption by pulmonary epithelial cells was investigated with the H441 human airway epithelial

cell line. These cells have been demonstrated to represent a suitable model for electrophysiological ion transport studies, and exhibit a robust Na^+ -absorbing phenotype when cultured at air/liquid interface in the presence of glucocorticoids (Lazrak and Matalon, 2003; Clunes *et al.*, 2004; Ramminger *et al.*, 2004; Woollhead *et al.*, 2005; Albert *et al.*, 2008; Brown *et al.*, 2008; Mace *et al.*, 2008; Althaus *et al.*, 2009; 2010; Nie *et al.*, 2009; Tan *et al.*, 2011). The administration of the H_2S -donating molecule, NaHS (300 μM , apical application), to H441 monolayers in Ussing chambers rapidly decreased transepithelial short-circuit currents from $12.8 \pm 3 \mu\text{A}\cdot\text{cm}^{-2}$ to $6.0 \pm 1.9 \mu\text{A}\cdot\text{cm}^{-2}$ ($P < 0.05$; $n = 5$) within 5 min (Figure 1A). Comparing the current-fractions sensitive to the ENaC blocker amiloride, before and after NaHS application, revealed that NaHS significantly decreased amiloride-sensitive currents (I_{ami}) by approximately 60% from $10.2 \pm 1.8 \mu\text{A}\cdot\text{cm}^{-2}$ to $3.8 \pm 0.9 \mu\text{A}\cdot\text{cm}^{-2}$ ($n = 5$; $P < 0.05$) (Figure 1A, B). To prove that the observed decreases in ion currents were not the result of damage to the monolayers, the effect of NaHS on transepithelial resistance, which would decrease upon epithelial damage, was measured (Figure 1C). Transepithelial resistance was $200.2 \pm 25.4 \Omega\cdot\text{cm}^{-2}$ before and $243.8 \pm 25 \Omega\cdot\text{cm}^{-2}$ after NaHS administration ($n = 5$; $P < 0.01$). In the presence of amiloride (10 μM), the effect of NaHS was lost (Figure 1D). I_{sc} values, in the presence of amiloride, were $5 \pm 2.7 \mu\text{A}\cdot\text{cm}^{-2}$ before, and $5.75 \pm 2.8 \mu\text{A}\cdot\text{cm}^{-2}$ after NaHS ($n = 4$; $P = 0.06$). The effect of NaHS on I_{ami} was concentration-dependent, with an IC_{50} of 146 μM ($n = 3$; Figure 1E). Interestingly, the effect of NaHS on I_{ami} was rapidly reversed after wash-out of the drug (Figure 1F, G). Values of I_{ami} before NaHS application ($8.33 \pm 1 \mu\text{A}\cdot\text{cm}^{-2}$) and after washout ($6.4 \pm 1.5 \mu\text{A}\cdot\text{cm}^{-2}$) were not significantly different ($n = 7$; $P = 0.1$).

In addition, the effect of NaHS on I_{ami} was also investigated on native tracheal preparations of pigs and mice in Ussing chambers (Figure 2). The administration of NaHS reversibly reduced I_{ami} of pig tracheae (Figure 2A, B) by $52 \pm 10\%$ ($n = 3$, $P < 0.01$). Similar results were obtained from recordings on mice tracheae (Figure 2C, D), where NaHS reversibly decreased I_{ami} by $66 \pm 3\%$ ($n = 3$, $P < 0.01$).

Effect of H_2S on cellular ATP levels

An important cellular target for H_2S is the mitochondrial respiratory chain. It has previously been demonstrated that H_2S can reversibly inhibit cytochrome c oxidase (Collman *et al.*, 2009), which eventually leads to metabolic depletion of cells. Because the activity of Na^+/K^+ -ATPase is tightly linked to cellular ATP levels, it may be speculated that H_2S inhibits I_{ami} indirectly by decreasing the concentration of cellular ATP. ATP concentrations of H441 monolayers, which were incubated with or without NaHS for 15 min, were therefore measured with a colorimetric approach. Control monolayers contained $18.35 \pm 0.68 \mu\text{M}$ ATP ($n = 5$), which was not significantly different from monolayers treated with 300 μM NaHS ($16.51 \pm 1.27 \mu\text{M}$ ATP; $n = 6$, one-way ANOVA) or 1 mM NaHS ($18.07 \pm 0.58 \mu\text{M}$ ATP; $n = 6$, one-way ANOVA). The mitochondrial uncoupler 2,4-DNP significantly decreased ATP levels to $13.42 \pm 0.78 \mu\text{M}$ ($n = 6$, $P < 0.05$, one-way ANOVA), which indicates that changes in ATP concentrations could be detected with the setup employed.

In general, transepithelial Na^+ transport is the result of the interplay of mainly two Na^+ -transporting molecules in epi-

Figure 1

Effects of NaHS on Na⁺ absorption by H441 monolayers. (A) Representative current trace. First, amiloride (10 μM, indicated with 'a') was applied to the apical bath to estimate baseline amiloride-sensitive Na⁺ currents. After wash-out of amiloride, NaHS (300 μM) was applied to the apical bath (blue bar), which resulted in a rapid current decrease. After 5 min, amiloride (10 μM) was applied. (B) Statistical analysis of experiments shown in (A). Depicted are amiloride-sensitive currents (I_{ami}) that were obtained from differences in current values before and after application of amiloride. (C) Transepithelial resistance (R_T) was not decreased by NaHS. (D) In the presence of amiloride, there was no effect of NaHS on short-circuit currents. (E) NaHS concentration-dependently decreased I_{ami} . Data were obtained from H441 monolayers exposed to different concentrations of NaHS ($n = 3$ for each concentration). The IC_{50} value was obtained from a sigmoidal concentration-response fit according to the Hill equation. (F) Reversibility of the NaHS effect. Experiments started analogous to those shown in (A). After removal of NaHS, currents nearly increased back to baseline levels. Amiloride was applied again and the I_{ami} values before and after NaHS were statistically compared (G). * $P < 0.05$; ** $P < 0.01$.

thelial cells: Na⁺ channels located at the apical membrane of the epithelial cells and the Na⁺/K⁺-ATPase located at the basolateral membrane. Inhibition of either transport system will decrease overall transepithelial Na⁺ transport. Therefore, experiments were performed to elucidate whether the observed inhibition of I_{ami} by NaHS was due to inhibition of amiloride-sensitive Na⁺ channels, such as ENaC, or inhibition of the Na⁺/K⁺-ATPase.

Effects of H₂S on amiloride-sensitive Na⁺ channels

To investigate if NaHS inhibits amiloride-sensitive Na⁺ channels, whole-cell patch-clamp experiments were performed on single H441 cells (Figure 3A, B). The application of amiloride (100 μM) decreased approximately 80% of the inward current at -60 mV holding potential. The amiloride-sensitive fraction

Figure 2

Effects of NaHS on Na^+ absorption by native tracheal preparations. (A) Original current trace of an Ussing chamber recording of native tracheal epithelium from pig. First, baseline amiloride-sensitive currents were estimated with amiloride ($10 \mu\text{M}$, a). After wash-out, NaHS ($300 \mu\text{M}$, apical side) was applied for 8 min, and then amiloride was applied. After 15 min of wash-out, amiloride was applied again. (B) Statistical analysis from experiments shown in (A). Depicted are normalized values of amiloride-sensitive currents (I_{ami}) that were obtained as ratios from values of I_{ami} with respect to baseline I_{ami} . NaHS significantly decreased I_{ami} ($n = 3$, $**P < 0.01$). Furthermore, after wash-out of NaHS, values of I_{ami} increased again and were not significantly different from baseline levels ($n = 3$, $P = 0.95$). (C, D) Similar experiments as shown in (A and B) on native tracheal preparations of mice ($n = 3$). NaHS also significantly reduced I_{ami} within 5 min ($n = 3$, $*P < 0.01$). After wash-out of NaHS, I_{ami} was not significantly different from baseline levels ($n = 3$, $P = 0.4$).

(I_{ami}), however, was not affected by the administration of NaHS (1 mM). Normalized values of I_{ami} were 0.81 ± 0.12 under control conditions, and 0.83 ± 0.17 after NaHS exposure ($n = 6$; $P = 0.92$). These data indicate that NaHS does not inhibit amiloride-sensitive Na^+ channels in H441 cells. To confirm this observation, TEVC recordings were performed on *Xenopus* oocytes, which expressed combinations of amiloride-sensitive ENaC subunits (Figure 3C, D). The classical ENaC consists of three subunits, α , β and γ , which probably assemble as a trimer in the plasma membrane (Jasti *et al.*, 2007). However, an additional subunit, δ , is expressed – at least at the mRNA level – in H441 cells (Wesch *et al.*, 2010), which can also form robust amiloride-sensitive Na^+ currents when co-expressed with β and γ (Wesch *et al.*, 2010). The administration of NaHS (1 mM), however, did not affect the I_{ami} of oocytes expressing either the amiloride-sensitive $\alpha\beta\gamma$ or $\delta\beta\gamma$ subunits of ENaC. Normalized values of I_{ami} for the $\alpha\beta\gamma$ ENaC expressing oocytes were 0.96 ± 0.05 under control conditions, and 0.88 ± 0.07 after NaHS exposure ($n = 8/9$; $P = 0.37$). Values for $\delta\beta\gamma$ ENaC expressing oocytes

were 1.0 ± 0.02 (control) and 1.07 ± 0.05 (NaHS; $n = 7$; $P = 0.25$).

Effects of H_2S on basolateral Na^+/K^+ -ATPase currents of H441 monolayers

As NaHS had no effect on amiloride-sensitive Na^+ channels, it was hypothesized that NaHS, instead, impairs the activity of the basolaterally located Na^+/K^+ -ATPase. Given that H_2S is membrane permeable (Mathai *et al.*, 2009), it is reasonable to assume apically applied H_2S has an effect on a basolateral enzyme. To test this hypothesis, the effect of NaHS on functionally isolated basolateral membranes of H441 monolayers was investigated in Ussing chambers (Figure 4). Firstly, amiloride was applied to the apical bath to block apical Na^+ channels. Then, nystatin ($75 \mu\text{M}$) was added to the apical bath to permeabilize the apical membrane. This resulted in an increase in I_{sc} , which is mainly generated by the Na^+/K^+ -ATPase (Woollhead *et al.*, 2007; Althaus *et al.*, 2009; 2010). When the current was stable, ouabain (1 mM), an inhibitor of the Na^+/K^+ -ATPase, was applied to the basolateral bath, which

Figure 3

Effects of NaHS on amiloride-sensitive sodium channels. (A) Original transmembrane current (I_M) trace from a whole-cell patch clamp recording of a single H441 cell. The holding potential was -60 mV. Amiloride-sensitive Na⁺ currents were estimated by the application of amiloride (100 μ M, indicated with 'a'). After wash-out of amiloride, NaHS (1 mM, blue bar) was applied. After 2 min of NaHS exposure, amiloride was applied. The transient current decrease upon NaHS application represents an artefact by the perfusion system, which was also present in control recordings without NaHS (data not shown). (B) Statistical evaluation of experiments shown in (A). Depicted are normalized amiloride-sensitive currents (normalized I_{ami}), which were obtained by the ratio of amiloride-sensitive currents after exposure, to either vehicle (control, recording not shown) or NaHS, to the baseline amiloride-sensitive current (estimated at the beginning of each experiment). (C) Representative current trace of a TEVC recording that investigated the effect of NaHS on heterologously expressed $\alpha\beta\gamma$ ENaCs in *Xenopus* oocytes. Experiments started with the perfusion of amiloride (10 μ M). After wash-out of amiloride, I_M increased, which represents ENaC activity. The application of NaHS (1 mM) had no effect on I_M . After exposure to NaHS, amiloride was applied again. (D) Statistical evaluation of experiments shown in (C). Shown are means of normalized I_{ami} . Normalization was as described in (B). There was no effect of NaHS on normalized I_{ami} of oocytes expressing either $\alpha\beta\gamma$ ENaC or $\delta\beta\gamma$ ENaC.

resulted in a large decrease in the I_{SC} (Figure 4A). The difference in I_{SC} values before and after addition of ouabain represents the activity of the Na⁺/K⁺-ATPase (I_{ouab}). I_{ouab} values were 13.8 ± 0.49 μ A $\cdot$ cm⁻² in control experiments, and 5.8 ± 0.58 μ A $\cdot$ cm⁻² in experiments where NaHS was applied before ouabain ($n = 5$, $P < 0.01$) (Figure 4B, C). This indicates that NaHS led to a decrease in Na⁺/K⁺-ATPase activity by approx. 60%.

Measurement of membrane abundance of the Na⁺/K⁺-ATPase

Changing the abundance of transporting molecules in the plasma membrane of epithelial cells is a general mechanism that regulates transepithelial ion transport processes. The observed inhibition of Na⁺/K⁺-ATPase currents in H441 monolayers might thus be due to NaHS-induced internalization of Na⁺/K⁺-ATPase molecules from the basolateral plasma membrane.

The abundance of Na⁺/K⁺-ATPase proteins in the basolateral membrane was investigated using a surface protein biotinylation approach followed by Western blotting (Figure 5A, B). There were no significant changes in membrane abundance of the Na⁺/K⁺-ATPase, even after a 60-min incubation with NaHS. This finding was confirmed by Ussing chamber studies with an endocytosis inhibitor (dynasore; Figure 5C). Pretreatment of monolayers with dynasore (70 μ M, 1 h incubation) did not significantly affect I_{NaHS} (6.0 ± 1.29 μ A $\cdot$ cm⁻²) compared with control experiments (DMSO-treated monolayers; 8.0 ± 2.12 μ A $\cdot$ cm⁻²; $n = 4$; $P = 0.46$).

Effects of H₂S on cytosolic Ca²⁺ concentrations and Na⁺/K⁺-ATPase activity

As there was no detectable change in the abundance of the Na⁺/K⁺-ATPase in the membrane, H₂S may instead affect Na⁺/K⁺-ATPase activity. In previous studies it has been demon-

Figure 4

Effects of NaHS on basolateral Na⁺/K⁺-ATPase activity in H441 monolayers. (A) Representative current trace of apically permeabilized H441 monolayers in Ussing chambers. Firstly, amiloride (10 μM) was applied to block apical ENaCs. Nystatin (75 μM, yellow bar) was subsequently applied to the apical bath. The resulting increase in short-circuit current (I_{sc}) represents the activity of the basolateral Na⁺/K⁺-ATPase. Na⁺/K⁺-ATPase activity was finally measured as I_{sc} values sensitive to the basolateral application of ouabain (1 mM), and denoted I_{ouab} . (B) Similar experiment to that shown in (A). After permeabilization with nystatin, NaHS (300 μM) was apically applied, which resulted in a current decrease. Note the current fraction, which is sensitive to ouabain, is decreased compared with the experiment as shown in (A). (C) Statistical analysis of experiments as shown in (A and B). I_{ouab} values without and with NaHS were compared. * $P < 0.01$.

strated that H₂S elicits Ca²⁺ signals (Lee *et al.*, 2006; Pouokam and Diener, 2011) that may activate downstream PKC and thereby decrease the activity of the Na⁺/K⁺-ATPase (Bertorello *et al.*, 1991).

Putative changes in cytosolic Ca²⁺ concentrations of H441 cells were measured using Fura-2 (Figure 6). The application of NaHS (1 mM) led to a slight and reversible increase in

Fura-2 ratios from 0.472 ± 0.007 to 0.557 ± 0.011 ($n = 40$; $P < 0.01$; Figure 6). CPA (10 μM), which was used as a positive control and inhibits sarcoplasmic reticulum Ca²⁺-ATPase, led to a strong increase in Fura-2 ratios from 0.47 ± 0.007 to 0.868 ± 0.041 ($n = 40$; $P < 0.01$; Figure 6).

As NaHS increased cytosolic Ca²⁺ concentrations, different inhibitors of Ca²⁺-dependent PKC were employed (Figure 7).

Figure 5

Effects of NaHS on the abundance of Na⁺/K⁺-ATPase in the membrane. (A) Western blot showing the abundance of Na⁺/K⁺-ATPase, in the basolateral membrane (basolateral) or in the non-bound predominantly intracellular protein fraction (intracellular), after different exposure times to NaHS (1 mM). As a control, the same blot was analysed for β actin, which should only be present in the intracellular fraction and not in the basolateral biotinylated fraction. (B) Graph of biotinylated Na⁺/K⁺-ATPase protein abundance by using integrative fluorescence analysis of Odyssey images from $n = 5$ independent samples. There was no significant difference between groups ($n = 5$; $P = 0.8991$, one-way ANOVA). (C) Data from Ussing chamber experiments that were performed with the endocytosis inhibitor dynasore. Monolayers were incubated with dynasore (70 μM , 1 h incubation) or DMSO (control) and the NaHS-induced current decrease (I_{NaHS}) was compared.

However, there were no significant changes in NaHS-induced currents when H441 monolayers were pretreated with Go6983 (5 μM ; Figure 7A–C), staurosporine (200 nM; Figure 7C), a myristoylated PKC peptide inhibitor (60 μM ; Figure 7D), or H-7 (20 μM ; Figure 7E). Further, the application of CPA (10 μM , apical application), which strongly increased cytosolic calcium concentrations in H441 cells (Figure 6), did not significantly change amiloride-sensitive Na⁺ currents (Figure 7F, G). Values of I_{ami} were $22.2 \pm 1.53 \mu A \cdot cm^{-2}$ before, and $22.7 \pm 1.67 \mu A \cdot cm^{-2}$ after administration of CPA ($n = 5$; $P = 0.41$; Figure 8F, G).

In addition, the activity of purified Na⁺/K⁺-ATPase (from porcine brain) was determined by measuring ouabain-sensitive production of inorganic phosphorus due to ATP hydrolysis. Under control conditions, purified Na⁺/K⁺-ATPase liberated $60.01 \pm 9.7 \mu M$ of phosphorus within 15 min at 37°C. This was not significantly different from the amount of phosphorus that was released in the presence of 1 mM NaHS ($59.67 \pm 1.98 \mu M$; $n = 5$, $P = 0.97$).

Modulation of basolateral K⁺ channels and its effects on transepithelial Na⁺ transport

Important regulators of transepithelial Na⁺ transport, including the Na⁺/K⁺-ATPase, are K⁺ channels, which are located in the basolateral membrane of epithelial cells. Basolateral K⁺ channels critically affect the membrane potential of the apical membrane and are important for the recycling of K⁺ ions, which are pumped into the cells by the Na⁺/K⁺-ATPase (Greenwood *et al.*, 2009). Thus, an inhibition of K⁺ channel activity will decrease transepithelial Na⁺ transport processes due to impairment of electrochemical gradients in the epithelial cells.

The activity of the Na⁺/K⁺-ATPase was measured by apical permeabilization exactly as described earlier (Figure 8). Under these conditions, basolateral application of the non-selective K⁺ channel inhibitor lidocaine (3 mM) decreased the ouabain-sensitive fraction of the I_{SC} , I_{ouab} , from $11.5 \pm 1.7 \mu A \cdot cm^{-2}$ to $6.8 \pm 1.2 \mu A \cdot cm^{-2}$ ($n = 5$, $P = 0.05$; Figure 8A, B). Additionally,

Figure 6

Effects of NaHS on cytosolic Ca^{2+} concentrations. Changes in cytosolic Ca^{2+} were measured with Fura-2. (A) Fura-2 ratios (340 nm/380 nm) of H441 cells. Depicted are the means $\pm$ SEM of $n = 40$ cells. NaHS (1 mM, blue bar) led to a reversible increase in Fura-2 ratios. CPA was used as a positive control. (B) Statistical analysis from data shown in (A). Depicted are Fura-2 ratios before NaHS application (pre NaHS), 15 min after NaHS (NaHS), 10 min after wash-out of NaHS (post NaHS), and 5 min after CPA-administration (CPA). ** $P < 0.01$.

inhibiting basolateral K^+ channels with lidocaine (3 mM) reduced amiloride-sensitive currents, and thus Na^+ transport of intact H441 monolayers from $12.0 \pm 1.4 \mu\text{A}\cdot\text{cm}^{-2}$ to $9.5 \pm 1 \mu\text{A}\cdot\text{cm}^{-2}$ ($n = 5$, $P < 0.05$).

Effects of H_2S on basolateral K^+ channels

Modulation of K^+ channel activity can indirectly affect Na^+ transport and Na^+/K^+ -ATPase currents in H441 cells, therefore, we investigated whether or not H_2S has an effect on K^+ channels in the basolateral membrane of H441 monolayers (Figure 8C–E). Monolayers were exposed to an apical to basolateral K^+ gradient. To exclude any contribution of the main Na^+ transporting molecules, amiloride and ouabain were also applied. Monolayers were apically permeabilized with nystatin, which resulted in a current increase from $7.0 \pm 1.2 \mu\text{A}\cdot\text{cm}^{-2}$ to $11.1 \pm 1.3 \mu\text{A}\cdot\text{cm}^{-2}$ ($n = 10$, $P < 0.005$). This current increase was sensitive to the non-specific K^+ channel blocker lidocaine (3 mM, Figure 8C). These conditions reflect the movement of K^+ ions via basolaterally located K^+ channels. The lidocaine-sensitive fraction of the I_{SC} (I_{lido}) under control conditions was $3.4 \pm 0.6 \mu\text{A}\cdot\text{cm}^{-2}$. NaHS significantly decreased I_{lido} to $0.4 \pm 0.4 \mu\text{A}\cdot\text{cm}^{-2}$ ($n = 5$, $P < 0.01$) (Figure 8D, E).

Additionally, the type of basolateral K^+ channels affected by H_2S was investigated and their contribution to the indirect inhibition of transepithelial Na^+ transport. Inhibitors of the three classes of K^+ channels were employed and their effects on I_{ami} investigated (Figure 9A). The basolateral application of glibenclamide (100 μM), a blocker of ATP-sensitive K^+ (K_{ATP}) channels, did not change I_{ami} of H441 monolayers. Normalized values of I_{ami} were 1 before and 1.06 ± 0.05 after application of glibenclamide ($n = 5$, $P = 0.25$, Figure 9A). By contrast, basolateral exposure to XE991 (100 μM), a blocker of voltage-gated K^+ (K_{V}) channels (Greenwood *et al.*, 2009), significantly decreased normalized I_{ami} from 1 to 0.73 ± 0.04

($n = 4$, $P < 0.01$; Figure 9A). Lastly, tetrapentylammonium (TPeA), a drug that preferentially inhibits calcium-dependent K^+ (K_{Ca}) channels dramatically decreased Na^+ transport when applied to the basolateral compartment (300 μM). Normalized values of I_{ami} significantly decreased from 1 to 0.43 ± 0.03 ($n = 5$, $P < 0.01$; Figure 9A). These data indicate that ~25% of the Na^+ current in H441 monolayers is maintained by active K_{V} channels and ~65% by active K_{Ca} channels, rather than K_{ATP} channels. Subsequently, the effects of H_2S on K_{V} and K_{Ca} channels were investigated. In the presence of the K_{V} inhibitor XE991 (100 μM , basolateral side), NaHS (300 μM , apical) still decreased I_{ami} to the same extent as in control recordings without XE991 (Figure 9B, C). The NaHS-induced currents, which were calculated as relative inhibition of I_{ami} (Figure 9C), were $44.65 \pm 3.98\%$ under control conditions and $44.3 \pm 1.94\%$ in the presence of XE991 ($n = 4$, $P = 0.94$; Figure 9C). By contrast, TPeA decreased I_{ami} to the same extent as NaHS (Figure 9D, E) and significantly reduced NaHS-induced currents from $51.01 \pm 2.32\%$ to $34.38 \pm 2.79\%$ ($n = 5$, $P < 0.01$; Figure 9F). These data indicate that H_2S preferentially inhibits K_{Ca} channels, and thus indirectly decreases amiloride-sensitive transepithelial Na^+ absorption.

Discussion and conclusions

The results presented here demonstrate that H_2S decreases transepithelial Na^+ absorption across human H441 airway epithelial cells (Figure 1) and native tracheal preparations of pigs and mice (Figure 2). The IC_{50} value for the donor molecule NaHS was determined as 146 μM , which will correspond to an IC_{50} of free H_2S of ~50 μM . The 'real' physiological concentration of H_2S is still debatable, because a variety of H_2S concentrations in tissues have been determined – ranging from <1 μM up to more than 100 μM (for

Figure 7

Effects of NaHS on Ca²⁺/protein kinase C signalling. (A, B) Representative current traces of H441 cells in Ussing chambers. Cells were incubated with solvent (DMSO), or the PKC inhibitor Go6983 (5 μM), 1 h before the start of experiments at 37°C. In Ussing chamber experiments, all drugs were also present in the apical and basolateral perfusion solutions. Note that Go6983 generally decreased short-circuit currents; however, the effect of NaHS (300 μM) was still present. 'a' = amiloride (10 μM). (C–E) Statistical analysis of experiments similar to those shown in (A). Depicted are NaHS-mediated currents (I_{NaHS}) relative to total amiloride-sensitive currents (I_{ami}). Go6983 (5 μM) and staurosporine (200 nM) did not significantly affect I_{NaHS} ($n = 5$, $P = 0.2955$, one-way ANOVA). Similarly, the PKC peptide inhibitor (60 μM , $n = 4$; $P = 0.42$) or H-7 (20 μM ; $n = 5$; $P = 0.79$) did not significantly affect I_{NaHS} . (F) The effect of CPA on ion currents of H441 monolayers in Ussing chambers. Firstly, amiloride (a) was applied to estimate baseline I_{ami} . After wash-out, CPA (10 μM) was applied to the apical compartment and after 10 min, I_{ami} was determined again. (G) There were not significant changes in I_{ami} induced by CPA ($n = 5$; $P = 0.41$).

Figure 8

Regulation of the Na^+/K^+ -ATPase by K^+ channels and effects of NaHS on basolateral K^+ channels in H441 cells. (A) Representative current trace of an experiment similar to those explained in Figure 4. Note that the basolateral application of lidocaine (3 mM) reduced ouabain-sensitive currents (I_{ouab}) on apically permeabilized H441 monolayers. (B) Statistical evaluation of experiments shown in (A). Basolateral application of lidocaine significantly decreased I_{ouab} compared with control experiments without lidocaine (current trace not shown). * $P < 0.05$. (C) H441 monolayers in Ussing chambers were apically bathed with a high K^+ solution containing amiloride (10 μM). The basolateral bath contained low K^+ and ouabain (1 mM). Monolayers were perfused for at least 10 min with these solutions (not shown). After plateau conditions had been reached, nystatin (75 μM) was apically applied to permeabilize the apical membrane. The resulting current increase was sensitive to the basolateral application of lidocaine (3 mM). (D) Similar experiment to those shown in (A). After permeabilization with nystatin, NaHS (300 μM) was applied to the apical bath. After plateau conditions had been reached, lidocaine (3 mM) was then applied to the basolateral bath. (E) Statistical analysis from experiments shown in (A and B). Depicted are lidocaine-sensitive currents (I_{lido}) and the NaHS-sensitive current (I_{NaHS}).

review see Olson, 2011). However, it has to be mentioned that most studies determined H_2S concentrations in plasma or whole-tissue homogenates. Even though the current view is that physiological concentrations of H_2S are in the nano-

molar to lower micromolar range (Olson, 2011), local H_2S concentrations might be higher – depending on the place and activity of H_2S -generating enzymes. Whether or not endogenously synthesized H_2S is a real physiological regulator of

Figure 9

Contribution of K_{ATP}, K_V and K_{Ca} channels to H₂S-mediated effects. (A) Effects of basolaterally applied K⁺ channel inhibitors on amiloride-sensitive currents (I_{ami}) of H441 monolayers in Ussing chambers. Depicted are normalized values of I_{ami} , which were obtained by normalization of I_{ami} with inhibitors (one inhibitor per monolayer) to baseline I_{ami} without inhibitors (normalized to '1', shown as dashed line). Glibenclamide (100 μ M, Glib.) did not affect I_{ami} ($n = 5$, $P = 0.25$). XE991 (100 μ M) and TPeA both significantly decreased I_{ami} ($n = 4$, and $n = 5$, respectively; $^{**}P < 0.01$). (B) Original current trace from H441 monolayers in Ussing chambers. The basolateral application of XE991 (100 μ M) decreased amiloride-sensitive currents (amiloride = a); however, there was still a prominent effect of NaHS (300 μ M, apical application). (C) Statistical analysis from experiments shown in (B) and from control recordings without XE991 (not shown). Depicted are NaHS-mediated currents (I_{NaHS}), which were normalized (as explained in Figure 7). XE991 had no significant effect on the I_{NaHS} ($n = 4$, $P = 0.94$). (D) Similar recordings to those shown in (B), with basolateral application of TPeA (300 μ M). (E) Statistical analysis of experiments shown in (D). Depicted are normalized values of I_{ami} , which were obtained by normalization of I_{ami} with TPeA, NaHS or a combination of both to baseline I_{ami} without drugs (normalized to '1', shown as dashed line). Statistics represent the comparison between normalized I_{ami} during drug exposure with respect to baseline normalized I_{ami} . Note that TPeA decreased I_{ami} to a similar extent as NaHS when applied without TPeA (original traces not shown); $^{**}P < 0.01$. (F) TPeA significantly decreased the NaHS-mediated currents (I_{NaHS} , $n = 5$, $^{**}P < 0.01$).

transepithelial Na^+ transport is an important question that needs to be addressed. We were able to detect mRNA transcripts for the H_2S -generating enzymes CBS and CSE in H441 cells (data not shown). However, administration of the CSE inhibitor β -cyanoalanine (500 μM apical), the CBS inhibitor amino-oxyacetate (1 mM, apical) or a combination of both, in concentrations that were demonstrated to inhibit CBS/CSE (Mok *et al.*, 2004; Hughes *et al.*, 2009), did not significantly increase amiloride-sensitive Na^+ absorption by H441 cells within 30 min (data not shown). This does not speak in favour of a tonic, inhibitory effect of H_2S that is produced directly in the epithelium. Nevertheless, H_2S production might be low under basal conditions or H_2S might originate from other lung compartments, such as the endothelium or even the plasma, and might thereby affect epithelial Na^+ transport. This important question warrants further investigations.

Amiloride-sensitive Na^+ channels, such as ENaC, were not directly affected by H_2S (Figure 3), whereas Na^+/K^+ -ATPase currents were decreased (Figure 4). This observation is in line with a recent study that described decreased Na^+/K^+ -ATPase currents due to NaHS in distal colonic epithelia (Pouokam and Diener, 2011). The Na^+/K^+ -ATPase creates the Na^+ gradient that drives the entry of Na^+ ions through Na^+ channels located at the apical membrane of epithelial cells. Thus, although in the present study, H_2S did not directly inhibit Na^+ channels, the inhibition of the Na^+/K^+ -ATPase by H_2S will indirectly decrease apical Na^+ uptake through those ion channels and decrease amiloride-sensitive transepithelial Na^+ transport (Figures 1 and 2).

The decreased Na^+/K^+ -ATPase currents in H441 cells were not the result of endocytosis of Na^+/K^+ -ATPase molecules from the plasma membrane, because the abundance of the Na^+/K^+ -ATPase in the membrane was unchanged (Figure 5).

The H_2S -induced inhibition of Na^+ transport by H441 cells was not accompanied by a decrease in cellular ATP, which might have represented a mechanism for an indirect Na^+/K^+ -ATPase inhibition and thus a decreased Na^+ transport. This finding is of particular importance because H_2S concentrations employed in the present study ($\sim 100 \mu\text{M}$ of free H_2S) are regarded to be in a range that might inhibit mitochondrial cytochrome c oxidase (Olson, 2011). However, the present results indicate that H_2S can impair Na^+/K^+ -ATPase activity independently of metabolic depletion and is thus a physiological, rather than a toxicological observation. This finding is in accordance with previous studies, which demonstrated that only a drastic depletion of ATP would alter Na^+/K^+ -ATPase activity (Boldyrev *et al.*, 1991; Woollhead *et al.*, 2005). Nevertheless, it remains possible that H_2S changes the ATP : AMP ratio, which will activate AMP-activated protein kinase (AMPK) and eventually inhibit the Na^+/K^+ -ATPase (Woollhead *et al.*, 2005). However, in H441 cells, AMPK inhibits the Na^+/K^+ -ATPase due to activation of PKC and endocytosis (data not shown). As both signalling pathways did not contribute to the H_2S effect (Figures 5 and 7), a contribution of AMPK to the H_2S induced Na^+/K^+ -ATPase inhibition is unlikely.

As the abundance of Na^+/K^+ -ATPase in the membrane was unaffected by H_2S , the decreased ouabain-sensitive current across the basolateral membrane is likely to be the result of decreased Na^+/K^+ -ATPase activity. Therefore, signalling path-

ways that lead to alterations in Na^+/K^+ -ATPase activity were investigated. H_2S elicited a small and reversible increase in cytosolic Ca^{2+} concentrations (Figure 6). This is in line with observations on rat colonic epithelia (Hennig and Diener, 2009). However, this increase in Ca^{2+} concentration did not account for the observed inhibition of Na^+ transport, because CPA had no effect on I_{ami} (Figure 7). Furthermore, the H_2S -mediated current inhibition was not affected by inhibitors of PKC or broad-spectrum kinase inhibitors (Figure 7). A putative direct interaction of H_2S with the Na^+/K^+ -ATPase, as indicated by measurement of ATP hydrolysis *in vitro*, was also not observed.

The present results show that H_2S blocked K^+ channels located at the basolateral membrane of the epithelial cells (Figure 8). Due to their effect on electrochemical gradients, K^+ channels are important regulators of transepithelial Na^+ fluxes (Greenwood *et al.*, 2009). A block of basolateral K^+ conductance will eventually depolarize the apical membrane potential. This will result in a decreased electrical driving-force for Na^+ entry through apical ion channels. Furthermore, there is a coupling between the Na^+/K^+ -ATPase and K^+ channels, which recycle the K^+ ions that enter the cells due to Na^+/K^+ -ATPase activity. It has been demonstrated that the activity of the Na^+/K^+ -ATPase is coupled to K^+ flux at the basolateral membrane (Beck *et al.*, 1994; Maurer *et al.*, 1998). This coupling might prevent loss of K^+ , when Na^+/K^+ -ATPase activity is low. *Vice versa*, it is hypothesized that inhibition of K^+ channels also decreases Na^+/K^+ -ATPase activity to prevent a K^+ loading of cells. This possibility is supported by the finding that the block of basolateral K^+ channels (with lidocaine, Inglis *et al.*, 2007) rapidly inhibits ouabain-sensitive currents of apically permeabilized H441 monolayers (Figure 8). Both events, depolarizing the apical membrane as well as indirectly impairing Na^+/K^+ -ATPase activity, will result in inhibition of transepithelial Na^+ transport.

In accordance with this assumption, a block of basolateral K^+ channels decreased Na^+ absorption across intact H441 monolayers (Figure 9). K_{ATP} channels were not active in the basolateral membrane of H441 monolayers, because the inhibitor glibenclamide was ineffective. Thus, K_{ATP} channels, which were shown to be sensitive to H_2S (Tang *et al.*, 2010), cannot account for the H_2S -mediated effects. Experiments with XE991 and TPcA (inhibitors of K_{V} and K_{Ca} channels, respectively) revealed that Na^+ transport across H441 cells is maintained by active K_{V} and K_{Ca} channels. The effect of H_2S was not sensitive to XE991, however, the inhibition of K_{Ca} channels mimicked and decreased the effect of H_2S on Na^+ transport (Figure 9). This finding is in accordance to studies by Kemp and colleagues, who demonstrated inhibition of human BK_{Ca} channels by H_2S (Telezhkin *et al.*, 2009). Although the precise mechanism of H_2S 's action on these channels is unknown, the effects of H_2S on BK_{Ca} channels were observed in cell-free membrane patches (Telezhkin *et al.*, 2010), and are probably independent of soluble signalling mediators. There is recent evidence that H_2S is able to convert SH-groups of proteins to SSH-groups, a mechanism that has been termed S-sulphydration (Mustafa *et al.*, 2009). S-sulphydration has been described for intermediate and short conductance K_{Ca} channels (Mustafa *et al.*, 2009). Hence, we speculate that the observed inhibition of K_{Ca} channels, due to H_2S , is the result of S-sulphydration.

Taken together, the data presented in this study indicate that H₂S blocks K_{Ca} channels located in the basolateral membrane and indirectly impairs transepithelial Na⁺ absorption by possibly altering the apical membrane potential, as well as Na⁺/K⁺-ATPase activity.

The finding that H₂S decreased pulmonary Na⁺ absorption has important pharmacological implications. In the distal lung, decreased Na⁺ absorption across the alveolar epithelium leads to decreased alveolar fluid clearance and the promotion of oedema formation. Interestingly, a characteristic of patients with H₂S poisoning is the development of pulmonary oedema, which was also observed in patients after prolonged exposure to low concentrations of H₂S (Cordasco and Stone, 1973). The results presented herein have provided a novel molecular mechanism for this observation.

Furthermore, H₂S is, due to its anti-inflammatory properties, suggested as a potential therapeutic option for the treatment of inflammatory lung diseases, including ALI (Esechie *et al.*, 2009; Otulakowski and Kavanagh, 2010). Although H₂S gas is unlikely to become a therapeutic tool, there are various H₂S-donating molecules (Olson, 2011) that would allow the pharmacological administration of H₂S. However, aside from inflammation, the development of pulmonary oedema is a hallmark of patients with ALI, or the more severe acute respiratory distress syndrome (ARDS; Ware and Matthay, 2000). Oedema occurrence in ALI/ARDS patients is *inter alia* associated with an impaired alveolar fluid clearance (Ware and Matthay, 2001). Impairment of transepithelial Na⁺ transport by H₂S is an important observation that needs to be taken into consideration when considering H₂S as a therapeutic option in ALI/ARDS (Otulakowski and Kavanagh, 2010), even when evaluating H₂S concentrations that do not lead to metabolic depletion.

Apart from the potential deleterious effects of H₂S with respect to oedema formation, H₂S might, by contrast, represent an interesting pharmacological target in situations where a hyperactive Na⁺ transport is present. In airway epithelia, increased Na⁺ absorption can promote cystic fibrosis-like lung disease (Mall *et al.*, 2004). In the kidneys, a hereditary form of hypertension (Liddle syndrome) is reasoned by an ENaC mutation that leads to Na⁺ hyperabsorption from the primary urine and consequently increases blood volume and pressure (Firsov *et al.*, 1996; Kellenberger and Schild, 2002). In both cases, blocking Na⁺ transport by increasing endogenous H₂S production might provide a new pharmacological approach.

In conclusion, the data presented here suggest that the gasotransmitter H₂S interferes with basolateral K⁺ channels in lung epithelial cells, and thereby indirectly impairs transepithelial Na⁺ transport. H₂S might therefore be a novel regulator of pulmonary Na⁺ absorption, and its signalling mechanisms might represent novel pharmacological targets for the investigation of drugs that regulate pulmonary Na⁺ and water homeostasis.

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Conflict of interest

The authors declare no conflict of interest.

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