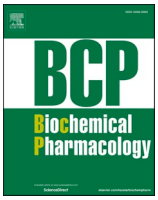
Biochemical Pharmacology 215 (2023) 115754

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm



The bitter taste receptor (TAS2R) agonist denatonium promotes a strong relaxation of rat corpus cavernosum

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ARTICLE INFO

Keywords:
Bitter taste receptors
TAS2R agonists
Denatonium
Smooth muscle relaxation
Rat corpus cavernosum

ABSTRACT

Bitter taste receptors (TAS2R) are found in numerous extra-oral tissues, including smooth muscle (SM) cells in both vascular and visceral tissues. Upon activation, TAS2R stimulate the relaxation of the SM. Nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling pathway is involved in penile erection, and type 5 phosphodiesterase (PDE5) inhibitors, a cGMP-specific hydrolase are used as first-line treatments for erectile dysfunction (ED). Nevertheless, PDE5 inhibitors are ineffective in a considerable number of patients, prompting research into alternative pharmacological targets for ED. Since TAS2R agonists regulate SM contractility, this study investigates the role of TAS2Rs in rat corpus cavernosum (CC). We performed immunohistochemistry to detect TAS2R10, isometric force recordings for TAS2R agonists denatonium and chloroquine, the slow-release H_2S donor GYY 4137, the NO donor SNAP, the β-adrenoceptor agonist isoproterenol and electrical field stimulation (EFS), as well as measurement of endogenous hydrogen sulfide (H_2S) production. The immunofluorescence staining indicated that TAS2R10 was broadly expressed in the CC SM and to some extent in the nerve fibers. Denatonium, chloroquine, SNAP, and isoproterenol cause potent dose-dependent SM relaxations. H_2S production was decreased by NO and H_2S synthase inhibitors, while it was enhanced by denatonium. In addition, denatonium increased the relaxations induced by GYY 4137 and SNAP but failed to modify EFS- and isoproterenol-induced responses. These results suggest neuronal and SM TAS2R10 expression in the rat CC, where denatonium induces a strong SM relaxation *per se* and promotes the H_2S- and NO-mediated inhibitory gaseous neurotransmission. Thus, TAS2R10 might represent a valuable therapeutic target in ED.

1. Introduction

Bitter taste receptors (TAS2R) are G protein-coupled receptors (GPCR) located on the surface of cells in the tongue, which play a role in gustatory taste perception [1]. Humans have a family of 25 type A G protein-coupled receptors (GPCRs) for TAS2Rs, whereas rats and mice have 35 TAS2Rs [2–4]. TAS2R also plays important extra-oral functions, including the relaxation of vascular and visceral smooth muscle (SM) of the respiratory, gastrointestinal, and urinary tracts [2,4,5]. In fact, airway SM cells express TAS2R that mediates relaxations in both mouse and human-isolated airway samples [6]. The potent bronchorelaxation in human airway SM caused by TAS2R activation suggested the possible use of TAS2R agonists in the bronchodilator therapy of asthma and/or chronic obstructive pulmonary disease [7]. TAS2R agonists also relax rat

Abbreviations: cAMP, cyclic adenosine monophosphate; CC, corpus cavernosum; cGMP, cyclic guanosine monophosphate; CSE, cystathionine γ-lyase; EFS, electrical field stimulation; ED, erectile dysfunction; GPCRs, G protein-coupled receptors; GYY 4137, P-(4-Methoxyphenyl)-P-4-morpholinylphosphinodithioic acid morpholine salt; H_2S, hydrogen sulfide; NANC, non-adrenergic non-cholinergic; NO, nitric oxide; NOS, nitric oxide synthase; PDE4, phosphodiesterase type 4; PDE5, phosphodiesterase type 5; PhE, phenylephrine; SM, smooth muscle; SNAP, (S)-Nitroso-N-acetylpenicillamine; TAS2R, bitter taste receptors; TAS2R10, bitter taste receptor 10; VOCC, voltage-operated calcium channels.

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https://doi.org/10.1016/j.bcp.2023.115754
Received 29 May 2023; Received in revised form 15 August 2023; Accepted 16 August 2023
Available online 18 August 2023

and mouse ileum SM in vitro and inhibited gastrointestinal motility in vivo, thus indicating that TAS2R agonists can be used as anti-spasmodic drugs [8]. In the bladder, chloroquine and denatonium cause relaxations of human and mouse detrusor, and chloroquine alleviates the symptoms of an overactive bladder in mice with partial obstruction of the bladder outlet, therefore, TAS2Rs have the potential to be a novel target for the development of drugs aimed at treating the symptoms associated with an overactive bladder [5]. In SM cells, TAS2Rs activation triggers a cascade of events involving G proteins, phospholipase C, and inositol trisphosphate (IP_3), ultimately resulting in the release of Ca^{2+} from IP3-sensitive Ca^{2+} stores. Increased intracellular Ca^{2+} concentration might activate large-conductance Ca^{2+}-activated K^+ channels (BK) which results in hyperpolarization of the cell membrane and/or inhibition of membrane voltage-operated Ca^{2+} channels (VOCC) [9].

In the corpus cavernosum (CC), non-adrenergic non-cholinergic (NANC) nerve-mediated vasorelaxation is caused by gaseous transmitters nitric oxide (NO) and hydrogen sulfide (H_2S) [10–12]. NO is known to play a key role in regulating nerve-dependent penile vasorelaxation since NO synthase (NOS) inhibitors selectively block neurogenic erectile responses being the neuronal isoform of NOS localized in the penile innervation [13–15]. NO induces SM relaxation by activating soluble guanylyl cyclase, which increases the concentration of intracellular cyclic guanosine monophosphate (cGMP). The elevated cGMP levels lead to a decrease in cytosolic Ca^{2+} levels and modifications in protein phosphorylation, ultimately causing SM relaxation [16]. The CC expresses phosphodiesterase type 5 (PDE5), an enzyme that selectively breaks down cyclic guanosine monophosphate (cGMP). PDE5 inhibitors such as sildenafil, vardenafil, and tadalafil increase the concentration of intracellular cGMP in cavernous tissue, leading to penile erection. Consequently, these inhibitors are commonly used in the treatment of erectile dysfunction (ED) [17,18]. In addition to NO, H_2S is another gaseous transmitter vasodilator that is involved in the neurogenic-dependent relaxation of human CC smooth muscle [19]. Endogenous H_2S production was significantly decreased due to low activities of H_2S synthesis enzymes, cystathionine γ-lyase (CSE), and cystathionine β-synthase in a rat model of diabetes accompanied by ED [20]. The potent vasodilatory and pro-erectile effect of H_2S on cavernous tissue is produced through cyclic adenosine monophosphate (cAMP)-dependent mechanisms [12]. PDE4, which selectively hydrolyzes cAMP, is expressed in the nerve cells of the CC smooth muscle and plays a key role in regulating rat CC tone. PDE4 inhibition and consequent increases in cAMP levels produce a potent SM relaxation by facilitating gasotransmitters-induced nerve-mediated response [21]. Interestingly, an interplay between endogenous H_2S and NO has been reported to be implicated in erectile function [22–25]. H_2S exerts its pro-erectile effects by enhancing the NO pathway, as H_2S levels are closely linked to the activity of the NO enzyme and the cGMP levels in the CC tissue. This indicates that, in CC tissues, H_2S has an important role in erectile function modulating the NO enzyme activity, which brings out a synergic role of both gasotransmitters regulating erectile function [22–25]. Thus, in rat CC, PDE4 inhibitors modulate, in a positive feedback fashion, nerve-mediated relaxation induced by both gaseous molecules, therefore indicating a pivotal role for neuronal PDE4 in penile erection [21].

PDE5 inhibitors are currently used in ED treatment. However, they fail in at least 30% of patients, especially in men with diabetes [26–29], so it is necessary to search for new therapeutic targets. TAS2R agonists induce vascular and visceral SM relaxation so TAS2R has been suggested as a target for pathologies related to impaired smooth muscle contractility [8]. Therefore, the present aims to investigate the expression and function of TAS2R in rat CC smooth muscle.

2. Materials and methods

2.1. Animals

Male Wistar rats aged 17–18 weeks and weighing between 270 and 360 g were used in the experimental protocols, which were carried out at the Animal Facility of the Department of Physiology at Complutense University of Madrid. The rats were housed in a 12:12 h light/dark cycle and provided with ad libitum access to food and water until the study. To perform euthanasia, the rats were first subjected to isoflurane inhalation (~5%) followed by cervical dislocation. The Institutional Animal Care and Use Committee of Complutense University of Madrid approved all protocols involving animals, which were conducted in compliance with the European Union Directive 2010/63/EU.

2.2. Corpus cavernosum extraction

After euthanizing the rats, the crura of the corpus cavernosum were excised at the attachment site to the pubic ramus. The penis was then gently detached and transferred to a physiological saline solution (PSS) at 4 °C, which had the following composition (in mM): NaCl 119, $NaHCO_3$ 24.9, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, ethylenediamine tetraacetic acid (EDTA) 0.027, glucose 11 and $CaCl_2$ 1.5, continuously gassed with a mixture of 5% CO_2 and 95% O_2 to maintain pH at 7.4. Next, strips of CC were obtained by carefully dissecting the tunica albuginea and surrounding connective tissues. The cavernous tissue samples were then maintained in PSS at a temperature of 4 °C to preserve their physiological properties.

2.3. Western blot

Total protein from rat CC was extracted and quantified by the Lowry method (DC Protein Assay Kit, Bio-Rad, Madrid, Spain). 50 μg was separated using a 10% polyacrylamide gel (SDS-PAGE) electrophoresis. Next, the gels were transferred to nitrocellulose membranes and incubated overnight at 4 °C with a 1:50 dilution of antibody anti-TAS2R10 (PA5-39708, Invitrogen, Madrid, Spain). Mouse anti-β-actin blots were used as a loading control (sc-47778, Santa Cruz Biotechnology Inc. Heidelberg, Germany). Secondary antibody (1:5000 dilution, m-IgG2a BP-HRP, sc-542731, Santa Cruz Biotechnology Inc. Heidelberg, Germany) were then added to washed membranes and kept for 60 min at room temperature (RT); the blots were revealed with ECL mixture (ECL Select-kit, GE Healthcare, Madrid, Spain) by chemiluminescence (Image-Quant LAS 500, GE Healthcare, Madrid, Spain).

2.4. Double-labeling immunofluorescence assays

Rat CC samples were fixed in 4% paraformaldehyde (prepared in PBS 1X) for four hours, followed by washing in PBS 1X. Next, samples were set in 30% of sucrose until the tissue sinks. After dehydration, the tissue was embedded in an OCT compound for frozen sectioning. Sections of 5 μm thickness were incubated in a blocking buffer containing PBS 1X, 5% BSA, 0.3% Triton X-100, and 10% normal goat serum for 2–3 h. Then, the sections were incubated with rabbit anti-TAS2R10 antibody (1:150 dilution, PA5-39708, Invitrogen, Madrid, Spain) and mouse anti-protein gene product 9.5 antibody (1:50 dilution, ab8189 Abcam, Cambridge, UK) for 48 h at 4 °C. After washing in PBS 1X, the sections were incubated with fluorochrome-labelled secondary antibodies (Alexa Fluor 594 goat anti-rabbit A11037 and Alexa Fluor 488 goat anti-mouse A11029, 1:200 dilution Invitrogen, Madrid, Spain) for 3 h at room temperature. The nuclei were stained with DAPI (ProLong Gold antifade reagent with DAPI, P36935 Molecular Probes, Oregón, USA), and the sections were mounted for visualization under a microscope.

2.5. Endogenous H2S measurement

Endogenous H_2S was quantified as previously described [30]. In summary, CC strips were incubated for 1 h with DL-propargylglycine (PPG, 1 mM), N^G-nitro-L-arginine (L-NOARG, 100 μM), or denatonium (0.1, 1 and 10 μM) to investigate the role of the cystathionine gamma lyase (CSE), the NO synthase (NOS), and the TAS2R agonist, respectively [18]. After incubation, CC samples were homogenized (1:10 w/v) in cooled 50 mM potassium phosphate buffer, pH 6.8. Each chamber was prepared with 50 mg of the homogenates and 1 ml of incubation solution (100 mM potassium phosphate buffer, 2 mM pyridoxal 5′-phosphate, 10 mM L-cysteine, pH 7.4). To start the reactions the chambers were then transferred from ice to a prewarmed shaking water bath and kept at 37 °C. After 30 min of incubation, zinc acetate (1%) was introduced the chambers to catch generated H_2S. The reaction was ended by protein denaturation adding 500% (w/v) of trichloroacetic acid and next, was mixed with 0.5 ml of N,N-dimethyl-p-phenylenediamine sulfate (20 mM in 7.2 M HCl) followed by 0.4 ml of $FeCl_3$ (30 mM in 1.2 M HCl). After 20 min, the final solution was placed in a spectrophotometer and measured at a wavelength of 670 nm. H_2S concentration was determined against a standard NaHS curve. Results are stated as nanomoles of H_2S per mg of soluble protein in 20 min (nmol/mg.min-^{1}). The protein was quantified with Lowry assay (DC Protein Assay Kit, Bio-Rad, Madrid, Spain).

2.6. Isometric force recordings

Two CC strips, each measuring 3 mm in length and 2 mm in width, were obtained from each penis and placed horizontally in myographs (DMT, Muscle Strip System – 820MS, Danish Myotechnology, Denmark). The myographs were filled with 5 ml of PSS and maintained at a temperature of 37 °C, while the pH was adjusted to 7.4 by continuous aeration with 95% to 5% O_2/CO_2. One end of the sample was secured to a micrometer screw, while the other was connected to an isometric force transducer for the measurement of isometric force. The data acquisition was carried out using a PowerLab v7.0 system (ADInstruments Inc, Oxford, UK). After being mounted, the strips were allowed to equilibrate for 60 min at a passive tension of 4.0 mN in a myograph filled with 5 ml of PSS and aerated continuously with 95% to 5% O_2/CO_2 to maintain a pH of 7.4. The PSS solution was replaced every 20 min, and the contractile function of the CC preparations was assessed using a 124 mM potassium-rich PSS (K-PSS 124 mM). The crura CC strips were contracted by exposing them to phenylephrine (PhE, 3 μM) and concentration-dependent relaxation curves were then obtained for TAS2R agonists, denatonium and chloroquine, the NO donor SNAP, and the β-adrenoceptor agonist isoproterenol. To perform the electrical field stimulation (EFS) experiments, Crura CC Strip samples were incubated for one hour with guanethidine (10 μM) and atropine (1 μM) to block noradrenergic neurotransmission and muscarinic receptors, respectively. Under these experimental conditions, the CC samples were precontracted with 3 μM PhE, and frequency-dependent relaxation curves were generated in the presence or absence of a threshold concentration of denatonium (0.1 μM). EFS stimuli were generated by applying a rectangular pulse (frequency range of 0.5–16 Hz, 1 ms duration, 20 s trains, and current output of 75 mA) every 4 min through two platinum electrodes positioned on either side of the samples' longitudinal axis (CS20 stimulator, Cibertec, Barcelona, Spain).

2.7. Compounds and solutions

Compounds utilized were atropine, chloroquine, denatonium, DL-propargylglycine (PPG), guanethidine, isoproterenol, N^G-nitro-L-arginine (L-NOARG), and phenylephrine (PhE) all from Sigma (St Louis, MO, USA). P-(4-Methoxyphenyl)-P-4-morpholinylphosphinodithioic acid morpholine salt (GYY 4137) and (S)-Nitroso-N-acetylpenicillamine (SNAP) were provided from Tocris (Bristol, UK). PPG, GYY 4137 and SNAP were dissolved in dimethylsulphoxide. The other drugs were solubilized in purified water. Based on earlier experiments, the solvents utilized were determined to have no impact on the contractility of the CC strips. PSS was freshly prepared and contained (in mM): NaCl 119, KCl 4.7, $NaHCO_3$ 24.9, $MgSO_4$ 1.2, KH_2PO_4 1.2, ethylenediamine tetraacetic acid (EDTA) 0.027, glucose 11 and $CaCl_2$ 1.5. To maintain a pH of 7.4, the organ bath containing PSS was constantly aerated with a mixture of 95% O_2 and 5% CO_2. The K^+-enriched PSS (KPSS) was freshly prepared and equimolar amounts of KCl for NaCl were exchanged.

2.8. Data analysis

Sensitivity to denatonium, chloroquine, isoproterenol, and SNAP was expressed as pD_2 ($pD_2 = -\log EC_{50}$). The EC_{50} value represents the concentration of a drug required to elicit 50% of the maximum response. pD_2 was obtained by non-linear regression analysis. The degree of relaxation was quantified as a percentage of the contraction elicited by 3 μM PhE. The results are presented as the mean ± SD of *n* (number of rats). Statistical analysis was conducted using paired Student's *t*-test or one-way analysis of variance (ANOVA) as appropriate to determine differences between groups. The threshold for statistical significance was set at $P < 0.05$. GraphPad Prism 8.30 (San Diego, CA) was used to perform the data analysis.

3. Results

3.1. Expression of TAS2R10

The expression of TAS2R in rat CC samples (n = 5) was examined through double immunofluorescence using a selective antibody for TAS2R10, in combination with a pan-neuronal marker protein gene product (PGP) 9.5. TAS2R10 expression was primarily observed in the SM cells. In addition, a slight TAS2R10 stain was observed to co-localize with PGP 9.5 in the nerve cells of the CC tissue (Fig. 1A-D) and dorsal penile artery (Fig. 1E-H). Without the primary antibodies, the samples did not show any IR (Fig. 1I-J). Furthermore, TAS2R10 expression was confirmed by western blot analysis, where a band at 35 kDa, consistent with the molecular weight of TAS2R10, was detected in the entire sample (Fig. 1K).

3.2. Functional studies

Rat crura CC strips were equilibrated to a tension of 0.35 ± 0.05 mN and exposed to PhE (3 μM), which induced a sustained SM contraction of 3.9 ± 0.1 mN (n = 23).

3.2.1. Relaxations to denatonium, chloroquine, isoproterenol, and SNAP

Denatonium, chloroquine, SNAP, and isoproterenol cause a potent dose-dependent CC SM relaxation (pD_2 and Emax values of 6.3 ± 0.6 and 87.7 ± 1.9%, 4.9 ± 0.3 and 100 ± 1.6%, 5.5 ± 0.4 and 88.5 ± 9.2% and 7.5 ± 0.3 and 88.3 ± 1.2% for denatonium, chloroquine, SNAP and isoproterenol, respectively, n = 7–8) (Fig. 2A-D).

3.2.2. Effects of threshold denatonium concentrations on GYY 4137-, SNAP-, EFS- and isoproterenol-induced relaxations

The slow-release H_2S donor GYY 4137 (10 nM) and the NO donor SNAP (10 nM) induced relaxations of CC strips, which were increased in the presence of threshold concentrations (0.1 μM) of denatonium (GYY 4137- and SNAP-induced relaxations of 25.3 ± 3.4% and 19.8 ± 5.4% and 37.7 ± 2.4%** and 30.3 ± 3.2%*, in the absence and presence of denatonium, respectively, n = 4. *$P < 0.05$ and **$P < 0.01$ vs control (paired Student's *t*-test) (Fig. 2E-F).

Maintaining NANC conditions, EFS elicited frequency-dependent relaxation of rat CC strips contracted with PhE. The EFS-induced relaxation did not increase by denatonium (Table 1) (Fig. 3). Moreover, denatonium failed to modify the isoproterenol responses (pD_2 and

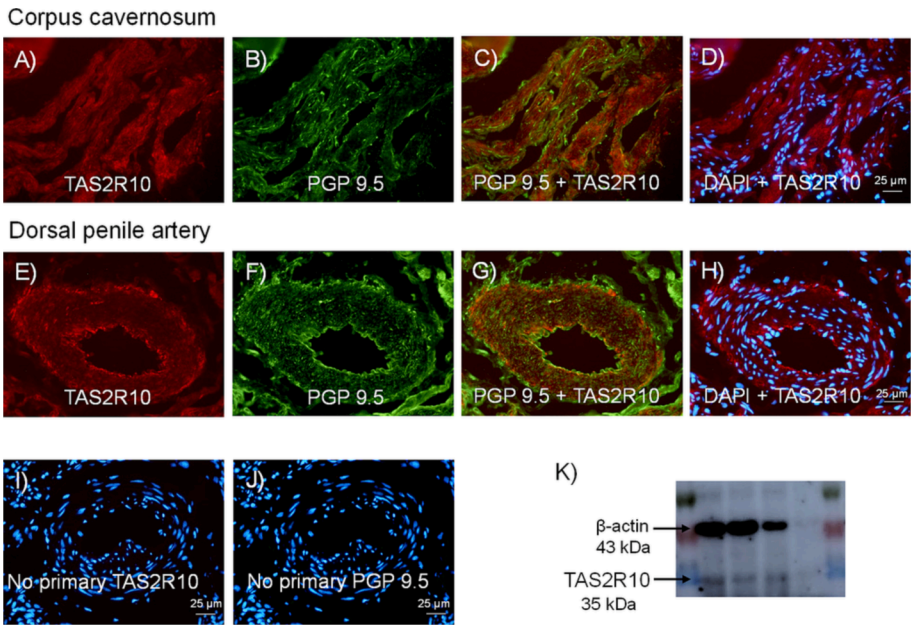

Fig. 1. TAS2R10 expression in the rat erectile tissue smooth muscle fibers. Immunofluorescence labelling in corpus cavernosum (CC) (A-D) and dorsal penile artery (E-H) of the rat. TAS2R10 protein expression (red areas) in CC (A) and dorsal penile artery sections (E) of the rat. Protein gene product 9.5 (PGP9.5) expression (green areas) exhibiting cavernous tissue nerves in CC (B) and dorsal penile artery (F) sections of the rat. Merged immunofluorescence labeling for TAS2R10 and PGP 9.5 in rat erectile tissue sections showing TAS2R10 expression within nerve fibers running parallel to SM fibers (yellow areas) (C and G). DAPI immunofluorescence staining labelling cell nuclei (blue areas) (D and H). Scale bars (25 µm). Negative controls exhibited a lack of immunoreactivity when rat erectile tissue sections were incubated without the primary antibody (I and J) (n = 5). Western blot of rat CC smooth muscle homogenates exhibiting a band at 35 kDa, which corresponds to the expected TAS2R10 molecular weight (K) (n = 5). Lanes correspond to different technical replicates (n = 3).

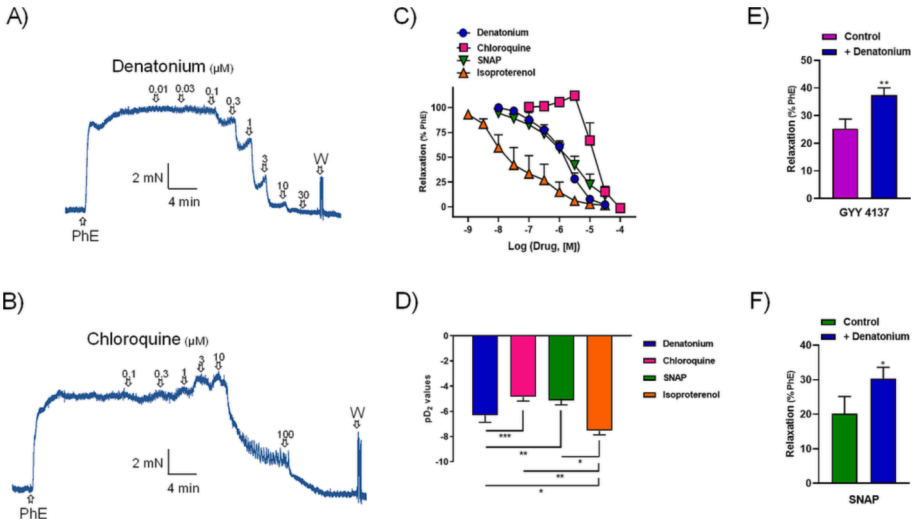

Fig. 2. Relaxation induced by denatonium and chloroquine in rat corpus cavernosum (CC). Isometric tension recordings showing the relaxations induced by denatonium (0.01——30 µM) (A) and chloroquine (0.1——100 µM) (B) on 3 µM phenylephrine (PhE)-contracted rat CC strips. The vertical bar shows tension (mN) whereas the horizontal bar shows time (min). W: wash. Dose-dependent relaxation curves to denatonium (0.01——30 µM) (blue circles), chloroquine (0.1——100 µM) (red squares), SNAP (0.01——30 µM) (green triangles), and isoproterenol (1 nM – 30 µM) (orange triangles) in rat CC samples (C). pD_2 values for denatonium (blue bar), chloroquine (red bar), SNAP (green bar), and isoproterenol (orange bar) (D). Results are expressed as mean ± SD of 7–8 rats. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs control (one-way ANOVA followed by Bonferroni post hoc test to determine differences between groups). The graph shows the relaxation induced by GYY 4137 (10 nM) (E) and SNAP (10 nM) (F) in untreated (control, purple and green bar, respectively) and treated with 0.1 µM denatonium (blue bar) in rat CC strips. Results are expressed as mean ± SD of 4 rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs control (paired Student's *t*-test).

Table 1
Effect of 0.1 µM denatonium on relaxations to electrical field stimulation (EFS, 1 ms duration, 0.5–16 Hz, 20 s trains, with constant current output adjusted to 75 mA) in rat corpus cavernosum strips.

EFS (Hz)						
	0.5	*1*	*2*	*4*	*8*	*16*
Control	5 ± 5	10 ± 7	28 ± 21	53 ± 17	72 ± 13	79 ± 11
Denatonium	9 ± 8	20 ± 13	42 ± 19	65 ± 17	77 ± 12	80 ± 13

Results are expressed as inhibition percentage of 3 µM phenylephrine-induced precontraction and represent the mean ± SD of 9 experiments.

Emax values of 7.5 ± 0.3 and 94.3 ± 3% and 7.3 ± 0.8 and 195 ± 2.4%, in the absence or presence of denatonium, respectively, n = 6).

3.3. Effect of NOS and CSE blockade and denatonium on H_2S production

The levels of H_2S generated by rat CC samples were determined to be 9.0 ± 0.8 nmol/mg.min^{-1}. After the inhibition of CSE and NOS using PPG (1 mM) and L-NOARG (100 µM), respectively, the H_2S levels were reduced. However, increasing concentrations of the TAS2R agonist denatonium (0.1–10 µM) restored the H_2S levels in a dose-dependent manner (Table 2) (Fig. 4).

4. Discussion

The current study suggests the presence of bitter taste receptors TAS2R10 playing a key role in regulating rat CC tone. TAS2R10 is

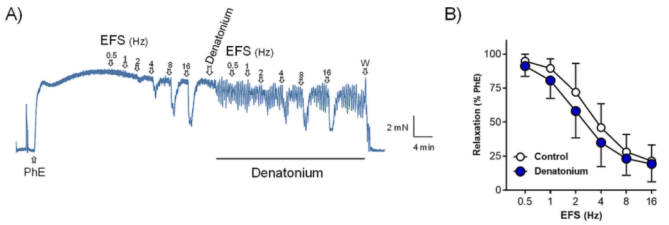

Fig. 3. Denatonium did not change nerve-mediated non-adrenergic non-cholinergic relaxation in rat corpus cavernosum (CC). Original isometric force recordings exhibiting frequency-dependent relaxation in response to electrical field stimulation (EFS, 0.5–16 Hz,1 ms in duration, and 20-second trains) in the absence (control) and presence of denatonium (0.1 μM). EFS was performed on rat CC strips contracted with phenylephrine (PhE, 3 μM) pretreated with guanethidine (10 μM) and atropine (1 μM). The vertical bar displays tension (mN) and the horizontal bar displays time (min). W: wash (A). The graph shows frequency-dependent relaxation curves to EFS in untreated (control, open circles) and treated with 0.1 μM denatonium (closed circles) in rat CC strips (B). Results are expressed as mean ± SD of 9 rats.

Table 2
Effect of CSE and NO synthase blockade and denatonium on endogenous H_2S production in rat corpus cavernosum samples.

	n	*H_2S level (nmol/mg.min^{-1})*
Control	5	9.0 ± 0.8
PPG (1 mM)	5	3.8 ± 0.4***
L-NOARG (100 μM)	5	6.4 ± 0.6*
Denatonium (0.1 μM)	4	9.8 ± 0.6
Denatonium (1 μM)	4	12.6 ± 0.9*
Denatonium (10 μM)	4	12.3 ± 0.9*
PPG + L-NOARG + Denatonium (1 μM)	4	7.8 ± 0.9

Results are expressed as mean ± SD of *n* experiments. *$P < 0.05$ and ***$P < 0.001$ versus control value (analysis of variance followed by Bonferroni method).

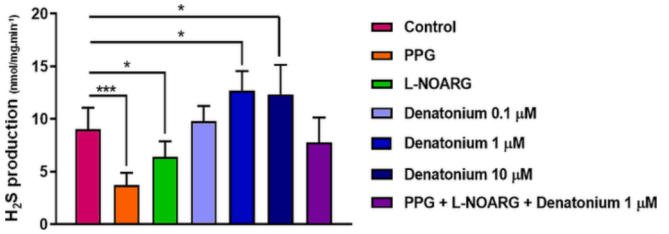

Fig. 4. The TAS2R10 agonist denatonium increases endogenous H_2S production in rat corpus cavernosum. Quantification of H_2S levels produced in the absence (control conditions) and in presence of DL-propargylglycine (PPG, 1 mM), N^G-nitro-L-arginine (L-NOARG, 100 μM) inhibitors of CSE and NO synthase, respectively, and in presence of denatonium (0.1, 1 and 10 μM). Bars represent mean ± SD. of 4–5 rats. *$P < 0.05$ and ***$P < 0.001$ vs control (one-way ANOVA followed by Bonferroni post hoc test).

widely expressed in the SM of CC and to a lesser extent in the nerve fibers. TAS2R agonists induce strong SM relaxation and promote the production of endogenous gaseous inhibitory neurotransmitters. This conclusion is maintained by the following findings: (i) A marked TAS2R10 expression in SM and the nerve fibers cells of the CC. (ii) TAS2R agonists denatonium and chloroquine caused a dose-dependent CC SM relaxation. (iii) Increasing concentrations of denatonium augmented endogenous H_2S generation, which was reduced by the blockade of NO and H_2S synthases. (iv) Threshold denatonium concentrations failed to modify both EFS- and isoproterenol-induced relaxations.

TAS2R10 is one of the TAS2R subtypes most widely expressed in both visceral and vascular SM cells [9,31]. Thus, in human airways SM cells TAS2R10, together with TAS2R14 and TAS2R31, show the most abundant expression, and TAS2R activation by bitter tastants causes a potent bronchodilator action [6]. Moreover, in human and mouse uterine SM cells TAS2R10, together with TAS2R4 and 5, has also been identified where TAS2R agonists cause relaxation of myometrium [32]. Concerning the vascular SM cells, TAS2R10, as well as TAS2R3, TAS2R4, and TAS2R14 expression has been detected in human pulmonary arteries and guinea-pig aorta, with levels comparable to that of the α_{1A}-adrenoceptor. In these tissues, TAS2R agonists induce an endothelium-independent vasorelaxation [33]. In the current study, in rat CC, the western blot demonstrated the presence of TAS2R10 protein expression in the whole tissue. Additionally, the double immunofluorescence labeling revealed that TAS2R10 IR was primarily localized in smooth muscle (SM) cells of both the CC and dorsal penile artery. Moreover, the immunofluorescence analysis showed a minor TAS2R10 staining co-localized with the neuronal marker PGP 9.5 in the nerve cells of the cavernous tissue samples, thus indicating the involvement of these receptors regulating SM contractility.

In the rat CC, expression of TAS2R10 in SM is consistent with the functional effects of TAS2R agonists, which cause strong relaxations of the cavernous SM. Nevertheless, the relaxation patterns induced by denatonium and chloroquine are distinct, thus indicating the involvement of different signaling pathways. Whereas denatonium induced a sole dose-dependent relaxant effect, chloroquine showed a biphasic response in which firstly, on PhE-induced tone, a small contractile response at lower concentrations (from 0.1 at 10 μM) was obtained to be followed by a maximal relaxation between 30 and 100 μM. These results are consistent partly with those found in guinea-pig trachea where both TAS2R agonists induced relaxations via different signaling pathways [34]. The relaxation induced by denatonium was found to be dependent on the specific type of intracellular signaling triggered by the agonist, whereas the response induced by chloroquine seemed to be either independent of signaling or via mechanisms that can counteract a wider range of signaling pathways. In rat CC, however, the involvement of different signaling pathways of TAS2R agonists is not seemed dependent on the constrictor agent used, due that SM relaxations induced by denatonium and chloroquine being obtained in samples contracted with PhE. In addition, the maximum relaxation for denatonium and chloroquine was similar to that of the β-adrenoceptor agonist isoproterenol, however, with markedly lower potencies. Thus, whereas isoproterenol exhibited a relaxant sensitivity in the nanomolar range (30 nM), TAS2R agonists showed potencies in the micromolar range (EC_{50} values of 0.5 and 16 μM for denatonium and chloroquine, respectively). These potencies, nevertheless, were higher than those reported in the literature for TAS2R agonists [1]. In rat CC, denatonium, a known selective activator of TAS2R10, displayed a higher potency than the reported potency of this agonist for hTAS2R10 transfected human embryonic kidney (HEK)-293 T cells (EC50: 120 μM) [1]. Although there are no known antagonists for TAS2Rs, the response of the rat CC to the tested agonists provides valuable information on which receptors are being activated. Thus, our findings indicate that the relaxations induced by denatonium in the rat CC are likely mediated through the activation of TAS2R10. One possible explanation for the higher potency of denatonium in the rat CC compared to its reported potency in transfected human cells is the potential interaction between TAS2R10 and the concomitant activation of TAS2R4. Given that denatonium is 100-fold more potent for hTAS2R10 than hTAS2R4, our findings suggest that the observed relaxation elicited by denatonium in rat CC is primarily mediated via the TAS2R10 receptor. The fact that denatonium cause a CC SM relaxation with a higher potency than that showed by the NO donor SNAP (EC_{50}: 8 μM) suggests that TAS2R10 activation might be a valuable therapeutic tool for pathologies related to impaired cavernous SM contractility.

On the other hand, the potency of chloroquine in rat CC was high (EC_{50}: 16 μM) versus that exhibited for hTAS2R3 in transfected HEK-293 T cells (EC_{50}: 173 μM) [1]. Since chloroquine is 1,000-fold less

potent for hTAS2R10 [1], it is postulated that the relaxation induced by chloroquine in rat CC is likely mediated by the activation of TAS2R3. The involvement of different TAS2R subtypes in the response to denatonium and chloroquine might explain the display of distinct patterns of relaxation exhibited for these agonists in the current study. Further studies will be necessary to investigate, together with TAS2R10, the involvement of TAS2R3 in erectile tissue vasorelaxation.

In erectile tissue, a marked PDE 5 expression has been described which regulates endogenous cGMP levels linked to the capacity to reach a penile erection [17,35,36]. cGMP favors the release of NO from nitrergic neurons in rabbit CC, thus highlighting the key role of NO/cGMP signaling pathway in nerve-mediated penile vasorelaxation [37]. Together with PDE5, a PDE4-IR, cAMP-specific PDE is also detected in smooth muscle and vascular endothelium co-localized with PKA of human cavernous arteries, thus indicating an interaction between cAMP- and cGMP-dependent pathways in the regulation of penile smooth muscle tone [35,38,39]. NO [13–15] and H_2S [19,20] play a pivotal role nerve-mediated relaxation human CC smooth muscle and an interplay between both endogenous gaseous inhibitory neurotransmitters has been described to be involved in erectile function [23–25]. In the current study, endogenous H_2S production decreased in presence of the H_2S and NO synthesis inhibitors. Despite the limited specificity and membrane permeability of the CSE inhibitor PPG, and the potential interference of other reactive sulfur species in H_2S quantification by spectrophotometry, the current results suggest a significant increase in CSE-dependent H_2S production in response to TAS2R10 activation. Moreover, endogenous CSE-generated H_2S production also involves a NO synthetic pathway, thus highlighting the existence of synergy between both gaseous neurotransmitters in CC tissue [23–25]. Nevertheless, H_2S production in the CC sample was totally reestablished and increased in a concentration-dependent fashion, above control levels, in response to increasing denatonium concentrations, thus providing indications that neuronal TAS2R10 activation promotes the synthesis and release of inhibitory gaseous neurotransmitters. Furthermore, the fact that threshold concentrations of denatonium increased GYY 4137- and SNAP-induced relaxations suggests that neuronal TAS2R10 activation would promote H_2S- and NO-mediated relaxation. However, denatonium failed to consistently enhance EFS-induced response, which could be explained by the limited presence of prejunctional TAS2R10.

β-adrenoceptor agonists are also involved in CC relaxation [40,41]. The presence of atypical β- and β_3-adrenergic receptors has been demonstrated, respectively, in rabbit and human CC tissue, and both receptor subtypes lead to adenylyl cyclase signaling cascade activation [41,42]. An interaction between PDE4 inhibitors and beta-adrenoceptor agonists has been reported in rat CC. Roflumilast, a selective PDE4 inhibitor, induces CC SM relaxation and potentiates the response induced by β-adrenoceptor activation [21]. In our study, threshold concentrations of denatonium failed to modify the relaxation curve to isoproterenol, thus indicating that the β-adrenoceptor signaling pathways do not seem to involve in the relaxation.

From these results, we suggest the involvement of prejunctional and postjunctional TAS2R10 in the regulation of CC SM contractility. In fact, denatonium induces a potent SM relaxation *per se*, through SM TAS2R10 activation. In addition, increased endogenous H_2S production in the CC sample in response to increasing concentrations of denatonium suggests that neuronal TAS2R10 activation would promote the synthesis and release of inhibitory gaseous neurotransmitters. Indeed, in CC tissue, CSE-dependent endogenous H_2S production also involves a synthetic NO pathway, highlighting the existence of synergy between both gasotransmitters. Neuronal TAS2R10 activation likely increases neuronal Ca^{2+} current via plasma membrane VOCC, with subsequent Ca^{2+} influx, as consequence of membrane depolarization in response to the arrival of an action potential at the motor nerve ending. The increase in neuronal cytosolic Ca^{2+}, via interaction with calmodulin, would stimulate CSE and neuronal NOS favoring the synthesis of H_2S and NO from L-cysteine and L-arginine, respectively, and their release from the nerve producing SM relaxation.

Because ED, overactive bladder, and bladder outlet obstruction can coexist and since CC, prostate, urethra, and bladder share common pathways, drugs that target simultaneously in these organs may offer synergic and valuable pharmacological action for patients presenting these conditions [43–47]. In fact, TAS2R activation promotes relaxations of the human detrusor SM and TAS2R agonists suppress the overactive bladder symptoms of mice with partial bladder outlet obstruction [5], thus indicating the possible usefulness of denatonium, alone or in combination with the PDE5 inhibitors currently used, for SM relaxation of erectile tissues in ED.

In conclusion, the current results show neuronal and SM TAS2R10 expression in the rat CC, where denatonium induces a potent SM relaxation and promotes the H_2S- and NO-mediated inhibitory gaseous neurotransmission, so that TAS2R10 activation may be a valuable therapeutic target for the development of efficacious compounds for erectile tissue vasorelaxation in ED.

The authors declare no potential conflicts of interest.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This study was supported by the grant PR75/18-21562 from the Universidad Complutense de Madrid (Santander-UCM). The authors thank Mr. Francisco Puente and Mr. Manuel Perales for their assistance within the laboratory.

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