Hydrogen sulfide stimulates CFTR in *Xenopus* oocytes by activation of the cAMP/PKA signalling axis

Alexander Perniss1,2, Kathrin Preiss1, Marcel Nier1 & Mike Althaus1,3

Hydrogen sulfide (H2S) has been recognized as a signalling molecule which affects the activity of ion channels and transporters in epithelial cells. The cystic fibrosis transmembrane conductance regulator (CFTR) is an epithelial anion channel and a key regulator of electrolyte and fluid homeostasis. In this study, we investigated the regulation of CFTR by H2S. Human CFTR was heterologously expressed in *Xenopus* oocytes and its activity was electrophysiologically measured by microelectrode recordings. The H2S-forming sulphur salt Na2S as well as the slow-releasing H2S-liberating compound GYY4137 increased transmembrane currents of CFTR-expressing oocytes. Na2S had no effect on native, non-injected oocytes. The effect of Na2S was blocked by the CFTR inhibitor CFTR_inh172, the adenylyl cyclase inhibitor MDL 12330A, and the protein kinase A antagonist cAMPS-Rp. Na2S potentiated CFTR stimulation by forskolin, but not that by IBMX. Na2S enhanced CFTR stimulation by membrane-permeable 8Br-cAMP under inhibition of adenylyl cyclase-mediated cAMP production by MDL 12330A. These data indicate that H2S activates CFTR in *Xenopus* oocytes by inhibiting phosphodiesterase activity and subsequent stimulation of CFTR by cAMP-dependent protein kinase A. In epithelia, an increased CFTR activity may correspond to a pro-secretory response to H2S which may be endogenously produced by the epithelium or H2S-generating microflora.

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride and bicarbonate conducting anion channel which is found in many vertebrate epithelia and essential for the epithelial regulation of electrolyte and fluid homeostasis. Gene mutations in CFTR cause cystic fibrosis, the most common autosomal-recessive disorder in Caucasians, with a disease incidence of around 1 in 1000–3000 in northern Europeans1. The CFTR protein contains two membrane-spanning regions (each consisting of six transmembrane domains) functioning as the channel pore, which are connected to two intracellular nucleotide binding domains (NBD1 and NBD2) as well as a regulatory (R) domain2. NBD1 and NBD2 regulate the opening and closing of the channel by binding and hydrolysing ATP23, whereas the R-domain initiates the transitions in channel conformation by protein kinase A (PKA)-dependent phosphorylation2. Although the NBDs and R-domain contain various phosphorylation sites which control biogenesis, trafficking, interaction with other proteins and channel open probability2, PKA is the primary regulator of CFTR activity. At the cellular level, CFTR is hence mainly regulated by cAMP-coupled signalling events.

CFTR is located in the apical membrane of epithelial cells, where it primarily represents a conductance for chloride ions and facilitates transepithelial movement of chloride4. CFTR allows chloride permeation in and out of the cells and the direction of chloride flux is determined by the gradient for chloride ions between the cytoplasm and luminal extracellular fluid, as well as the apical membrane potential of the epithelial cells5. Whereas CFTR mediates chloride secretion in the intestine, pancreas, secretory coils of sweat glands or serous cells of airway submucosal glands6,7, it absorbs chloride in the ducts of sweat glands8 or, as recently suggested, in the airway surface epithelium9. Its physiological importance is also reflected in the consequence of CFTR malfunction and cystic fibrosis phenotype which includes pancreas insufficiency, airway mucus obstruction, meconium ileus and high sweat chloride concentrations1. Under physiological conditions, multiple cellular signalling cascades regulate the activity or membrane abundance of CFTR10, thus allowing for a precise regulation of chloride flux and, eventually, electrolyte and fluid homeostasis.

1Institute for Animal Physiology, Justus-Liebig-University, Giessen, Germany. 2Present address: Institute for Anatomy and Cell Biology, Justus-Liebig-University, Giessen, Germany. 3School of Biology, Newcastle University, Newcastle upon Tyne, United Kingdom. Correspondence and requests for materials should be addressed to M.A. (email: Mike.Althaus@newcastle.ac.uk)
Hydrogen sulfide (H₂S) is a well-known environmental chemical threat with a characteristic odour of rotten eggs; however, research over the past decade has revealed that H₂S is also an important cellular signalling molecule. H₂S is involved in a variety of physiological and patho-physiological processes (for review see ref.13), and H₂S-liberating compounds are currently explored for a therapeutic potential13. Physiological concentrations of H₂S are likely in the nano- to low micro-molar range and depend on its production, intracellular storage and mitochondrial degradation14. H₂S is enzymatically generated within the metabolism of L-cysteine by cystathionine-γ-lyase, cystathionine-β-synthase or 3-mercaptopyruvate sulfurtransferase. Enzymatically generated H₂S can either be stored as sulfane sulfur (oxidative formation of protein polysulfides)12,13, or degraded by the sulfide oxidation pathway in mitochondria14.

In this study we investigated the regulation of CFTR by H₂S. Using heterologous expression of human CFTR in Xenopus oocytes, we provide evidence that low-micromolar H₂S concentrations decrease phosphodiesterase activity. This activates the cAMP/PKA signalling axis and triggers activation of CFTR.

Results

Hydrogen sulfide stimulates CFTR in Xenopus oocytes. Human CFTR was heterologously expressed in Xenopus laevis oocytes and channel activity was measured electrophysiologically by two-electrode voltage-clamp (TEVC) microelectrode recordings. In order to investigate the potential influence of H₂S on heterologously expressed CFTR, the H₂S-forming sulphur salt Na₂S was employed (Fig. 1). The application of 50 µM Na₂S to CFTR-expressing oocytes significantly increased transmembrane currents from −0.059 ± 0.015 µA to −0.395 ± 0.11 µA (n = 6; N = 3; P = 0.0313; Fig. 1a,b). This effect was transient, as transmembrane currents began to decline in the presence of Na₂S. Furthermore, the effect was fully reversible upon removal of Na₂S. In order to confirm expression of CFTR, the cAMP-elevating compounds forskolin (5 µM) and IBMX (100 µM) were applied to the oocyte’s superfusate with forskolin/IBMX elicited a significant increase in transmembrane current from −0.076 ± 0.015 µA to −1.055 ± 0.186 µA (n = 6; N = 3; P = 0.0030; Fig. 1a,b). Native oocytes which did not express CFTR did not respond to forskolin/IBMX (Fig. 1c). Transmembrane currents of these oocytes were −0.054 ± 0.026 µA before, and −0.056 ± 0.028 µA after application of the drugs (n = 8; N = 3; P = 0.7525).

In sum, these data indicate that H₂S activates human CFTR which is heterologously expressed in Xenopus oocytes.
Figure 1. Hydrogen sulfide stimulates CFTR in Xenopus oocytes. (a) A representative current trace of a TEVC recording of a CFTR-expressing oocyte. The application of Na₂S (50µM, black bar) as well as forskolin (fsk.; 5µM) and IBMX (100µM; grey bars) led to an increase in transmembrane current signals (I_{m}). (b) Statistical analysis of data obtained from experiments as shown in panel a. Depicted are values of I_{m} (before drug application or peak values after drug application) from individual experiments (grey symbols) as well as means ± SEM (*P ≤ 0.05, Wilcoxon signed rank test; **P ≤ 0.01, Student’s paired t-test). (c) Summarised data from experiments as similar to those shown in panels a and b, using native, non-CFTR-expressing oocytes. Values of I_{m} were taken at time point where CFTR-expressing oocytes of the same donor had the maximal response to the drugs. Depicted are means ± SEM (**P ≤ 0.01, Student’s paired t-test). (d) A representative current trace of a TEVC recording of a CFTR-expressing oocyte. After application of Na₂S (50µM, black bar), the CFTR inhibitor CFTR_inh172 (CFTR_inh.; 25µM) was additionally applied. This readily inhibited values of I_{m}. (e) Statistical analysis of data obtained from experiments as shown in panel d. Depicted are values of I_{m} (before drug application or peak values after drug application) from individual experiments (grey symbols) as well as means ± SEM (**P ≤ 0.01, Wilcoxon signed rank test). (f) Evaporative loss of H₂S was measured by monitoring the concentration of H₂S in the employed buffers solutions by the formation of methylene blue. Depicted are values for methylene blue absorbance at 670 nm over time. Na₂S (50µM) exposure is indicated by the black bar. (g) A representative current trace of a TEVC recording of a CFTR-expressing oocyte. Both, GYY4137 (500µM, grey bar) as well as Na₂S (50µM, black bar) stimulated I_{m}. (h) Statistical analysis of data obtained from experiments as shown in panel g. Depicted are values of I_{m} (peak values after drug application) from individual experiments (grey symbols) as well as means ± SEM (*P ≤ 0.05, Wilcoxon signed rank test). Numbers of experiments (n) are indicated in parentheses.
The application of forskolin/IBMX elicited a significant and transient increase in transmembrane current (Fig. 2a) from \(-0.064 \pm 0.009\mu A\) to \(-1.364 \pm 0.284\mu A\) (n = 11; N = 5; P = 0.0009). This effect was fully reversible upon removal of the drugs. A second application of forskolin/IBMX again stimulated CFTR activity from \(-0.081 \pm 0.022\mu A\) to \(-0.604 \pm 0.114\mu A\) (n = 11; N = 5; P = 0.0038; Fig. 2a). The second effect of forskolin/IBMX was normalised to the effect of the first forskolin/IBMX application and defined as ‘normalised CFTR activity’. Without any additional treatment, control oocytes had thus a normalised CFTR activity of \(0.42 \pm 0.04\) (n = 11; N = 5). We then applied increasing concentrations of Na₂S after the first application of forskolin/IBMX (Fig. 2a). Interestingly, 50 µM Na₂S which elicited robust currents in previous experiments (Fig. 1) did not significantly stimulate transmembrane currents after the oocytes had been exposed to forskolin/IBMX (Fig. 2a,c). Only a high dose of 300 µM Na₂S triggered a small increase in transmembrane currents from \(-0.033 \pm 0.010\mu A\) to \(-0.051 \pm 0.009\mu A\) (n = 8; N = 3; P = 0.0423; Fig. 2a,c). However, despite the lack of an effect of Na₂S after previous exposure of the oocytes to forskolin/IBMX, Na₂S enhanced the second effect of forskolin/IBMX. Normalised CFTR activity dose-dependently increased due to application of Na₂S (Fig. 2a,d). This effect was inhibited by 25 µM of CFTR_inh.172 (Fig. 2b,d). Furthermore, there was only a minor current activation due to 50 µM Na₂S and forskolin/IBMX in native, non-CFTR expressing oocytes (Fig. 1c). These data suggest that Na₂S potentiates CFTR-activity which was elicited by forskolin/IBMX.

To investigate if the Na₂S-induced stimulation of CFTR involves AC and PKA, specific inhibitors of these enzymes were employed and Na₂S-induced currents (I_{Na2S}) were estimated with or without these drugs (Fig. 3). Na₂S (50 µM) was applied twice to CFTR-expressing oocyte in order to control for a potential desensitisation in response to repetitive Na₂S-exposure (Fig. 3a,b). The first I_{Na2S} was 0.321 ± 0.108 µA and not significantly different from the second I_{Na2S} which was 0.312 ± 0.087 µA (Fig. 3b; n = 6; N = 5; P = 0.854).

MDL 12330A was used as an inhibitor of AC. In control experiments, Na₂S (50 µM) was applied to CFTR-expressing oocytes, which led to an I_{Na2S} of 0.130 ± 0.040 µA (n = 6; N = 2). Afterwards, the oocytes were perfused with DMSO (0.1%; the solvent for MDL 12330A) and stimulated again with 50 µM Na₂S. This resulted in an I_{Na2S} of 0.085 ± 0.019 µA (n = 6; N = 2), which was not significantly different (P = 0.1395) from the first I_{Na2S} (Fig. 3c,d). By contrast, when MDL 12330A was applied, I_{Na2S} significantly decreased from 0.133 ± 0.035 µA to 0.011 ± 0.003 µA (n = 8; N = 2; P = 0.0056; Fig. 3c,d).

A similar experiment was performed with cAMPS-Rp – a PKA antagonist – which was directly injected into the oocytes during experiments. For control experiments, CFTR-expressing oocytes were stimulated with Na₂S (50 µM). Subsequently, 9.2 nl of an intracellular-analogous solution (IAS) was injected into the oocytes and the cells were stimulated a second time with Na₂S. This manoeuvre increased (although values did not reach statistical significance) I_{Na2S} from 0.255 ± 0.046 µA to 0.484 ± 0.126 µA (n = 9; N = 2; P = 0.0743; Fig. 3e,f). A similar observation has been reported in a previous study25, where a volume-increase in oocytes increased the activation of CFTR by cAMP-elevating compounds. By contrast, injection of the PKA antagonist cAMPS-Rp abrogated the second effect of Na₂S. Values of I_{Na2S} significantly decreased from 0.248 ± 0.061 µA to 0.011 ± 0.006 µA (n = 9; N = 2; P = 0.0039; Fig. 3e,f). Taken together, these data show that H₂S activates CFTR by cAMP- and PKA-mediated signalling in Xenopus oocytes.

**Hydrogen sulphide targets phosphodiesterase rather than adenylly cyclase.** An increase in intracellular cAMP concentrations could either be the result of enhanced cAMP production by AC, or inhibition of cAMP degradation by phosphodiesterase (PDE). H₂S might thus stimulate AC or inhibit PDE – both effects would result in accumulation of cAMP, a downstream activation of PKA and subsequent stimulation of CFTR. In order to discriminate between AC- and PDE-mediated contributions to CFTR activation, CFTR was stimulated with maximal effective concentrations of either forskolin (AC agonist) or IBMX (PDE inhibitor). If H₂S potentiated the effect of forskolin, but not that of IBMX, H₂S likely prevents cAMP degradation by PDE. If H₂S potentiated the effect of IBMX, but not that of forskolin, H₂S likely mediates forskolin stimulation by AC.

First, we investigated if H₂S affects the effect of forskolin alone (Fig. 4a). Since we were not able to additionally stimulate CFTR activity by increasing the forskolin concentration to 30 µM (data not shown), we considered the employed concentration of 5 µM as maximally effective, an observation which is consistent with a reported EC_{50} value of ~0.07 µM for forskolin in airway epithelia24. In control experiments, forskolin (5 µM) was applied to CFTR-expressing oocytes, which stimulated I_{forskolin} by 0.324 ± 0.056 µA (n = 13; N = 3). After wash-out, the oocytes were stimulated again with 5 µM forskolin. This resulted in a second stimulation of I_{forskolin} by 0.363 ± 0.068 µA (n = 13; N = 3; Fig. 4a). The second effect of forskolin was normalised to the effect of the first forskolin application and defined as ‘normalised forskolin effect’ (Fig. 4b). Under these control conditions, the normalised forskolin effect was 1.135 ± 0.146 (n = 13; N = 2). By contrast, oocytes which were treated with 50 µM Na₂S (together with the second application of forskolin) had a significantly enhanced normalised forskolin effect of 3.054 ± 0.405 (n = 13, N = 3, P < 0.0001, Gaussian approximation; Fig. 4a,b).

An identical protocol was employed with a high concentration of the PDE inhibitor IBMX (1 mM) and a ‘normalised IBMX effect’ was estimated (Fig. 4c,d). The normalised IBMX effect was 0.714 ± 0.080 (n = 10; N = 3) under control conditions, and not significantly different from that which was estimated in the presence of Na₂S which was 0.966 ± 0.178 (n = 10; N = 3; P = 0.2208). Na₂S thus enhanced the effect of the AC-agonist forskolin, but not that of the PDE-inhibitor IBMX. H₂S might therefore impair PDE-mediated cAMP degradation rather than AC-mediated cAMP production.

In order to confirm these observations a different strategy was employed (Fig. 4e–g). AC-mediated cAMP-production was blocked by application of the AC inhibitor MDL 12330A (20 µM) to CFTR-expressing oocytes. Subsequently, 100 µM of membrane-permeable 8-Br-cAMP was applied. This stimulated an increase in transmembrane current (I_{AAMP}) of 0.056 ± 0.0221 µA (n = 7; N = 4). After washout of all drugs, MDL 12330A (20 µM) was applied again and 50 µM Na₂S was added. Afterwards, 8Br-cAMP was additionally applied and I_{AAMP} significantly increased to 0.590 ± 0.154 µA (n = 7; N = 4; P = 0.0124; Fig. 4e,g). By contrast, there was no
difference between the first and second $I_{\text{AMP}}$ ($0.091 \pm 0.026 \mu A$ and $0.064 \pm 0.016 \mu A$; n = 5; N = 2; P = 0.1561) when the procedure was repeated without Na$_2$S (Fig. 4f,g). These data indicate that H$_2$S enhances the efficacy of 8-Br-cAMP.

In sum, these data provide evidence that H$_2$S inhibits endogenous PDE in *Xenopus* oocytes. This results in cAMP-mediated stimulation of CFTR-activity via downstream activation by PKA.

### Discussion

In this study we investigated the regulation of CFTR by H$_2$S. Previous studies using the mouse hippocampal cell line HT22 or rat vaginal epithelial preparations suggested that CFTR might be a target for H$_2$S. In order to elaborate on this hypothesis, human CFTR was heterologously expressed in *Xenopus* oocytes and functional CFTR expression was confirmed by application of the cAMP-elevating compounds forskolin and IBMX, which resulted in a transient increase in transmembrane currents which did not occur in native, non-injected oocytes. These observations are consistent with previously published functional electrophysiological analyses of human CFTR in *Xenopus* oocytes. The H$_2$S-liberating sulphur salt Na$_2$S elicited a transient current stimulation of CFTR-expressing oocytes which was readily inhibited by the CFTR inhibitor CFTR$_{\text{inh172}}$ and did not occur in native oocytes. Furthermore, the slow-releasing H$_2$S-liberating molecule GYY4137 also stimulated transmembrane currents in *Xenopus* oocytes. These data indicate that H$_2$S, released from Na$_2$S or GYY4137, stimulates CFTR activity.

We then elaborated on the signalling mechanisms which mediate the H$_2$S-induced activation of CFTR. We first stimulated CFTR-expressing oocytes with forskolin/IBMX and after removal of these drugs, cells were exposed to H$_2$S. Interestingly, a direct response to H$_2$S only occurred when high concentrations of Na$_2$S were employed. When forskolin/IBMX were applied twice to CFTR-expressing oocytes, the second activation by forskolin/IBMX was ~60%
Figure 3. H₂S stimulates CFTR via cAMP- and PKA-mediated signalling. (a) Representative current trace of a TEVC recordings of a CFTR-expressing oocyte. Transmembrane currents (I_m) were recorded and the oocyte was exposed twice to Na₂S (50 µM, black bar). (b) Statistical analysis of data obtained from experiments as shown in panel a. Depicted are values of the first (1) and second (2) Na₂S-induced current (I_{Na2S}) from individual experiments (grey symbols) as well as means ± SEM (n.s. = not significant, Student’s paired t-test). I_{Na2S} was calculated by subtracting the current before application of Na₂S from the peak value after application of Na₂S, resulting in positive values for I_{Na2S}. (c) Representative current traces of TEVC recordings of CFTR-expressing oocytes. Transmembrane currents (I_m) were recorded and oocytes were exposed twice to Na₂S (50 µM, black bar) DMSO (0.1%; left trace) or the AC inhibitor MDL 12330 A (MDL, 20 µM; right trace) were applied between the first and second stimulation with Na₂S (black arrowheads). (d) Statistical analysis of data obtained from experiments as shown in panel a. Depicted are values of the first (1) and second (2) Na₂S-induced current (I_{Na2S}) from individual experiments (grey symbols) as well as means ± SEM (**P ≤ 0.01, Student’s paired t-test). I_{Na2S} was calculated by subtracting the current before application of Na₂S from the peak value after application of Na₂S, resulting in positive values for I_{Na2S}. (e) Representative current traces of TEVC recordings of CFTR-expressing oocytes. Transmembrane currents (I_m) were recorded and oocytes were exposed twice to Na₂S (50 µM, black bar). The perfusion recording was stopped briefly between the first and second stimulation with Na₂S (grey lines). Then, an intracellular-analogue solution (IAS) or IAS containing the PK-antagonist cAMPS-Rp (87 µM) were injected into the oocytes before the second stimulation with Na₂S (black arrowheads). (f) Statistical analysis of data obtained from experiments as shown in panel a. Depicted are values of the first (1) and second (2) Na₂S-induced current (I_{Na2S}) from individual experiments (grey symbols) as well as means ± SEM (**P ≤ 0.01, Wilcoxon signed rank test). Numbers of experiments (n) are indicated in parentheses.
Figure 4. H₂S targets phosphodiesterase rather than adenylyl cyclase. (a) Representative current traces of TEVC recordings of CFTR-expressing oocytes. Transmembrane currents (Iₘ) were recorded and oocytes were either exposed twice to forskolin (fsk., 5µM; black bars; left trace) or first to forskolin and then to a combination of forskolin and 50µM Na₂S (grey bar; right trace). (b) Statistical analysis of data obtained from experiments as shown in panel a. Depicted are normalised values from individual experiments (grey symbols) as well as means ± SEM of forskolin (fsk.) effects. This represents the ratio of the first and second current stimulated by forskolin (**P ≤ 0.001, Mann-Whitney test). (c) Representative current traces of TEVC recordings of CFTR-expressing oocytes. Oocytes were either exposed twice to IBMX (1 mM; black bars; left trace) or first to IBMX and then to a combination of IBMX and 50µM Na₂S (grey bar; right trace). (d) Statistical analysis of data obtained from experiments as shown in panel c. Depicted are normalised values from individual experiments (grey symbols) as well as means ± SEM of IBMX effects. This represents the ratio of the first and second current stimulated by IBMX (Student’s unpaired t-test with Welch’s correction). (e, f) Representative current traces of TEVC recordings of CFTR-expressing oocytes. (f) Oocytes were exposed twice to membrane-permeable 8Br-cAMP (100µM; grey bars) in the presence of the AC-inhibitor MDL 12330 A (MDL, 20µM; black bars). The perfusion was stopped (indicated by the number symbol’) when 8-Br-cAMP was in the perfusion chambers in order to avoid massive consumption of this compound. Perfusion was started again at the time of drug removal. (e) The same protocol was employed, with the exception that Na₂S (50µM, black arrowhead) was applied before the second exposure to 8Br-cAMP. (g) Statistical analysis of data obtained from experiments as shown in panels e and f. Depicted are values of the first (1) and second (2) 8-Br-cAMP-induced current (Iₖ) from individual experiments (grey symbols) as well as means ± SEM (**P ≤ 0.01, Student’s paired t-test). Numbers of experiments (n) are indicated in parentheses.
These inconsistent findings suggest that the net-effect of H\(_2\)S on cAMP signalling depends not only on whether AC or PDE is targeted by H\(_2\)S, but also on the precise molecular repertoire of the PDE protein family which can generate nearly 100 different subtypes. The specific isoform expression in a cell might critically determine whether H\(_2\)S activates or inhibits cAMP-signalling. This is especially important, since H\(_2\)S will diffuse across cell membranes in an unspecific manner and hence specificity is likely not achieved by membrane receptors but possibly by the subtype repertoire of intracellular signalling molecules.

In epithelia, H\(_2\)S exerts pro-secretory or anti-absorptive effects and we recently suggested a concept by which epithelia use their electrolyte and liquid transport machinery as a defence mechanism in order to flush potential sources for harmful amounts of H\(_2\)S from the epithelial surfaces. The herein reported cAMP-mediated activation of CFTR would be consistent with a pro-secretory action on chloride-secreting epithelia. A recent study demonstrated a CFTR-mediated chloride secretion across rat vaginal epithelial preparations. The authors speculated that this might be due to an increase in cAMP and our findings would support this hypothesis. Since H\(_2\)S is not directly targeting CFTR, the PDE repertoire of epithelial cells will determine whether or not H\(_2\)S triggers CFTR-mediated electrolyte secretion.
Aside from the pro-secretory effects, H$_2$S prevents liquid absorption by sodium-transporting epithelia$^{48-50}$. In these epithelia, cAMP/PKA signalling stimulates sodium absorption by an increase in the membrane abundance of sodium transporting molecules such as the epithelial sodium channel (ENaC)$^{1,12}$. Interestingly, we recently showed that H$_2$S prevents this cAMP-mediated increase in ENaC abundance in sodium-absorbing lung epithelial cells by yet unidentified mechanisms$^{35}$. Furthermore, H$_2$S did not enhance cAMP-mediated chloride secretion in primarily sodium absorbing pig airway surface epithelia (data not shown). Nevertheless, this illustrates that – depending on the specific enzymatic repertoire of the CAMP signalling axis – H$_2$S might trigger cAMP-mediated electrolyte secretion in a fraction of epithelial cells, whilst simultaneously preventing enhanced electrolyte absorption in other cells. The herein reported data thus provide a step further in understanding the mechanisms of how H$_2$S elicits a switch from absorptive to secretory electrolyte and water transport in epithelia.

Methods

Isolation of *Xenopus laevis* oocytes. All animal experiments were performed in accordance with the German animal welfare law and had been declared to the Animal Welfare Officer of the University (Registration No.: M_478 and M_549). The animal housing facility was licensed by the local authorities (AZ: FD 62 - §11 ILU Tierphysiologie). The methods used to euthanize the animals humanely were consistent with the recommendations of the AVMA Guidelines for the Euthanasia of Animals. All procedures and experimental protocols were approved by the Animal Welfare Officer of the University as well as the regional council of Giessen (Registration No.: M_478 and M_549).

Oocytes of stages V/VI were isolated from freshly dissected *Xenopus laevis* ovaries and defolliculated exactly as previously described$^{33}$. Isolated oocytes were stored at 16 °C in an oocyte culture solution containing 90 mM NaCl, 1 mM KCl, 2 mM CaCl$_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 mM sodium pyruvate, 0.06 mM penicillin G and 0.02 mM streptomycin sulfate at pH 7.4.

CFTR-cRNA synthesis and injection into oocytes. The plasmid construct for human CFTR (in pGEM-HE) was a kind gift from Professor Blanche Schwappach (University of Göttingen, Germany). Plasmids were linearised with Mlu1 (Promega, Mannheim, Germany) and subsequently in vitro transcribed with the Ribomax MAX Large Scale RNA Production System (Promega) using T7 RNA polymerase. CFTR-cRNA was diluted with diethyl pyrocarbonate (DEPC)-treated water to a final concentration of 250 ng/µl. Fifty-one nanoliters of CFTR-cRNA were injected into oocytes with a Nanoliter-Injector (Drummond Scientific, Broomall, Pennsylvania, USA) yielding final concentrations of 12.5 ng RNA/oocyte. Injected oocytes were cultured for 2–5 days at 16 °C in the oocyte culture solution.

Microelectrode recordings (Two-Electrode Voltage-Clamp, TEVC). CFTR-expressing oocytes were placed in a Lucite chamber which was continuously perfused with oocyte ringer solution (ORS) containing 90 mM NaCl, 1 mM KCl, 2 mM CaCl$_2$, 5 mM HEPES at pH 7.4. Chlorinated silver wires served as recording electrodes and were mounted into borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) with an outer diameter of 1.2 mm, which were pulled to microelectrodes with a DMZ universal puller (Zeitzi-Instruments, Martinsried, Germany) and filled with 1 M KCl. Ag/AgCl wires were used as reference electrodes and placed directly into the recording chamber. The membrane voltage was clamped to −60 mV using a TEVC amplifier (Warner Instruments, Hamden, Connecticut, USA). Transmembrane currents ($I_M$) were low-pass filtered at 1000 Hertz (Frequency Devices 902, Haverhill, Massachusetts, USA) and continuously recorded with a strip chart recorder (Kipp&Zonen, Delft, The Netherlands).

Determination of evaporative loss of H$_2$S from buffer solutions. The equilibration of H$_2$S with its concentration in air will eventually lead to evaporative loss of this gas from the experimental buffer solutions$^{34}$. Therefore the relative concentration of H$_2$S in ORS was determined at various time points after administration of 50 µM Na$_2$S by the formation of methylene blue. Samples (300 µl) of the solutions were mixed with 500 µl of 4% zinc acetate and incubated on ice for at least 30 min. Afterwards, 200 µl of 0.1% dimethylphenyldiamine sulfate (in 5 M HCl) and 100 µl of 50 mM FeCl$_3$ were added. Samples were vortexed, centrifuged at 5000 × g and incubated for 5 min at room temperature. The absorption of methylene blue was measured at 670 nm with a Vis-spectrophotometer (Krueiss Optronic, Hamburg, Germany).

Chemicals and solutions. In order to apply H$_2$S, the sulfur salt Na$_2$S (Sigma, Taufkirchen, Germany) or the slow releasing H$_2$S donor GYY4137 (Santa Cruz, Biotechnology, Dallas, Texas, USA) were employed. Na$_2$S was prepared as a stock solution of 50 mM in ORS freshly before experiments and immediately diluted to final working concentrations in order to prevent evaporative loss of H$_2$S from the experimental solutions. Stock solutions of 100 mM GYY4137 were prepared in H$_2$O and stored at −20 °C. Forskolin (MoBiTec, Göttingen, Germany) was used as a stimulator of adenyl cyclase and stock solutions of 10 mM were prepared in dimethyl sulfoxide (DMSO, Sigma) and stored at −20 °C. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Sigma, Taufkirchen, Germany) was dissolved to 100 mM in DMSO and stored at +4 °C. The adenyl cyclase inhibitor MDL 12330 A hydrochloride (MDL; Tocris Bioscience, Bristol, UK) was dissolved to 20 mM in DMSO and stored at +4 °C. cAMPS-Rp (Tocris Bioscience) was used as a competitive antagonist of cAMP-induced Protein Kinase A (PKA). Stock solutions of cAMPS-Rp were prepared to 10 mM in H$_2$O and stored at −20 °C. cAMPS-Rp was injected into *Xenopus* oocytes during TEVC experiments. Therefore, stock solutions of cAMPS-Rp were diluted 1:1 with an intracellular-analogous solution (IAS) which contained 20 mM NaCl, 130 mM KCl, 2 mM MgCl$_2$, and 5 mM HEPES at pH 7.3. This mixture (9.2 nl) was injected into oocytes, leading to concentrations of ~87 µM cAMPS-Rp per oocyte. Corresponding control experiments were performed with IAS. The membrane permeable cAMP-analogue 8-Br-cAMP (Tocris Bioscience) was prepared as a 10 mM stock solution in H$_2$O and stored at −20 °C.
Drug application. Drugs were generally applied using a gravity-driven perfusion system. In order to reduce the amount of drugs needed, the membrane-permeable cAMP-analogue 8-Br-cAMP was washed into the perfusion chamber and the perfusion was stopped afterwards. The PKA inhibitor cAMPS-Rp was directly injected into the oocytes since this strategy was established earlier and achieved adequate inhibition of PKA.15

Statistical analysis. For electrophysiological transmembrane recordings, outward-anion currents are defined as negative current signals and depicted in all figures as downward-deflections of the current traces. Data are presented as individual data points (grey symbols) as well as means ± standard error of the mean (SEM). The number of individual oocytes is indicated with ‘n’ whereas the number of donor frogs is represented by ‘N’. Statistical analysis of data was performed with GraphPad Prism version 5 (La Jolla, California, USA). Data were analysed for normal distribution by the Kolmogorov-Smirnov test. For paired experiments, Student’s paired t-test or non-parametric Wilcoxon matched-pairs test (two-tailed) were used. Independent experiments were compared with Student’s unpaired t-test or non-parametric Mann-Whitney test (two-tailed). Multiple comparison analysis was performed by Kruskal-Wallis test followed by Dunn’s Multiple Comparison Test. P-values ≤ 0.05 were regarded as statistically significant and marked with an asterisk (*). P-values ≤ 0.01 and ≤ 0.001 were marked with ** and *** respectively.

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Author Contributions
MA designed experiments, AP, KP and MN performed experiments. All authors analysed and interpreted data. MA wrote the manuscript and prepared figures. All authors reviewed the manuscript.

Additional Information
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