

The Role of L-Cysteine/H₂S Pathway in CaSRs-Mediated Relaxations in Mouse Bladder Tissue

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Received February 12, 2025

Accepted May 27, 2025

Summary

The activation of Calcium-Sensing Receptors (CaSRs) reduces detrusor activity in bladder tissues. Also, hydrogen sulfide (H₂S) produces in bladder tissue and regulates the bladder smooth muscles tone. However, there is no evidence of the interaction between CaSRs and H₂S in bladder tissue. The aim of this study is to investigate the possible contribution of L-cysteine/H₂S pathway in CaSRs-mediated relaxation responses in isolated mouse bladder tissue. CaCl₂ (1, 2, 3, 5, 10 mM) was applied to isolated mouse bladder tissues pre-contracted with carbachol (1 µM). CaCl₂-induced relaxations were performed in the presence of PAG (10 mM), AOAA (1 mM), and Calhex-231 (5 µM), cystathionine-gamma-lyase (CSE), cystathionine-beta-synthase (CBS) and CaSR inhibitor, respectively. L-cysteine (1 µM-10 mM), an H₂S substrate, was used to induced a concentration-dependent relaxant response in isolated bladder tissues pre-contracted with carbachol. L-cysteine induced relaxations were performed in the presence of PAG (CSE inhibitor, 10 mM), AOAA (CBS inhibitor, 1 mM) and Calhex-231 (CaSR inhibitor, 5 µM). CaCl₂-induced relaxations were decreased by PAG and AOAA. Also, Calhex-231 decreased the CaCl₂-induced relaxant responses. L-cysteine-induced relaxant responses were reduced in the presence of PAG (10 mM) and AOAA (1 mM). Calhex-231 (5 µM) caused a significant decrease in L-cysteine-induced relaxations. Also, Calhex-231 reduced the increase in H₂S production in the presence of L-cysteine. In addition, CaCl₂ increased basal H₂S generation, and PAG (10 mM), AOAA (1 mM) and Calhex-231 (5 µM) reduced the increase in H₂S production stimulated with CaCl₂. In conclusion, CSE and CBS-derived endogenous H₂S formation may, at least in part, contribute to CaSR-mediated relaxation responses, and CaSRs

involve in endogenous H₂S relaxation responses in isolated mouse bladder tissue.

Key words

Bladder • CaSRs • Calhex-231 • Hydrogen sulfide • L-cysteine • Mouse

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Introduction

Calcium-sensing receptors (CaSRs) are found in many tissues and induce numerous functions [1]. CaSRs were firstly expressed from the bovine parathyroid gland, where they regulate parathyroid hormone-dependent extracellular calcium homeostasis [2]. CaSRs are G protein-coupled receptors (GPCRs) located on the cell membrane and sense the levels of extracellular Ca²⁺, and activate signaling pathways that modulate calcium homeostasis. The activation of CaSRs by increased extracellular Ca²⁺ or with other CaSRs agonists triggers the phosphatidylinositol-specific phospholipase C (PLC), initiating the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃), inhibition of adenylyl cyclase and activation of MAPK/ERK1/2 pathway [3,4].

Recent studies have demonstrated that tissues previously considered non-calcitropic, such as vascular smooth muscle may play a role in modulating Ca²⁺ homeostasis [5,6]. Also, it has been shown that

CaSRs produced hyperpolarization and vasodilation in mesenteric artery tissues of rat and rabbit [7,8]. CaSRs is also expressed in vascular endothelium [9] and in smooth muscle cells [10,11]. Alam *et al.* suggested that CaSRs-mediated relaxation may contribute to multiple components within the mesenteric vasculature [12]. Bukoski and co-workers have reported that increasing extracellular $[Ca^{2+}]$ within the physiological range (from 1 to 5 mM) induces relaxation in rat mesenteric arteries and also showed that the Ca^{2+} -induced relaxation was independent of a functional endothelium [13]. Moreover, Ca^{2+} -induced relaxation was also attenuated by ibuprofen and capsaicin, suggesting that Ca^{2+} activates CaSRs on perivascular nerves, leading to the release of a diffusible substance which in turn activates large conductance calcium-activated potassium (BKCa) in vascular smooth muscle cells (VSMCs) [14].

In addition, Calhex-231 (a negative allosteric modulator of CaSR) antagonized the effects of $CaCl_2$ and calindol which are positive allosteric modulators of CaSRs [15]. Also, it has been shown that stimulation of endothelial CaSRs induces nitric oxide (NO)- and endothelium derived hyperpolarizing factor (EDHF)-mediated vasorelaxation in pre-contracted arteries. Furthermore, the increase in extracellular Ca^{2+} concentration or calcimimetics stimulates endothelial CaSRs and produces NO, which causes vasorelaxation *via* stimulation of BKCa channels in VSMCs [16-20]. Hydrogen sulfide (H_2S) was known as a toxic gas in the past. For the first time, H_2S was synthesized in brain tissue of mammals, and recognized a gaseous neurotransmitter such as NO and carbon monoxide [21]. H_2S is synthesized through cystathionine gamma lyase (CSE), cystathionine beta synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) enzymes in various tissues [21-23]. H_2S synthesis occurs in the bladder tissue and urothelium of various species, such as mice, rats, pigs, and human, where it plays a role in the regulation of muscle tone and is associated with conditions including overactive bladder [24-29]. Eto and Kimura have been shown that CBS are involved in the regulation of its activity in the presence of Ca^{2+} and calmodulin in brain tissue [30].

Also, recent studies suggest that the elevation in intracellular Ca^{2+} increases CSE activity and H_2S generation in vascular smooth muscle cells [31,32]. In addition, it has been reported that CSE-induced H_2S synthesis is enhanced by the activation of CaSRs with $CaCl_2$ in vascular tissues [33]. Furthermore, Wang *et*

al. found that the CaSRs-induced upregulation of CSE expression and the production of endogenous H_2S are related to the PLC-IP3 receptor and calcium-calmodulin (CaM) signaling pathways [34]. However, it is not known that CaSRs regulates detrusor activity by which mechanism. Wu *et al.* reported that CaSRs are expressed in the rat bladder urothelium and the activation of these receptors reduces detrusor activity [35]. To our knowledge, there are no studies investigating the possible interaction between CaSRs and H_2S pathway in bladder tissue. For the first time, we investigated the role of the L-cysteine/ H_2S pathway in CaSRs-mediated responses in mouse bladder tissue. Our data first demonstrate that there is an interaction between L-cysteine/ H_2S pathway and CaSRs, and, CSE/CBS-induced endogenous H_2S may partly contribute to the relaxation responses due to CaSRs activation in mouse bladder.

Materials and Methods

Animals

Swiss albino male mice were used in the experiments. All experimental protocols were approved by the Cukurova University Local Ethics Committee of Animal Experiments (the approval number 3/11/04.05.2023). The animals were kept under a 12 h light/dark cycle and allowed free access to food and water. The present study was followed by the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Bethesda, MA, USA; NIH Publication No. 85-23 revised 1996).

Tissue preparation

Male Swiss albino mice, weighing 20-25 g, were used for these experiments. They were killed by stunning and cervical dislocation. The bladder tissue was carefully removed. Strips (0.5 mm wide and 4-5 mm long) from the midportion of the urinary bladder with urothelium were mounted in a (5 ml) organ bath filled with Krebs solution (in mM: NaCl 118.1, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2 \cdot 6H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.5). The bath medium was maintained at 37 °C and gassed with a mixture of 95 % O_2 and 5 % CO_2 at pH 7.4. Muscle strips were allowed to equilibrate for 60 min, during which the medium was changed every 15 min. Changes in muscle length were recorded isometrically *via* an isometric transducer (MP35).

Experimental protocol

In this study, the interaction between CaSRs and the H₂S pathway was investigated. In the first experiments, the contribution of endogenous H₂S to the CaCl₂-induced relaxations was investigated. Firstly, tissues were contracted with carbachol (1 μ M) to assess the viability of bladder strips. After the contractions to carbachol were obtained, tissues were washed and incubated for 30 min in Krebs solutions. Then, the tissues were re-contracted with carbachol (1 μ M), and after the contractile responses were reached a plateau, bolus CaCl₂ (1, 2, 3, 5 and 10 mM) was applied for relaxation responses. To demonstrate the role of CaSRs in the CaCl₂-induced relaxations, experiments were run in the presence of Calhex-231, a CaSRs – specific inhibitor. For this purpose, after the contractile responses to carbachol were obtained, tissues were washed and incubated with 5 μ M Calhex-231 for 30 min and then responses to CaCl₂ were obtained in the same manner. Also, the contribution of endogenous H₂S to the relaxant responses to CaCl₂ (1, 2, 3, 5 and 10 mM) was investigated in the presence of propargylglycine (PAG: CSE inhibitor; 10 mM) or aminooxyacetic acid (AOAA: CBS inhibitor; 1 mM). For this purpose, the tissues were washed and incubated with PAG (10 mM) or AOAA (1 mM) for 60 and 30 min, respectively, and then responses to CaCl₂ were obtained in the same manner. Furthermore, the contribution of CaSRs to the relaxant responses to L-cysteine (H₂S substrate) was investigated. Firstly, the bladder tissues were contracted with carbachol (1 μ M) and relaxation responses were obtained by applying cumulatively L-cysteine (1 μ M-10 mM) to the tissues. After the first series of relaxation responses to L-cysteine were obtained, tissues were incubated for 30 min with Krebs solutions and the second series of relaxations were recorded in the same manner. In mouse bladder tissue, to confirm that L-cysteine-induced relaxations are dependent on the endogenous H₂S, the effects of CSE and CBS enzyme inhibitors on these relaxations were investigated. For this purpose, after the first series of L-cysteine relaxations, the tissues were incubated with PAG (10 mM) or AOAA (1 mM) for 60 and 30 min, respectively, and then the second series of responses to L-cysteine were obtained in the same manner. The involvement of CaSRs in L-cysteine-induced relaxations was investigated in the presence of Calhex-231, a CaSRs inhibitor. After the relaxant responses to L-cysteine (1 μ M-10mM) were obtained, tissues were washed and incubated with 5 μ M Calhex-231 for 30 min,

and then responses to L-cysteine (1 μ M-10 mM) were obtained in the same manner.

Measurement of endogenous H₂S release in mouse bladder strips

H₂S levels were measured as described in our previous studies [29,36]. In the presence of Fe³⁺, H₂S reacts with color developing agent to form stable methylene blue, and methylene blue has the maximum absorption peak at 665 nm. The H₂S content can be calculated by measuring its absorption value. H₂S production in bladder tissue samples was determined with a commercially available H₂S colorimetric assay kit (Elabsience Biotechnology Co., Ltd., Wuhan, China) through the reaction between H₂S and zinc acetate, N, N-dimethyl-p-phenylenediamine, and ammonium ferric sulfate. Protein concentration was determined by using a bicinchoninic acid assay kit (Sigma Chemical Co., St. Louis, MO, USA). Bladder tissues at 10 % (w/v) concentration were homogenized in normal saline (0.9 %) at 4 °C. And then centrifuged for 10 min at 4 °C at 10000× g to remove insoluble material, and the supernatant was collected. The supernatant solution was mixed with an equal volume of reagents 1 and 2. After centrifugation, the sediment was dissolved in reagents 1, 3, and 4. The supernatant obtained after centrifugation was mixed with reagent 5. The absorbance of solutions was measured after 20 min at a wavelength of 665 nm and H₂S concentrations in bladder tissues, expressed as nmol/mg protein.

Drugs

The following drugs were used; amino-oxyacetic acid (o-carboxymethyl), dl-propargylglycine, carbachol, L-cysteine (Sigma Chemical Co., St Louis, MO, USA), Calhex-231 (4-Chloro-N-[(1S,2S)-2-[[[(1R)-1-(1-naphthalenyl)ethyl]amino]cyclohexyl]-benzamide hydrochloride) (CAYMAN Chemical Company, USA) and; CaCl₂ (MERCK). All drugs were dissolved in distilled water except calhex-321, which was dissolved in dimethyl sulphoxide (DMSO) up to 1 mM; further dilutions were made in distilled water. DMSO per se did not affect the tone of the strips. The final concentration of DMSO was less than 0.001 M.

Statistical analysis

The relaxant responses to CaCl₂ and L-cysteine were expressed as a percentage of the carbachol-induced contraction. Due to CaCl₂ was applied to the tissues as

a single series, the single-series relaxations of the inhibitor groups were compared with those in the control group. Also, since a difference was observed between the first and second relaxation series in the L-cysteine control group, the relaxant responses to L-cysteine obtained in the presence of specific inhibitors were compared to the second series of the control group. Student unpaired *t*-tests and analysis of variance (ANOVA) were used for statistical comparison of mean values, and corrected for multiple comparisons (Bonferroni corrections). All data are presented as means \pm standard error of the mean (SEM), and “n” refers to the number of tissues used in each experiment. Maximum relaxant response (E_{\max}) was expressed as the relaxation induced by CaCl_2 and L-cysteine. The sensitivities of the bladder tissues to CaCl_2 and L-cysteine were calculated as the effective concentration the elicits 50 % of the maximal response by using nonlinear regression curve fit and expressed as pEC_{50} (-Log M) (GraphPAD Software, version 5.00, San Diego, USA). $P < 0.05$ was considered to be statistically significant.

Results

The role of L-cysteine/H₂S pathway on CaCl₂-induced relaxations

To elucidate the relaxant effect of CaCl_2 on carbachol-induced contractions in mouse bladder strips, the effects of CaCl_2 were studied. After a steady-state

contraction was obtained with carbachol (1 μM), CaCl_2 was applied at 1, 2, 3, 5 and 10 mM concentrations to the bladder tissues. CaCl_2 caused concentration-dependent relaxations in bladder tissues pre-contracted with carbachol (1 μM) (Fig. 1A). To determine the involvement of L-cysteine/H₂S pathway in the relaxant action of CaCl_2 in mouse bladder tissues, we investigated the inhibitory effects of PAG (10 mM) and AOAA (1 mM), CSE and CBS inhibitor, respectively, on relaxations induced by CaCl_2 (1, 2, 3, 5 and 10 mM). Pre-incubation of bladder strips with PAG and AOAA significantly reduced the relaxant responses to CaCl_2 ($P < 0.05$; Fig. 1A, B, C). E_{\max} to CaCl_2 were significantly decreased by PAG and AOAA from $37.40 \pm 1.30\%$ to $18.87 \pm 4.37\%$ and $18.20 \pm 4.34\%$, respectively ($P < 0.05$). But there was no significant difference in pEC_{50} values for CaCl_2 between control (2.89 ± 0.39), PAG (2.25 ± 0.40) and AOAA (2.72 ± 0.37) groups.

To confirm that the relaxant effect of CaCl_2 is mediated through CaSR, we examined the effect of Calhex-231, a CaSRs inhibitor, on CaCl_2 -induced relaxations in the bladder tissues. Pre-incubation of bladder tissues with Calhex-231 (5 μM) significantly reduced the relaxant responses to CaCl_2 ($P < 0.05$; Fig. 1A and 1D). E_{\max} to CaCl_2 were significantly decreased by Calhex-231 from $37.40 \pm 1.30\%$ to $19.75 \pm 4.57\%$ ($P < 0.05$). However, there was no significant difference in the pEC_{50} values for CaCl_2 between control (2.89 ± 0.39) and Calhex-231 (2.61 ± 0.37) groups.

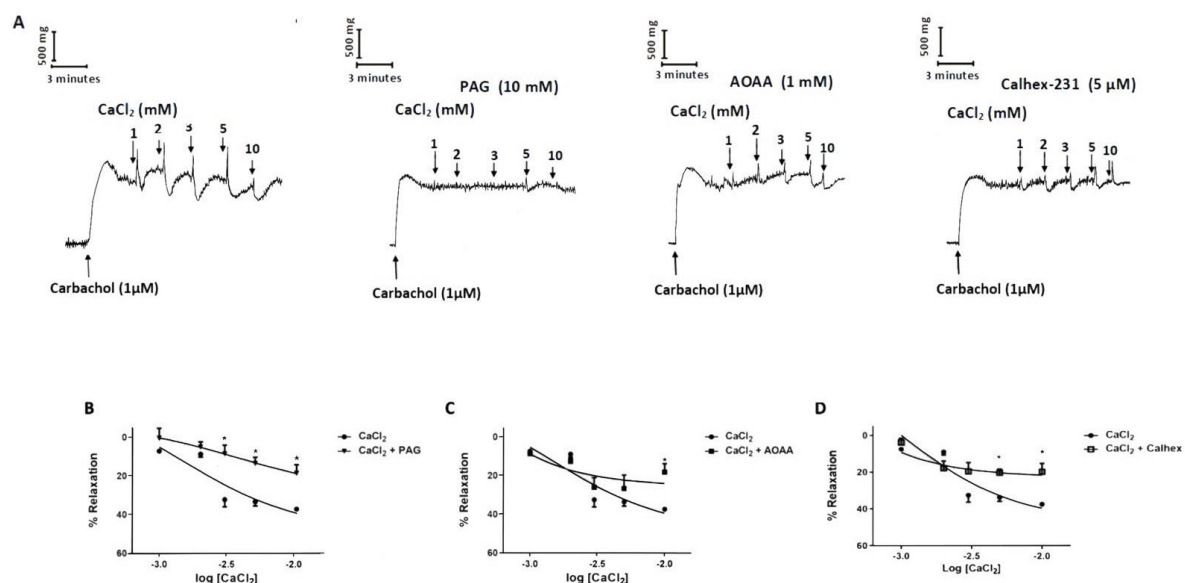


Fig. 1. The role of L-cysteine/H₂S pathway on CaCl_2 -induced relaxations. Representative traces CaCl_2 -induced relaxations (A). Graph showing that CaCl_2 -induced relaxations in the presence of PAG (Cystathionine-γ-lyase (CSE) inhibitor, 10 mM) (B), AOAA (Cystathionine beta synthase (CBS) inhibitor, 1 mM) (C), and Calhex-231 (Calcium Sensing Receptors (CaSRs) inhibitor, 5 μM) (D). All values are mean \pm S.E.M. (n=6). * $P < 0.05$ significantly different from the control; unpaired *t*-test followed by Bonferroni's comparison test.

The role of CaSR on L-cysteine-induced relaxations

To investigate the involvement of CaSR in relaxant responses to endogenous H₂S substrate L-cysteine in bladder strips, we studied the inhibitory effects of Calhex-231, a CaSRs-specific inhibitor, on relaxations induced by L-cysteine. After a steady-state of contraction was obtained with carbachol (1 μ M), L-cysteine was applied cumulatively at concentrations from 1 μ M to 10 mM. L-cysteine (1 μ M-10 mM) caused concentration-dependent relaxations in bladder strips pre-contracted with carbachol (1 μ M) (Fig. 2A).

To confirm that endogenous H₂S-dependent relaxation of L-cysteine, we studied the effect of H₂S synthesis inhibitors on L-cysteine-induced relaxations. PAG (10 mM), a CSE inhibitor and AOAA (1 mM), a CBS inhibitor, significantly decreased the relaxant responses to L-cysteine (1 μ M-10 mM) ($P < 0.05$; Fig. 2A, B and C). Also, pEC₅₀ for L-cysteine was significantly reduced by PAG from 3.88 ± 0.15 to 2.99 ± 0.15 ($P < 0.05$). The E_{max} for L-cysteine were significantly decreased by AOAA $79.27 \pm 5.47\%$ to $62.06 \pm 2.43\%$ ($P < 0.05$). However, there was no significant difference in pEC₅₀ values for L-cysteine between the control (3.88 ± 0.15) and AOAA (3.79 ± 0.15) groups.

To determine the involvement of CaSRs in the relaxant action of L-cysteine/H₂S in mouse bladder tissues, we investigated the inhibitory effects of Calhex-231 on relaxations induced by L-cysteine (1 μ M-10 mM). Pre-incubation of bladder strips with

Calhex-231 significantly reduced the relaxations at lower concentrations of L-cysteine ($P < 0.05$; Fig. 2A and 2D) but the E_{max} value did not change. The pEC₅₀ for L-cysteine was significantly reduced by Calhex-231 from 3.88 ± 0.15 to 3.31 ± 0.15 ($P < 0.05$).

Effects of CaCl₂ and Calhex-231 on H₂S generation in mouse bladder tissue

We studied the effects of CaCl₂ and Calhex-231 on H₂S generation. Mouse bladder tissue generated detectable amounts of basal H₂S (0.11 ± 0.02 nmol/mg). L-cysteine increased basal H₂S generation (0.28 ± 0.05 nmol/mg), and CSE inhibitor PAG (10 mM) and CBS inhibitor AOAA (1 mM) reduced the increase in H₂S production induced by L-cysteine from 0.28 ± 0.05 nmol/mg to 0.13 ± 0.03 nmol/mg and 0.13 ± 0.02 nmol/mg respectively, suggesting that mouse bladder tissue is capable of synthesizing H₂S from L-cysteine. Also, Calhex-231, a CaSRs-specific inhibitor, reduced the increase in H₂S production in the presence of L-cysteine (0.07 ± 0.02 nmol/mg). In addition, CaCl₂ increased basal H₂S generation (0.31 ± 0.04 nmol/mg), and PAG, AOAA and Calhex-231 reduced the increase in H₂S production induced by CaCl₂ from 0.32 ± 0.04 nmol/mg to 0.19 ± 0.01 nmol/mg, 0.18 ± 0.01 nmol/mg and 0.16 ± 0.02 nmol/mg respectively, (Fig. 3), suggesting an interaction between H₂S and CaSRs pathway, and the interaction may occur through the CSE and CBS enzyme in the mouse bladder tissue.

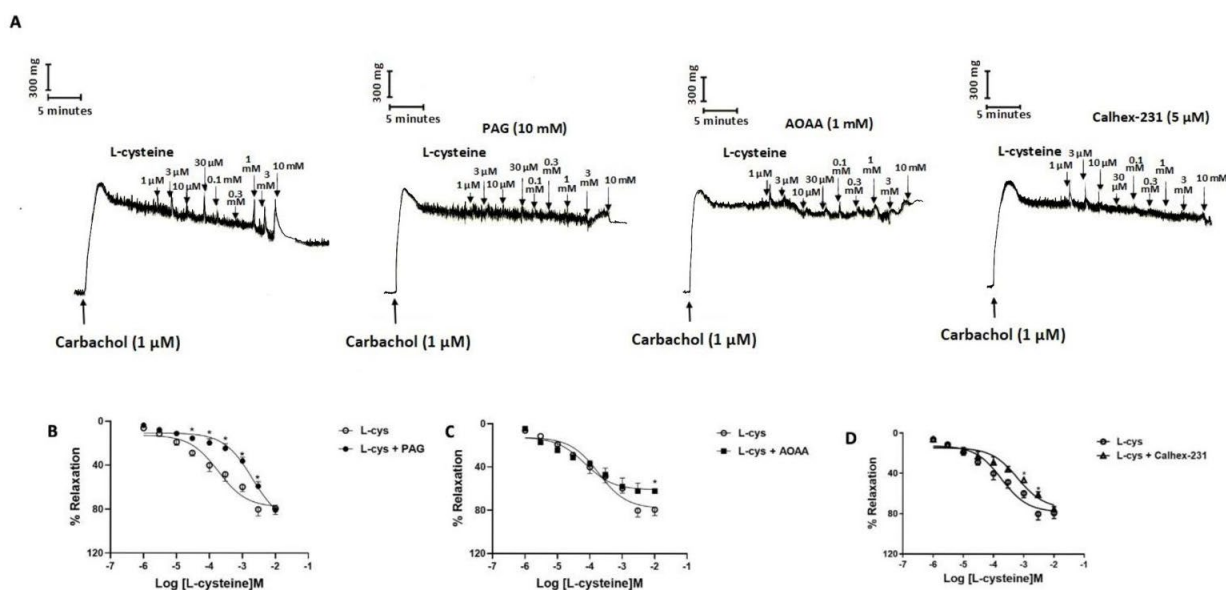


Fig. 2. The role of Calcium Sensing Receptors (CaSRs) on L-cysteine-induced relaxations. Representative traces L-cysteine-induced relaxations (A). Graph showing that L-cysteine-induced relaxations in the presence of PAG (Cystathionine-gamma-lyase (CSE) inhibitor, 10 mM) (B), AOAA (Cystathionine beta synthase (CBS) inhibitor, 1 mM) (C) and Calhex-231 (CaSR inhibitor, 5 μ M) (D) in mouse bladder strips. All values are mean \pm S.E.M. ($n=6$). * $P < 0.05$ significantly different from the control; unpaired t -test followed by Bonferroni's comparison test.

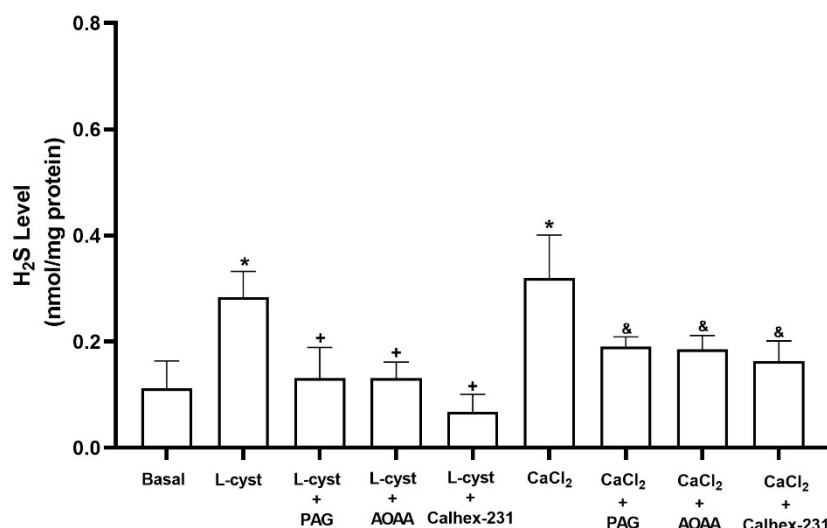


Fig. 3. The role of CBS, CSE and Calcium Sensing Receptors (CaSRs) inhibition on endogenous H₂S formation. The bar graph shows the effects of L-cysteine (L-cyst; 10 mM), and CaCl₂ (10 mM) in the absence or presence of PAG (Cystathionine-gamma-lyase (CSE) inhibitor, 10 mM), AOAA (Cystathionine beta synthase (CBS) inhibitor, 1 mM), and Calhex-231 (CaSRs inhibitor, 5 μ M). All values are mean \pm S.E.M. (n=4). * P<0.05 significantly different from basal; + P<0.05 significantly different from L-cysteine; & P<0.05 significantly different from CaCl₂; unpaired t-test followed by Bonferroni's comparison test.

Discussion

In the present study, we investigated the role of L-cysteine/H₂S pathway in CaSR-mediated responses in mouse bladder tissue. We found that 1) CaCl₂ produced relaxant responses. 2) CaCl₂-induced relaxations were inhibited by Calhex-231, a CaSR-specific inhibitor. 3) L-cysteine-induced-relaxations were inhibited by PAG and AOAA, CSE and CBS enzyme inhibitors, respectively. 4) PAG and AOAA reduced the relaxations to CaCl₂. 5) Calhex-231 reduced the relaxations to L-cysteine. 6) Calhex-231 reduced the increase in H₂S production in the presence of L-cysteine. In addition, CaCl₂ increased basal H₂S generation, and PAG, AOAA and Calhex-231 reduced the increase in H₂S production stimulated with CaCl₂. These findings suggest that there is an interaction between L-cysteine/H₂S pathway and CaSRs, and CSE/CBS-induced endogenous H₂S may partly contribute to the relaxation responses due to CaSRs activation in mouse bladder.

CaSRs, a G-protein coupled receptor, trigger intracellular signals *via* the modulation of a series of intracellular signaling proteins and modulate several physiological functions. The presence of CaSRs has been demonstrated in aortic endothelial cells and vascular smooth muscle cells including human artery [7,8,37,38]. Also, it has been reported that CaSRs are expressed in rat bladder urothelium and activation of these receptors reduces detrusor activity [28]. H₂S is a gaseous neurotransmitter that has a relaxing effect on vascular and extravascular smooth muscles. H₂S is synthesized endogenously from L-cysteine *via* CSE, CBS and 3-MST enzymes in mammalian tissues. It has been reported that H₂S is synthesized in mouse, rat, pig and human bladder

tissues [25,27,29,39]. H₂S produced a concentration-dependent contraction and relaxation response in isolated bladder tissues [25,40]. In the present study, we obtained concentration-dependent relaxation to L-cysteine in mouse bladder. Taken together, we propose that the effect of L-cysteine on bladder smooth muscle tone may vary depending on the type of pre-treatment and its concentration. It has been reported that H₂S synthesis is associated with an increase in the amount of intracellular Ca²⁺ in vascular smooth muscle cells and CaSRs activation increases CSE expression and H₂S synthesis in vascular smooth muscle [33]. However, it has not been previously investigated the interaction between H₂S and CaSRs in bladder smooth muscle tissue. In the present study, we investigated the role of L-cysteine/H₂S pathway in CaSRs-mediated responses in mouse bladder tissue. For this purpose, CaCl₂ was applied as a bolus into bladder tissues contracted by carbachol for CaSRs activation, and CaCl₂ produced concentration-dependent relaxations. To confirm that CaCl₂ relaxation is mediated *via* CaSRs, CaCl₂-induced relaxant responses were studied in the presence of Calhex-231, negative allosteric modulators of CaSRs or calcilytics [15,41,42]. CaCl₂ relaxations significantly decreased in the presence of Calhex-231, suggesting that CaCl₂ responses are dependent on CaSRs activation in bladder tissue. In consistent with our findings, it has been reported that CaCl₂-induced relaxant responses were significantly reduced in the presence of Calhex-231 in rat and rabbit mesenteric artery tissues, suggesting that CaSRs mediate CaCl₂ relaxations [8,14,17]. In our study, we aimed to investigate the role of endogenous H₂S in CaSRs-mediated relaxations, we studied the effects of PAG and AOAA, CSE and CBS enzyme inhibitor, respectively, on

CaCl₂-induced relaxant responses. PAG and AOAA caused a significant decrease in CaCl₂ relaxation responses. This finding functionally suggests that CaCl₂-mediated relaxations may partly dependent on CSE/CBS-induced endogenous H₂S formation. Consistent with this, we observed that CaCl₂ enhanced basal H₂S formation, and PAG, AOAA and Calhex-231 markedly reduced the augmentation in H₂S production in the presence of CaCl₂, indicating that the interaction between CaSRs and L-cysteine/H₂S pathway mainly occurs through the CSE and CBS enzymes in the mouse bladder tissues. Also, it has been reported that CSE-induced H₂S synthesis increases by CaSRs activation with CaCl₂ in VSMCs [33].

Furthermore, there may be a two-way interaction between CaSRs and H₂S, such as CaCl₂ increasing H₂S production through CSE/CBS enzyme activation and H₂S causing relaxation by activating CaSRs. Consequently, we studied the role of CaSRs in relaxant responses to L-cysteine/H₂S in bladder tissues. L-cysteine, H₂S substrate, caused a concentration-dependent relaxation response on carbachol-constricted isolated mouse bladder tissues. In the present study, inhibition of L-cysteine-induced relaxation responses in the presence of PAG and AOAA confirms that these relaxations are caused by endogenous H₂S. Also, these findings functionally demonstrate the role of CSE and CBS enzymes in endogenous H₂S synthesis in mouse bladder tissue. Similar to our findings, Fusco *et al.* showed that L-cysteine-induced relaxations were significantly reduced in the presence of PAG and AOAA in isolated human bladder tissue [27]. Also, expressions of CSE, CBS, and 3-MST enzymes and L-cysteine-mediated H₂S production were shown in mouse, rat, guinea pig, and human bladder tissues [24-26,39,43]. Consistent with studies, we recently reported the presence of endogenous H₂S-generating enzymes in mouse bladder tissue [29]. To determine the possible contribution of CaSRs to endogenous H₂S relaxation responses, the effect of Calhex-231 on L-cysteine-induced relaxant responses were investigated. In the presence of Calhex-231, a significant decrease in

L-cysteine-induced relaxations was observed, and Calhex-231 reduced the increase in H₂S production in the presence of L-cysteine, suggesting that endogenous H₂S responses may be associated with CaSRs in mouse bladder tissue. To our knowledge, this is the first report to show the involvement of CaSRs on endogenous L-cysteine/H₂S pathway in bladder tissues. Some studies also confirmed that CaSRs activation mediated the H₂S enzymes pathway in vascular smooth muscle cells [33,34]. Also, Zhong *et al.* demonstrated that CaSRs regulated the endogenous CSE/H₂S pathway to inhibit the proliferation of vascular smooth muscle cells in both diabetic and high glucose models [33]. In addition, it has been reported that CaSR modulates CSE/H₂S pathway and is associated with PLC-IP₃ receptor and calmodulin signaling which inhibits the proliferation of VSMCs *via* the Erk1/2 dependent signaling pathway in hyperhomocysteinemia [34]. Our results are in agreement with previous studies showing the contribution of CaSRs to endogenous H₂S-induced physiological responses. Further studies are needed to clarify the interaction between L-cysteine/H₂S and CaSRs pathway in bladder tissue.

In conclusion, these results suggest that there is an interaction between L-cysteine/H₂S and CaSRs through activation of CBS and CSE enzymes. Also, the mechanism of CaSRs-mediated relaxant responses involves, at least in part, CSE and CBS-generated H₂S in mouse bladder. There may be a two-way interaction between CaSRs and H₂S, such as CaCl₂ increasing H₂S production through CSE/CBS enzyme activation and H₂S causing relaxation *via* activation of CaSRs. This is the first time the interaction between CaSRs and L-cysteine/H₂S pathway has been demonstrated in bladder tissue.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This study was granted by the Çukurova University Research Foundation (TYL-2023-16387).

References

1. Brown EM, MacLeod RJ. Extracellular calcium sensing and extracellular calcium signaling. *Physiol Rev* 2001;81:239-297. <https://doi.org/10.1152/physrev.2001.81.1.239>
2. Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, et al. Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature* 1993;366:575-580. <https://doi.org/10.1038/366575a0>

3. Ward DT. Calcium receptor-mediated intracellular signalling. *Cell Calcium* 2004;35:217-228. <https://doi.org/10.1016/j.ceca.2003.10.017>
4. Tfelt-Hansen J, Brown EM. The calcium-sensing receptor in normal physiology and pathophysiology: a review. *Crit Rev Clin Lab Sci* 2005;2:35-70. <https://doi.org/10.1080/10408360590886606>
5. Hannan FM, Kallay E, Chang W, Brandi ML, Thakker RV. The calcium-sensing receptor in physiology and in calcitropic and noncalcitropic diseases. *Nat Rev Endocrinol* 2018;15:33-51. <https://doi.org/10.1038/s41574-018-0115-0>
6. Schepelmann M, Yarova PL, Lopez-Fernandez I, Davies TS, Brennan SC, Edwards PJ, Aggarwal A, et al. The vascular Ca²⁺-sensing receptor regulates blood vessel tone and blood pressure. *Am J Physiol Cell Physiol* 2016;310:C193-C204. <https://doi.org/10.1152/ajpcell.00248.2015>
7. Weston AH, Absi M, Ward DT, Ohanian J, Dodd RH, Dauban P, Petrel C, et al. Evidence in favor of a calcium-sensing receptor in arterial endothelial cells: studies with calindol and Calhex 231. *Circ Res* 2005;97:391-398. <https://doi.org/10.1161/01.RES.0000178787.59594.a0>
8. Greenberg HZE, Jahan KS, Shi J, Vanessa Ho WS, Albert AP. The calcilytics Calhex-231 and NPS 2143 and the calcimimetic Calindol reduce vascular reactivity via inhibition of voltage-gated Ca²⁺ channels. *Eur J Pharmacol* 2016;791:659-668. <https://doi.org/10.1016/j.ejphar.2016.10.008>
9. Berra Romani R, Raqeeb A, Laforenza U, Scaffino MF, Moccia F, Avelino-Cruz JE, Oldani A, et al. Cardiac microvascular endothelial cells express a functional Ca⁺ -sensing receptor. *J Vasc Res* 2009;46:73-82. <https://doi.org/10.1159/000140677>
10. Wonneberger K, Scofield MA, Wangemann P. Evidence for a calcium-sensing receptor in the vascular smooth muscle cells of the spiral modiolar artery. *J Membr Biol* 2000;175:203-212. <https://doi.org/10.1007/s002320001068>
11. Molostvov G, Fletcher S, Bland R, Zehnder D. Extracellular calcium-sensing receptor mediated signalling is involved in human vascular smooth muscle cell proliferation and apoptosis. *Cell Physiol Biochem* 2008;22:413-422. <https://doi.org/10.1159/000185484>
12. Alam MU, Kirton JP, Wilkinson FL, Towers E, Sinha S, Rouhi M, Vizard TN, et al. Calcification is associated with loss of functional calcium-sensing receptor in vascular smooth muscle cells. *Cardiovasc Res* 2009;81:260-268. <https://doi.org/10.1093/cvr/cvn279>
13. Bukoski RD, Bian K, Wang Y, Mupanomunda M. Perivascular sensory nerve Ca²⁺ receptor and Ca²⁺-induced relaxation of isolated arteries. *Hypertension* 1997;30:1431-1439. <https://doi.org/10.1161/01.HYP.30.6.1431>
14. Thakore P, Ho WS. Vascular actions of calcimimetics: role of Ca²(+) -sensing receptors versus Ca²(+) influx through L-type Ca²(+) channels. *Br J Pharmacol* 2011;162:749-762. <https://doi.org/10.1111/j.1476-5381.2010.01079.x>
15. Petrel C, Kessler A, Maslah F, Dauban P, Dodd RH, Rognan D, Ruat M. Modeling and mutagenesis of the binding site of Calhex 231, a novel negative allosteric modulator of the extracellular Ca(2+)-sensing receptor. *J Biol Chem* 2003;278:49487-49494. <https://doi.org/10.1074/jbc.M308010200>
16. Awumey EM, Bridges LE, Williams CL, Diz DI. Nitric-oxide synthase knockout modulates Ca²⁺-sensing receptor expression and signaling in mouse mesenteric arteries. *J Pharmacol Exp Ther* 2013;346:38-47. <https://doi.org/10.1124/jpet.113.205534>
17. Greenberg HZ, Shi J, Jahan KS, Martinucci MC, Gilbert SJ, Vanessa Ho WS, Albert AP. Stimulation of calcium-sensing receptors induces endothelium-dependent vasorelaxations via nitric oxide production and activation of IKCa channels. *Vascul Pharmacol* 2016;80:75-84. <https://doi.org/10.1016/j.vph.2016.01.001>
18. Loot AE, Pierson I, Syzonenko T, Elgheznavy A, Randriamboavonjy V, Zivković A, Stark H, Fleming I. Ca²⁺-sensing receptor cleavage by calpain partially accounts for altered vascular reactivity in mice fed a high-fat diet. *J Cardiovasc Pharmacol* 2013;61:528-535. <https://doi.org/10.1097/FJC.0b013e31828d0fa3>
19. Smajilovic S, Sheykhzade M, Holmegard HN, Haunso S, Tfelt-Hansen J. Calcimimetic, AMG 073, induces relaxation on isolated rat aorta. *Vascul Pharmacol* 2007;47:222-228. <https://doi.org/10.1016/j.vph.2007.06.010>
20. Ziegelstein RC, Xiong Y, He C, Hu Q. Expression of a functional extracellular calcium-sensing receptor in human aortic endothelial cells. *Biochem Biophys Res Commun* 2006;342:153-163. <https://doi.org/10.1016/j.bbrc.2006.01.135>

21. Abe K, Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* 1996;16:1066-1071. <https://doi.org/10.1523/JNEUROSCI.16-03-01066.1996>
22. Shibuya N, Tanaka M, Yoshida M, Ogasawara Y, Togawa T, Ishii K, Kimura H. 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. *Antioxid Redox Signal* 2009;11:703-714. <https://doi.org/10.1089/ars.2008.2253>
23. Tang C, Li X, Du J. Hydrogen sulfide as a new endogenous gaseous transmitter in the cardiovascular system. *Curr Vasc Pharmacol* 2006;4:17-22. <https://doi.org/10.2174/157016106775203144>
24. Matsunami M, Miki T, Nishiura K, Hayashi Y, Okawa Y, Nishikawa H, Sekiguchi F, et al. Involvement of the endogenous hydrogen sulfide/Ca(v) 3.2 T-type Ca²⁺ channel pathway in cystitis-related bladder pain in mice. *Br J Pharmacol* 2012;167:917-928. <https://doi.org/10.1111/j.1476-5381.2012.02060.x>
25. Gai JW, Wahafu W, Guo H, Liu M, Wang XC, Xiao YX, Zhang L, et al. Further evidence of endogenous hydrogen sulphide as a mediator of relaxation in human and rat bladder. *Asian J Androl* 2013;15:692-696. <https://doi.org/10.1038/aja.2013.32>
26. Fernandes VS, Ribeiro AS, Barahona MV, Orensanz LM, Martínez-Sáenz A, Recio P, Martínez AC, et al. Hydrogen sulfide mediated inhibitory neurotransmission to the pig bladder neck: role of KATP channels, sensory nerves and calcium signaling. *J Urol* 2013;190:746-756. <https://doi.org/10.1016/j.juro.2013.02.103>
27. Fusco F, di Villa Bianca Rd, Mitidieri E, Cirino G, Sorrentino R, Mirone V. Sildenafil effect on the human bladder involves the L-cysteine/hydrogen sulfide pathway: a novel mechanism of action of phosphodiesterase type 5 inhibitors. *Eur Urol* 2012;62:1174-1180. <https://doi.org/10.1016/j.eururo.2012.07.025>
28. di Villa Bianca Rd, Cirino G, Sorrentino R. Hydrogen Sulfide and Urogenital Tract. *Handb Exp Pharmacol* 2015;230:111-136. https://doi.org/10.1007/978-3-319-18144-8_5
29. Dalkir FT, Aydinoglu F, Ogulener N. The role of rhoA/rho-kinase and PKC in the inhibitory effect of L-cysteine/H₂S pathway on the carbachol-mediated contraction of mouse bladder smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol* 2023;396:2023-2038. <https://doi.org/10.1007/s00210-023-02440-6>
30. Eto K, Kimura H. A novel enhancing mechanism for hydrogen sulfide-producing activity of cystathionine beta-synthase. *J Biol Chem* 2002;277:42680-42685. <https://doi.org/10.1074/jbc.M205835200>
31. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, et al. H₂S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 2008;322:587-590. <https://doi.org/10.1126/science.1162667>
32. Fu M, Zhang W, Wu L, Yang G, Li H, Wang R. Hydrogen sulfide (H₂S) metabolism in mitochondria and its regulatory role in energy production. *Proc Natl Acad Sci U S A* 2012;109:2943-2948. <https://doi.org/10.1073/pnas.1115634109>
33. Zhong X, Wang Y, Wu J, Sun A, Yang F, Zheng D, Li T, et al. Calcium sensing receptor regulating smooth muscle cells proliferation through initiating cystathionine-gamma-lyase/hydrogen sulfide pathway in diabetic rat. *Cell Physiol Biochem* 2015;35:1582-1598. <https://doi.org/10.1159/000373973>
34. Wang Y, Wang X, Liang X, Wu J, Dong S, Li H, Jin M, et al. Inhibition of hydrogen sulfide on the proliferation of vascular smooth muscle cells involved in the modulation of calcium sensing receptor in high homocysteine. *Exp Cell Res* 2016;347:184-191. <https://doi.org/10.1016/j.yexcr.2016.08.004>
35. Wu WY, Lee SP, Chiang BJ, Lin WY, Chien CT. Urothelial Calcium-Sensing Receptor Modulates Micturition Function via Mediating Detrusor Activity and Ameliorates Bladder Hyperactivity in Rats. *Pharmaceuticals (Basel)* 2021;14:960. <https://doi.org/10.3390/ph14100960>
36. Aydinoglu F, Erdem EN, Toyran T, Ogulener N. Impairment of Endogenous H₂S Pathway due to Aging and Endothelium Denudation in Mouse Isolated Thoracic Aorta. *Physiol Res* 2025;74:59-68. <https://doi.org/10.33549/physiolres.935419>
37. Molostvov G, James S, Fletcher S, Bennett J, Lehnert H, Bland R, Zehnder D. Extracellular calcium-sensing receptor is functionally expressed in human artery. *Am J Physiol Renal Physiol* 2007;293:F946-F955. <https://doi.org/10.1152/ajprenal.00474.2006>
38. Smajilovic S, Hansen JL, Christoffersen TE, Lewin E, Sheikh SP, Terwilliger EF, Brown EM, et al. Extracellular calcium sensing in rat aortic vascular smooth muscle cells. *Biochem Biophys Res Commun* 2006;348:1215-1223. <https://doi.org/10.1016/j.bbrc.2006.07.192>

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39. Zou S, Shimizu T, Shimizu S, Higashi Y, Nakamura K, Ono H, Aratake T, et al. Possible role of hydrogen sulfide as an endogenous relaxation factor in the rat bladder and prostate. *Neurourol Urodyn* 2018;37:2519-2526. <https://doi.org/10.1002/nau.23788>
 40. Patacchini R, Santicioli P, Giuliani S, Maggi CA. Pharmacological investigation of hydrogen sulfide (H₂S) contractile activity in rat detrusor muscle. *Eur J Pharmacol* 2005;509:171-177. <https://doi.org/10.1016/j.ejphar.2005.01.005>
 41. Nemeth EF, Delmar EG, Heaton WL, Miller MA, Lambert LD, Conklin RL, Gowen M, et al. Calcilytic compounds: potent and selective Ca²⁺ receptor antagonists that stimulate secretion of parathyroid hormone. *J Pharmacol Exp Ther* 2001;299:323-331. [https://doi.org/10.1016/S0022-3565\(24\)29333-2](https://doi.org/10.1016/S0022-3565(24)29333-2)
 42. Kessler A, Faure H, Roussanne MC, Ferry S, Ruat M, Dauban P, Dodd RH. N(1)-Arylsulfonyl-N(2)-(1-(1-naphthyl)ethyl)-1,2-diaminocyclohexanes: a new class of calcilytic agents acting at the calcium-sensing receptor. *Chembiochem* 2004;5:1131-1136. <https://doi.org/10.1002/cbic.200400049>
 43. Wang W, Bo Q, Du J, Yu X, Zhu K, Cui J, Zhao H, et al. Endogenous H₂S sensitizes PAR4-induced bladder pain. *Am J Physiol Renal Physiol* 2018;14:F1077-F1086. <https://doi.org/10.1152/ajprenal.00526.2017>
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