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ORIGINAL PAPER

Effect of Hydrogen Sulfide on Cyclic AMP Production in Isolated Bovine and Porcine Neural Retinae

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Abstract Hydrogen sulfide (H_2S) has been reported to exert pharmacological effects on neural and non-neural tissues from several mammalian species. In the present study, we examined the role of the intracellular messenger, cyclic AMP in retinal response to H_2S donors, sodium hydrosulfide (NaHS) and sodium sulfide (Na_2S) in cows and pigs. Isolated bovine and porcine neural retinae were incubated in oxygenated Krebs buffer solution prior to exposure to varying concentrations of NaHS, Na_2S or the diterpene activator of adenylate cyclase, forskolin. After incubation at different time intervals, tissue homogenates were prepared for cyclic AMP assay using a well established methodology. In isolated bovine and porcine retinae, the combination of both phosphodiesterase inhibitor, IBMX (2 mM) and forskolin (10 μM) produced a synergistic increase ($P < 0.001$) in cyclic AMP concentrations over basal levels. NaHS (10 nM–100 μM) produced a time-dependent increase in cyclic AMP concentrations over basal levels which reached a maximum at 20 min in both bovine and porcine retinae. At this time point, both NaHS and Na_2S (10 nM–100 μM) caused a significant ($P < 0.05$) dose-dependent increase in cyclic AMP levels in bovine and porcine retinae. For instance, NaHS (100 nM) elicited a four-fold and three-fold increase in cyclic AMP concentrations in bovine and porcine retinae respectively whilst higher concentrations of Na_2S (100 μM) produced a much lesser effect in both species. In bovine and porcine retinae, the effects caused by forskolin (10 μM) on cyclic AMP production were not potentiated by addition of low or high concentrations of both NaHS and Na_2S. We conclude that H_2S donors can increase cyclic AMP production in isolated neural retinae from cows and pigs. Bovine retina appears to be more sensitive to the stimulatory effect of H_2S donors on cyclic nucleotide production than its porcine counterpart indicating that species differences exist in the magnitude of this response. Furthermore, effects produced by forskolin on cyclic AMP formation were not additive with those elicited by H_2S donors suggesting that these agents may share a common mechanism in their action on the adenylyl cyclase pathway.

Keywords Hydrogen sulfide · Cyclic AMP · Retina · Sodium sulfide · Sodium hydrosulfide

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Abbreviations

H_2S	Hydrogen sulfide
Na_2S	Sodium sulfide
NaHS	Sodium hydrosulfide
Cyclic AMP	Adenosine 3′,5′ cyclic monophosphate
PDE	Phosphodiesterase
COX	Cyclooxygenase

Introduction

In the past decade, interest in the non-toxic actions of hydrogen sulfide (H_2S) has led to several studies aimed at elucidating its potential physiological and pathological effects in mammalian tissues. H_2S, a colorless gas characterized by its pungent odor (commonly described as the smell of rotten eggs) has been known for decades only as an environmental pollutant with a broad toxicity spectrum [1, 2]. Recently, there is evidence that this "toxic" gas can serve as an endogenous neurotransmitter, a smooth muscle relaxant and a regulator of immune reactions [1–4]. Endogenous H_2S is generated in mammalian tissues from L-cysteine, a reaction catalyzed by two endogenous pyridoxal-5′-phosphate dependent-enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) [5–8], and is a component of foodstuffs, human feces [9, 10] and a product of bacterial and helminth metabolism [11, 12].

In the eye, the presence of the enzymes (CBS and CSE) responsible for H_2S biosynthesis has recently been reported in ocular tissues [13, 14]. Both CBS and CSE were shown to be highly localized in the retina indicating the presence of a functional trans-sulfuration pathway and thus suggesting a potential role of H_2S as a gaseous neuromodulator in this tissue. In the eye, toxicity associated with exposure to lethal concentrations of H_2S is mostly at the mucus membrane level leading to keratoconjunctivitis [15]. Furthermore, studies have reported that deficiency of CBS is often associated with many eye disorders including retinal degeneration, retinal detachment, optical atrophy and glaucoma [16, 17]. Evidence from our laboratory demonstrate that H_2S (using sodium hydrosulfide, NaHS and sodium sulfide, Na_2S as donors) can induce pharmacological effects in mammalian ocular tissues [18–20]. We found that both NaHS and Na_2S relaxed pre-contracted isolated porcine irides [18] and inhibited sympathetic and glutamatergic neurotransmission from isolated porcine and bovine anterior uvea and retina, an effect that was shown to be dependent on intramural biosynthesis of this gas [19, 20].

In the cardiovascular system, the pharmacological effects of H_2S has been reported to be mediated largely by ATP-sensitive potassium channels (K_{ATP}) [21, 22]. However, in neuronal tissues H_2S has been shown to stimulate the production of adenosine 3′,5′ cyclic monophosphate (cyclic AMP) and thus activate cyclic AMP-dependent processes [23]. Cyclic AMP is an intracellular second messenger that plays an essential role in numerous neuronal functions such as cell survival, axon regeneration and modulation of axonal guidance [24, 25]. Taken together, it appears that both K_{ATP} channels and cyclic AMP may mediate the pharmacological actions of H_2S in mammalian tissues. In the present study, we examined the effect of H_2S (using NaHS and Na_2S as donors) on cyclic AMP production in isolated bovine and porcine neural retinae. Parts of the data reported in this paper have been communicated in an abstract form [26].

Methods

Chemicals

NaHS, Na_2S, Ethylenediamine tetra-acetic acid solution (EDTA), Isobutylmethylxanthine (IBMX) and Forskolin were all purchased from Sigma–Aldrich, St. Louis, MO. Flurbiprofen was procured from Cayman Chemicals, Ann Arbor, MI. Stock solutions of IBMX, flurbiprofen and forskolin were dissolved in Dimethylsulfoxide (DMSO) whilst solutions of NaHS and Na_2S were prepared with distilled water. At pH 7.4 the concentration of H_2S solution is relatively stable [22, 27, 28]. All test agents were freshly prepared immediately before use on the day of the study.

Preparation of Retinal Tissues

Bovine and Porcine eyes were obtained from a local slaughterhouse (Fisher Ham & Meat Co., Houston, Texas) and transported to the laboratory on ice following decapitation of animals. The eyeballs were enucleated, and the anterior chambers were carefully removed. The resulting eye cups were inverted and placed in fresh oxygenated Krebs solution containing the following composition (mM): potassium chloride, 4.8; sodium chloride, 118; calcium chloride, 2.3, potassium dihydrogen phosphate, 1.2; sodium bicarbonate, 25; magnesium sulfate, 2.0; and dextrose, 10. The neural retinae were detached by gently movement and incubated immediately in freshly prepared oxygenated Krebs solution (pH 7.4) containing the cyclooxygenase (COX) inhibitor, flurbiprofen (3 μM), with or without the presence of the cyclic nucleotide phosphodiesterase (PDE) inhibitor, IBMX (2 mM) for 30 min. Time elapsed between animal sacrifice and retina preparation was less than 24 h.

Cyclic AMP Assay

The methodology employed for cyclic AMP assay was essentially the same as reported by [29] with some modifications. Immediately following the 30 min incubation, the isolated bovine or porcine neural retinae were transferred to 2 ml of freshly prepared Krebs solution (pH 7.4) containing flurbiprofen (3 μM) with or without IBMX (2 mM). In studies, were we examined the role of PDE on metabolism of cyclic AMP, isolated neural retinae without prior incubation with IBMX, were exposed

to either IBMX (2 mM), forskolin (10 μM) or a combination of both at the desired time of 20 min (this time scale was chosen based on results from our preliminary experiments that showed that maximal effect of agonists on cyclic AMP was achieved at this time). All subsequent experiments on the effect of H_2S donors and forskolin were performed in the presence of IBMX (2 mM). Tissues were treated with varying concentrations of, H_2S donors (NaHS, Na_2S) or the intracellular cyclic AMP-elevating agent forskolin (10 μM) and exposed for 20 min. Control tissues were exposed to an appropriate volume of vehicle (0.9% saline) for the same time period. In experiments were we examined the combined effect of forskolin and H_2S donors on cyclic AMP production, isolated neural retinae were exposed to forskolin (10 μM) for 20 min before the addition of the H_2S donors. After an additional 10 min the reaction was terminated by addition of 4 mM ice-cold EDTA. The tissue homogenates were boiled for 20 min and then centrifuged at 3,000 rpm for 10 min. Pellets obtained were dissolved in 1 N NaOH at 60°C for protein determination by method of Bradford and aliquots of the supernatant were employed for measurement of cyclic AMP content using a cyclic AMP enzyme immunoassay kit purchased from Cayman Chemicals, Ann Arbor, Michigan.

Data Analysis

Results are expressed as pmol/μg of protein. Values given are arithmetic means ± SEM. The means were determined from two separate experiments performed in triplicates. Significance of differences between control and treatment groups were evaluated using one-way analysis of variance (ANOVA) followed by Newman–Keuls comparison test. Drug treatment, time and interaction between drug treatment and time were assessed by two-way ANOVA followed by post-hoc Bonferroni test (Graph Pad Prism Software, San Diego, CA). A P value of <0.05 was considered as statistically significant.

Results

Effects of Activation of Adenylyl Cyclase and Inhibition of Phosphodiesterase on Cyclic AMP Levels in Neural Retina

Isobutylmethylxanthine is a phosphodiesterase (PDE) inhibitor that prevents the breakdown of accumulated intracellular cyclic AMP. In this study, we considered the possibility that PDE inhibition may contribute to the responses observed with forskolin (diterpene activator of adenylyl cyclase) in both bovine and porcine isolated neural retinae. Furthermore, PDE inhibition could also allow optimization of the yield of cyclic AMP in response to the H_2S donors. The PDE inhibitor, IBMX (2 mM) caused a two–three fold increase in basal cyclic AMP concentrations in bovine (Fig. 1a) and porcine (Fig. 1b) retinae respectively. Tissues stimulated with forskolin (10 μM) alone did not have any significant effect on cyclic AMP concentrations when compared to basal levels in both species (Fig. 1a, b). In the presence of IBMX, responses elicited by forskolin were enhanced significantly ($P < 0.001$) in both bovine (Fig. 1a) and porcine (Fig. 1b) retinae.

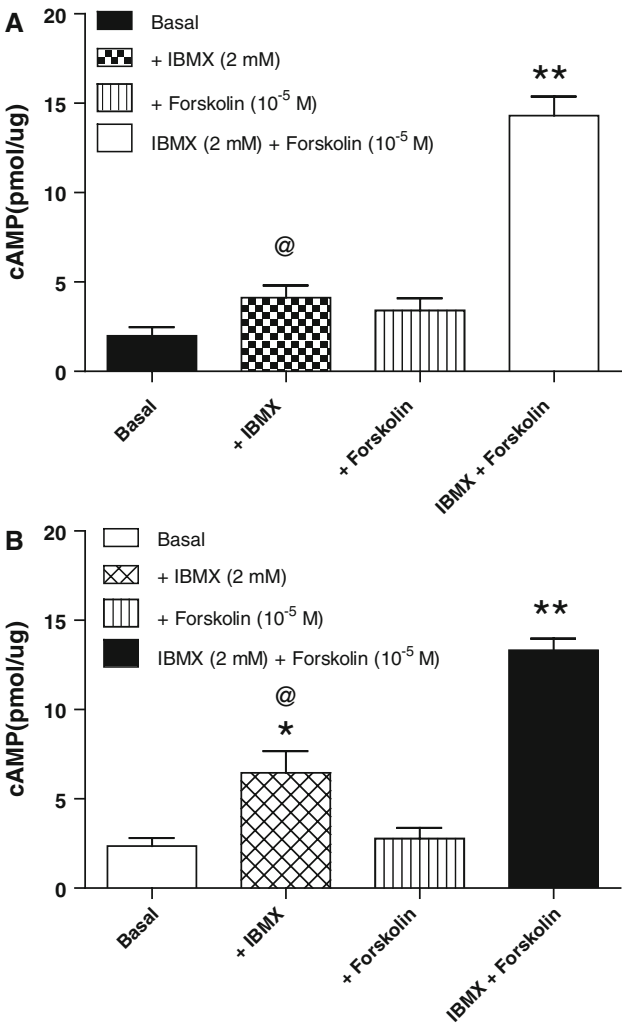

Fig. 1 Effects of PDE inhibition on cyclic AMP levels in isolated bovine (**a**) and porcine (**b**) retina: control, in the presence of IBMX (2 mM) and Forskolin (10 μM). *Vertical bars* represent means ± SEM; $n = 6$. $^{**}P < 0.001$, $^{*}P < 0.01$ significantly different from controls; $^{@}P < 0.01$ significantly different from IBMX-Forskolin treated groups

Effects of NaHS on Cyclic AMP Levels in Neural Retina

Since there is evidence that exogenous H_2S increases the production of cyclic AMP in neurons [30], we investigated the effects of the H_2S donor, NaHS on cyclic AMP formation in bovine and porcine neural retinae. Based on data obtained in studies described in Fig. 1, all subsequent experiments on the effect of H_2S donors were performed in the presence of IBMX (2 mM). As illustrated in Fig. 2, concentrations of 10 nM, 1 μM or 100 μM of NaHS produced a time-dependent significant ($P < 0.05$) increase in cyclic AMP levels over basal concentrations in both bovine (Fig. 2a) and porcine (Fig. 2b) retinae which reached a maximum at 20 min. In bovine retina, NaHS (100 μM) caused a five-fold and six-fold increase in cyclic AMP concentrations over basal levels at 5- and 20-min time intervals, respectively (Fig. 2a). In contrast, in porcine retina, NaHS (100 μM) elicited a two-fold and three-fold increase in cyclic AMP concentrations over basal levels at 5- and 20 min time intervals, respectively (Fig. 2b).

In subsequent experiments, we examined the effect of different concentrations of NaHS on cyclic AMP production using a 20 min incubation time for both bovine and porcine neural retinae. NaHS (10 nM–100 μM) produced a concentration-dependent significant ($P < 0.05$) increase in cyclic AMP levels reaching a maximal effect at 100 nM (Fig. 3a). At this concentration, NaHS (100 nM) caused a four-fold increase in cyclic AMP concentrations above basal levels in bovine retina. NaHS (10 nM–100 μM) also caused concentration-dependent significant ($P < 0.05$) increases in cyclic AMP levels in porcine retina with a maximal effect observed at 1 μM (Fig. 3b). In porcine retina, NaHS (1 μM) produced a three-fold increase in cyclic AMP concentrations above basal levels. The diterpene activator of adenylate cyclase, forskolin (10 μM) also produced a significant ($P < 0.001$) increase in cyclic AMP concentrations above basal levels (Fig. 3a, b).

Effects of Na_2S on Cyclic AMP Levels in Neural Retina

We next examined the effect of another H_2S donor, Na_2S on cyclic nucleotide production in bovine and porcine neural retinae. At 20 min of incubation, Na_2S (10 nM–100 μM) elicited a concentration-dependent increase in cyclic AMP levels for both bovine and porcine neural retinae (Fig. 4a, b). At a concentration of 100 μM, Na_2S caused a two-one half-fold increase in cyclic AMP concentration over basal levels in both bovine (Fig. 4a) and porcine (Fig. 4b) retinae. Upon stimulation with the positive control, forskolin (10 μM), both bovine and porcine retinae responded with a significant ($P < 0.001$) increase in cyclic AMP concentrations over basal levels (Fig. 4a, b).

Effects of H_2S Donors on Activation of Adenylyl Cyclase by Forskolin

We examined the combined effect of forskolin and H_2S donors on cyclic AMP production in both bovine and porcine retinae. In bovine retina, submaximal (10 nM) and maximal (100 nM) concentrations of NaHS were examined on forskolin (10 μM) stimulated cyclic AMP production (Fig. 5a). The response observed to forskolin was not enhanced by the presence of both low and high concentrations of NaHS (Fig. 5a). Likewise, in porcine retina, the effects caused by forskolin (10 μM) on cyclic AMP formation were not enhanced by addition of submaximal (10 nM) and maximal (1 μM) concentrations of NaHS (Fig. 5b).

We next investigated the combined effect of forskolin and Na_2S on cyclic AMP formation in both bovine and

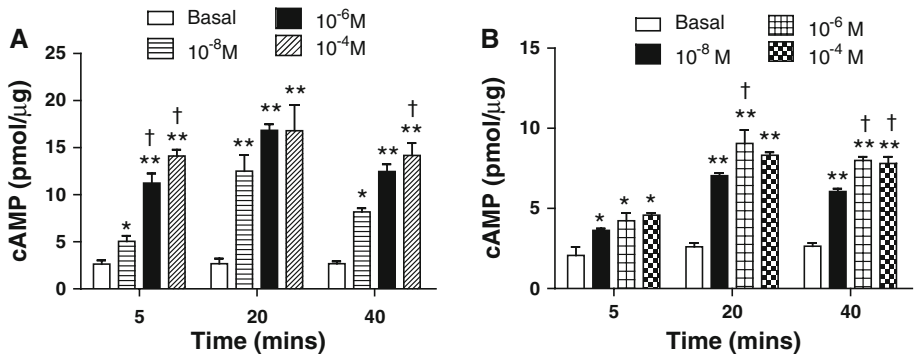

Fig. 2 Time-dependent effect of NaHS on cyclic AMP in isolated bovine (**a**) and porcine (**b**) retina: control and in the presence of NaHS (10 nM–100 μM). *Vertical bars* represent means ± SEM; $n = 6$. $^{**}P < 0.001$, $^{*}P < 0.01$ significantly different from controls; $^{\dagger}P < 0.01$ significantly different among drug-treated groups (10 nM). Two-way ANOVA: In bovine retina (**a**) time, $P < 0.004$; drug treatment, $P < 0.002$. For both groups, treatment and interaction (treatment × time of study) = NS; $P < 0.280$. In porcine retina (**b**) time, $P < 0.001$; drug treatment, $P < 0.001$. For both groups, treatment and interaction (treatment × time of study) = significant; $P < 0.01$

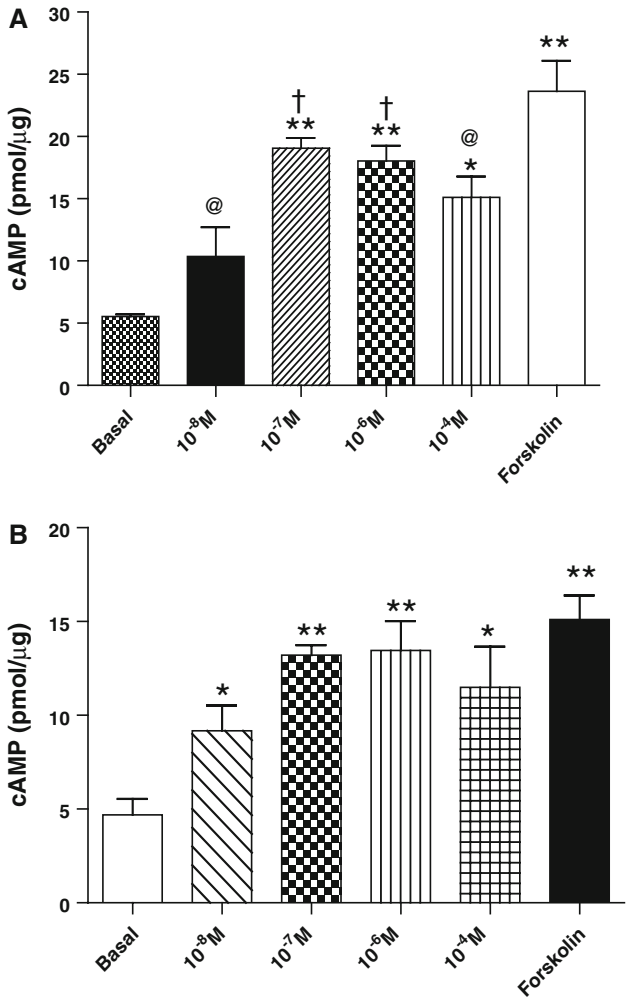

Fig. 3 Concentration-dependent effect of NaHS on cyclic AMP in isolated bovine (**a**) and porcine (**b**) retina: control, in the presence of NaHS (10 nM–100 μM) and Forskolin (10 μM). *Vertical bars* represent means ± SEM; $n = 6$. $^{**}P < 0.001$, $^{*}P < 0.01$ significantly different from controls; $^{@}P < 0.01$ significantly different from Forskolin treated groups; $^{\dagger}P < 0.01$ significantly different among drug-treated groups (10 nM)

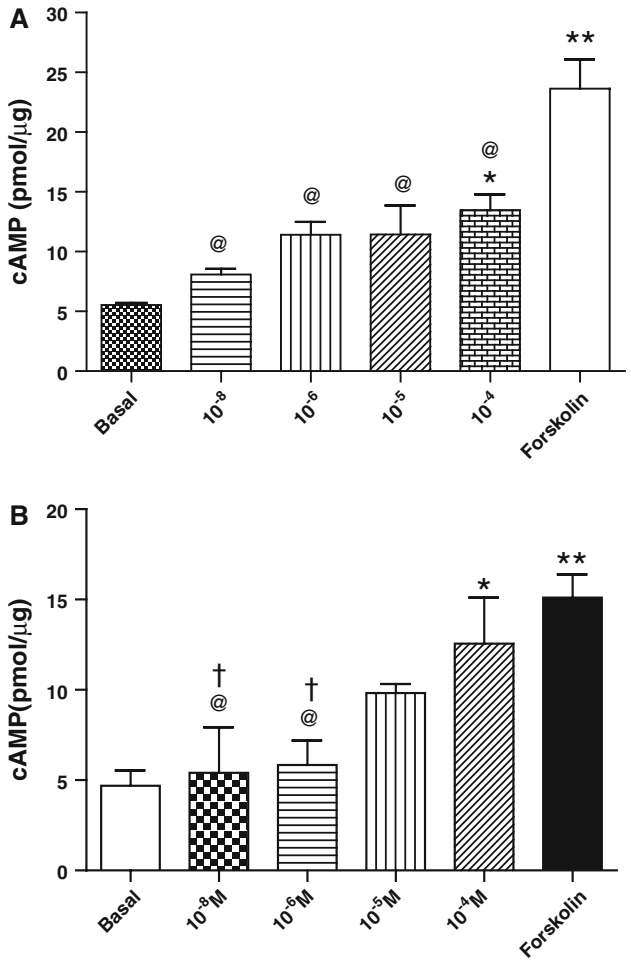

Fig. 4 Concentration-dependent effect of Na_2S on cyclic AMP in isolated bovine (**a**) and porcine (**b**) retina: control, in the presence of Na_2S (10 nM–100 μM) and Forskolin (10 μM). *Vertical bars* represent means ± SEM; $n = 6$. $^{**}P < 0.001$, $^{*}P < 0.01$ significantly different from controls; $^{@}P < 0.01$ significantly different from Forskolin treated groups; $^{\dagger}P < 0.01$ significantly different among drug-treated groups (100 μM)

porcine retinae. In bovine retina, the effects elicited by forskolin (10 μM) on cyclic AMP production were not potentiated by addition of submaximal (10 nM) and maximal (1 μM) concentrations of Na_2S (Fig. 6a). Similarly, in porcine retina, the effects produced by forskolin (10 μM) on cyclic AMP formation were not enhanced by submaximal (1 μM) and maximal (100 μM) concentrations of Na_2S (Fig. 6b).

Discussion

In the last two decades, there has been a surge of interest in the biological effects of H_2S, a gas that is now deemed to serve as a gaseous transmitter along with nitric oxide and carbon monoxide [1]. Indeed the existence of the H_2S biosynthetic enzymes, CBS and CSE, in the retina suggests a potential physiological role for H_2S in this tissue [13, 14]. In a previous study, we found that the H_2S donors, NaHS and Na_2S, can inhibit excitatory amino acid transmission from isolated bovine and porcine retinae. Furthermore, this effect was determined to be dependent, at least in part, on intramural biosynthesis of this gas [20]. Based on the known actions of this gas on the vasculature and brain, numerous investigators have reported that possible mechanistic effects may involve potassium-sensitive ATP (K_{ATP}) channels, reactive oxygen species (ROS), intracellular calcium and mitogen-activated protein (MAP) kinases [1, 2, 31]. However, the mechanism by which H_2S elicits its physiological effects in the retina has not been clearly elucidated.

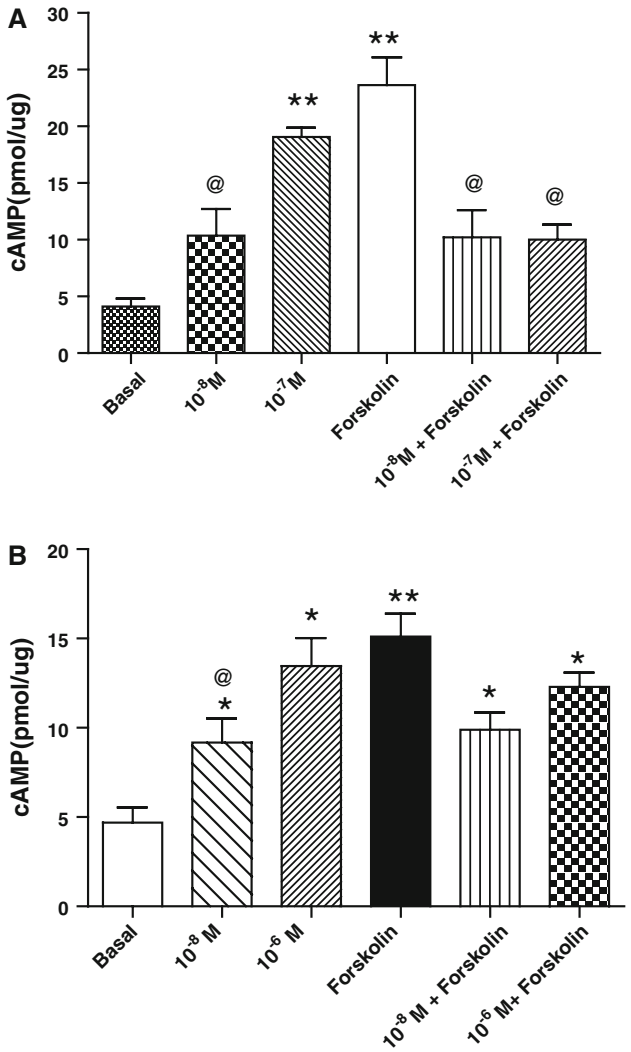

Fig. 5 Effects of NaHS on Forskolin (10 μM)-stimulated cyclic AMP production in isolated bovine (**a**) and porcine (**b**) retina: control, in the presence of NaHS (10 nM–1 μM) and Forskolin (10 μM). *Vertical bars* represent means ± SEM; $n = 6$. $^{**}P < 0.001$, $^{*}P < 0.01$ significantly different from controls; $^{@}P < 0.01$ significantly different from groups treated with forskolin alone

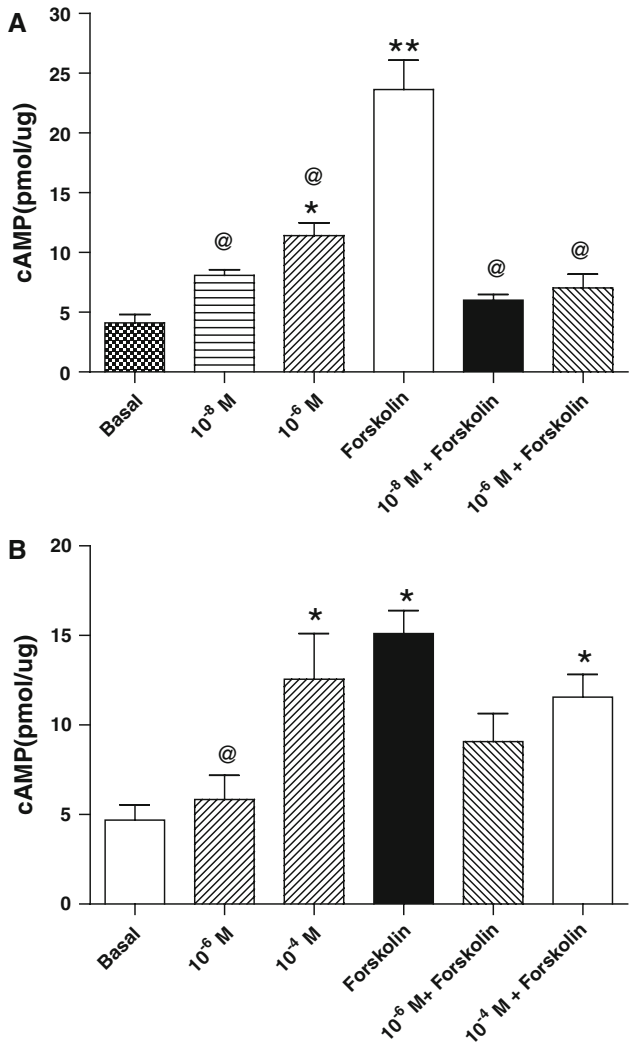

Fig. 6 Effects of Na_2S on Forskolin (10 μM)-stimulated cyclic AMP production in isolated bovine (**a**) and porcine (**b**) retina: control, in the presence of Na_2S (10 nM–100 μM) and Forskolin (10 μM). *Vertical bars* represent means ± SEM; $n = 6$. $^{**}P < 0.001$, $^{*}P < 0.01$ significantly different from controls; $^{@}P < 0.001$ significantly different from groups treated with forskolin alone

The cyclic nucleotide, adenosine 3′,5′ cyclic monophosphate (cyclic AMP) is a ubiquitous cellular second messenger that is important in many biological processes. It is used for intracellular signal transduction and has been reported to be involved in numerous neuronal functions including cell survival, axon regeneration, and modulation of axonal guidance [24, 25]. In the present study, we examined the role of PDE in the metabolism of cyclic AMP in isolated bovine and porcine neural retinae. Inhibition of PDE with IBMX enhanced the responses elicited by the diterpene activator of adenylyl cyclase, forskolin suggesting that catabolism of cyclic AMP does occur in these tissues. Indeed, there is evidence that PDE is present in several retinal cells such as the ganglion, bipolar, horizontal, amacrine and rod photoreceptors [32]. We included IBMX in all subsequent studies on the effects of H_2S donors on cyclic AMP production in the retina. Furthermore, we employed forskolin as a positive control in all assays performed in the present study.

Studies have reported that intracellularly, H_2S enhances *N*-methyl-D-aspartate (NMDA) receptor-mediated response via cyclic AMP production and that exogenous H_2S increases production of cyclic AMP in primary cultures of rat cerebral and cerebellar neurons, or in some neuronal and glial cell lines [30]. In the present study, we investigated the effect of H_2S donors on cyclic AMP production in isolated mammalian retinae. We report that the H_2S donor, NaHS produced a time-dependent increase in cyclic

AMP concentrations over basal levels in both isolated bovine and porcine retinae. Data from these experiments enabled us to determine an optimum time (20 min) for interaction between H_2S donors and the adenylate cyclase pathway. Both NaHS and Na_2S significantly increased cyclic AMP levels in bovine and porcine retinae in a concentration-related manner indicating that this nucleotide serves as a mediator of effects caused by H_2S in these tissues. Data from these studies also show that NaHS was more potent than Na_2S in stimulating cyclic AMP production in both bovine and porcine retinae. A similar change in sensitivity of tissues to NaHS and Na_2S has been reported by our laboratory in studies of sympathetic neurotransmission in porcine iris-ciliary body [19], amino acid transmission in bovine retina [20] and in the relaxation of isolated porcine irides to both H_2S donors [18]. In the present study, we also observed that the ability of H_2S donors to increase cyclic AMP concentrations was greater in bovine than porcine retina, suggesting that species differences exist in the response of this tissue to H_2S donors. Opere et al. [20] also reported that species differences exist in the effects caused by H_2S donors on glutamatergic transmission.

So far, data obtained from the present study shows that both H_2S donors and forskolin can increase the production of cyclic AMP in bovine and porcine retinae. To establish whether H_2S donors and forskolin utilize the same pathways for increasing cyclic AMP formation, we designed experiments that exposed retina to both agents simultaneously. We observed that in both bovine and porcine retinae, responses elicited by forskolin on cyclic formation was not additive with those produced by NaHS and Na_2S indicating that a common pathway may mediate the observed responses. It is, however, unclear whether the effects of H_2S donors on the adenylyl cyclase pathway are due to a direct and/or indirect action on this enzyme.

In conclusion, H_2S donors can cause a time- and dose-dependent increase in cyclic AMP production in isolated neural retina from cows and pigs. Bovine retina appears to be more sensitive to the stimulatory effect of H_2S donors on cyclic nucleotide production than its porcine counterpart indicating that species differences exist in the magnitude of this response. Effects produced by forskolin on cyclic AMP formation were not additive with those elicited by H_2S donors suggesting that these agents may share a common mechanism in their action on the adenylyl cyclase pathway. Taken together, our findings suggest that H_2S may play a regulatory role in signal transduction processes in mammalian retina.

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