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In vitro inhibition of phosphodiesterase type 4 enhances rat corpus cavernosum nerve-mediated relaxation induced by gasotransmitters

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ABSTRACT

Aims: Nitric oxide (NO) and hydrogen sulfide (H_2S) are involved in nerve-mediated corpus cavernosum (CC) relaxation. Expression of phosphodiesterase type 5 (PDE5) and type 4 (PDE4), cyclic guanosine monophosphate (cGMP)- and cyclic adenosine monophosphate (cAMP)-specific, respectively, has been described and PDE5- and PDE4-inhibitors induce cavernous smooth muscle relaxation. Whereas the NO/cGMP signaling pathway is well established in penile erection, the cAMP-mediated mechanism is not fully elucidated. The aim of this study is to investigate the localization and the functional significance of PDE4 in rat CC tone regulation.
Main methods: We performed immunohistochemistry for the detection of the PDE4A isoenzyme. Isometric tension recordings for roflumilast and tadalafil, PDE4 and PDE5 inhibitors, respectively, electrical field stimulation (EFS) and β-adrenoceptor agonist isoproterenol and endogenous H_2S production measurement.
Key findings: A marked PDE4A expression was detected mainly localized in the nerve cells of the cavernous smooth muscle. Furthermore, roflumilast and tadalafil exhibited strong corpus cavernous relaxations. Endogenous H_2S production was decreased by NO and H_2S synthase inhibitors and increased by roflumilast. Isoproterenol- and EFS-induced relaxations were increased by roflumilast.
Significance: These results indicate that PDE4A is mainly expressed within the nerves cells of the rat CC, where roflumilast induces a potent corpus cavernous relaxation per se and potentiates the response induced by β-adrenoceptor activation. The fact that roflumilast enhances H_2S production, as well as EFS-elicited responses suggests that PDE4 inhibitors modulate, in a positive feedback fashion, nerve-mediated relaxation induced by gasotransmitters, thus indicating a key role for neuronal PDE4 in penile erection.

1. Introduction

In the corpus cavernosum (CC), non-adrenergic non-cholinergic (NANC) inhibitory neurotransmission is mediated by nitric oxide (NO) [1,2]. In fact, the expression of NO synthase (NOS) has been demonstrated in neurons of animal models and in human penile tissue [3–7]. NO induces smooth muscle relaxation via activation of soluble guanylyl cyclase which leads to the increase of intracellular cyclic guanosine monophosphate (cGMP) concentration. This latter produces a decrease of cytosolic calcium levels and modifications in protein phosphorylation, thus causing smooth muscle relaxation [8]. Phosphodiesterase type 5 (PDE5), that selectively hydrolyzes cGMP, is widely expressed in CC and PDE5 inhibitors, including sildenafil, vardenafil and tadalafil promote penile erection by increasing the intracellular cGMP concentration in cavernous tissue. Therefore, they are used in erectile dysfunction treatment [9,10].

Hydrogen sulfide (H_2S), another inhibitory gaseous transmitter, also exerts a potent vasodilatory and pro-erectile action on the cavernosum tissue via cyclic adenosine monophosphate (cAMP)-dependent mechanisms [11]. Endogenous H_2S production deficiency, associated with low testosterone levels, is considered a predictor of erectile impairments in old rats [12]. Moreover, exogenous H_2S improves erectile function via inhibiting the RhoA/ROCK1 signaling pathway which leads to the suppression of the phenotypic modulation of CC smooth muscle cells [13].

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Phosphodiesterases (PDEs) are a group of hydrolyzing enzymes of cGMP and cAMP. Their blockade increases intracellular cGMP and/or cAMP concentration with the consequent activation of the protein kinase G (PKG) and protein kinase A (PKA), respectively, therefore inducing smooth muscle relaxation [14,15]. Of all 11 types of PDEs (PDE1-PDE11) isoforms, the PDE5 selectively hydrolyze cGMP whereas PDE4 is selective for the cAMP hydrolyzation [14,15]. PDE5 and PDE4 immunoreactivity (IR) has been detected in human cavernous smooth muscle cells and intracavernous resistance arteries. PDE5/cGMP and PDE4/cAMP-dependent mechanisms regulate the vascular tone of the erectile tissue [16]. Whereas the NO/cGMP signaling pathway is well established to be involved in nerve-mediated penile vasorelaxation, the cAMP pathway has been related to the endothelium-dependent vasorelaxation due to the presence of PDE4 within endothelial cells of the CC arteries [16]. No data exists, however, concerning the possible presence and the functional significance of PDE4 in nerve fibers of cavernous tissue. There are 4 isoforms of PDE4 (PDE4A, PDE4B, PDE4C and PDE4D) [14,15]. Immunosignals specific for PDE4A isoenzyme are prominent in the male and female urogenital system. In fact, a marked expression for PDE4A has been described in the human vagina [17], bladder neck [18] and urethra [19].

The first line of treatment for erectile dysfunction involves the use of PDE5 inhibitors, however, refractoriness to this treatment in some patients groups such as men with diabetes is high, either because they are insensitive to or contraindicated for this therapy [20–23]. Hence, alternative pharmacological targets are required to improve treatment for erectile dysfunction in these patients. In this context, the PKA signaling pathway could be a promising pharmacological target to treat these patients. In asthma and chronic obstructive pulmonary disease (COPD), roflumilast, a selective PDE4 inhibitor, improves lung function due to its bronchodilator and anti-inflammatory action [24–26]. Therefore, the present study investigates the role played by PDE4 and roflumilast in nerve-mediated relaxation in rat CC.

2. Materials and methods

2.1. Animals

Experimental protocols were performed using male Wistar rats, aging 17–18 weeks, with weight range between 260 and 370 g. The rats were accommodated within the Animal Facility of the Department of Physiology, Complutense University of Madrid. They had free access to water and food and kept 12:12 h light/dark cycles until used for study. Euthanasia was performed by isoflurane inhalation (~5%) and followed by cervical dislocation. All protocols using animals were performed according to the European Union Directive 2010/63/EU, approved by the Institutional Animal Care and Use Committee of the Complutense University of Madrid (PR75/18-21562).

2.2. Corpus cavernosum harvesting

Rats were slaughtered and the crura CC was cut in the line of attachment to the pubic ramus. The penis was carefully detached and placed in cold (4 °C) physiological saline solution (PSS) with 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 a pH at 7.4. CC strips were then harvested by dissection of the tunica albuginea and adjacent connective tissues. Cavernous tissue samples were kept in cooled PSS at 4 °C.

2.3. Western blot

Proteins were extracted from rat CC and 50 μg were separated by electrophoresis using a 10% polyacrylamide gel (SDS-PAGE). Next, the gels were transferred to nitrocellulose membranes and incubated overnight at 4 °C with a 1:50 dilution of anti-PDE4A antibody (Santa Cruz Biotechnology Inc. Heidelberg, Germany) or with a 1:50 dilution of anti-protein gene product 9.5 (anti-PGP 9.5 antibody, ab8189 Abcam, Cambridge, UK). The relative protein levels were normalized to the intensity of mouse anti-β-actin loading blots (Santa Cruz Biotechnology Inc. Heidelberg, Germany). Secondary antibodies (1:5000 dilution) 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) by chemiluminescence (Image-Quant LAS 500, GE Healthcare).

2.4. Double-labeling immunofluorescence assays

Rat CC samples were fixed overnight in 4% paraformaldehyde (prepared in phosphate-buffered saline 1×, PBS 1×). Next, samples were washed in PBS 1× and placed in 30% sucrose until the tissue sinks. Samples were embedded in the OCT compound for frozen sectioning with a microtome-cryostat. Sections of 5 μm thickness were obtained and incubated in blocking buffer (PBS 1×, 0.3% Triton X-100 and 10% normal goat serum) for 2-3 h. After that, sections were incubated with a 1:50 dilution of the mouse anti-PDE4 (anti-PDE4A H-7, Santa Cruz Biotechnology Inc. Heidelberg, Germany) and a 1:50 dilution of rabbit anti-PGP 9.5 (ab8189 Abcam, Cambridge, UK) for 48 h at 4 °C. Then, sections were washed in PBS 1×, and incubated with the fluorochrome-labeled secondary antibodies (Alexa Fluor 594 goat-antirabbit, and Alexa Fluor 488 goat-antimouse, 1:200 dilution, Abcam) during 3 h at RT. The nuclei were stained with DAPI (Invitrogen) and mounted for microscope visualization. The intensity of the immunofluorescence was measured on the original raw images using Image J (National Institutes of Health, Bethesda, Maryland, USA). Microphotographs were taken always using the same conditions.

2.5. Endogenous H_2S measurement

Endogenous H_2S was quantified as previously described [27]. In summary, CC samples were collected and 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). N^G-nitro-L-arginine (L-NOARG, 100 μM), DL-propargylglycine (PPG, 1 mM), or roflumilast (0.1, 1 and 10 μM) were added to the incubation solution to explore the role of the cystathionine gamma-lyase (CSE), NO synthase (NOS), and the selective PDE4 inhibitors, respectively [18]. To start the reactions the chamber was 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 into the chambers to catch generated H_2S. The reaction was ended by protein denaturation adding 500% (w/v) of trichloroacetic acid and then 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. The 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. The protein was quantified with Lowry assay (DC Protein Assay Kit, Bio-Rad, Madrid, Spain).

2.6. Isometric force measurements

Two crura CC strips (3 mm long and 2 mm wide) from each penis were acquired and placed horizontally in myographs (DMT, Muscle Strip System - 820MS, Danish Myotechnology, Denmark) filled up with 5 ml PSS kept at 37 °C and continuously aerated with 95% to 5% O_2/CO_2 to get a final pH of 7.4. The sample had one end tied to a micrometer screw and the other to an isometric force transducer. Isometric force measurements were obtained using a PowerLab v7.0 system (ADInstruments

Inc., Oxford, UK). The strips were subjected to equilibration for 60 min under a passive force of 4.0 mN. PSS solution was changed each 20 min. The contractile ability of the crura CC samples was checked using a 124 mM potassium-rich PSS (K-PSS 124 mM). Crura CC Strips were contracted with phenylephrine (PhE, 3 μM) and concentration-dependent relaxation curves were performed for the PDE4 and PDE5 selective inhibitors, roflumilast and tadalafil, respectively, and the β-adrenoceptor agonist isoproterenol. For the electrical field stimulation (EFS) assays CC samples were incubated for 1 h with guanethidine (10 μM) and atropine (1 μM) to block, respectively, the noradrenergic neurotransmission and muscarinic receptors. Keeping these conditions, samples were precontracted with 3 μM PhE and frequency-dependent relaxations curves were obtained in the absence or presence of a threshold concentration of roflumilast (0.3 nM). EFS stimuli were achieved by delivering a rectangular pulse (1 ms duration, 0.2–16 Hz, 20s trains, with constant current output adjusted to 75 mA), each 4 min, using a CS20 stimulator (Cibertec, Barcelona, Spain) connected to two platinum electrodes placed at each side of the long axis of the samples.

2.7. Drugs and solutions

Employed drugs were: phenylephrine (PhE), atropine, guanethidine, roflumilast, tadalafil, isoproterenol, DL-propargylglycine (PPG) and N^G-nitro-L-arginine (L-NOARG) all from Sigma (USA). Roflumilast and PPG were dissolved in dimethylsulphoxide. Tadalafil was dissolved in acetonitrile. All other drugs were dissolved in purified water. The used solvents have no effects on the contractility of the crura CC samples [7]. PSS was prepared daily and contains (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. The organ bath with PSS was continuously aerated with 95% to 5% of O_2/CO_2 to maintain pH 7.4. K^+-enriched PSS (KPSS) was prepared daily by replacing NaCl with KCl on an equimolar basis.

2.8. Data analysis

Sensitivity to isoproterenol, tadalafil and roflumilast is expressed in

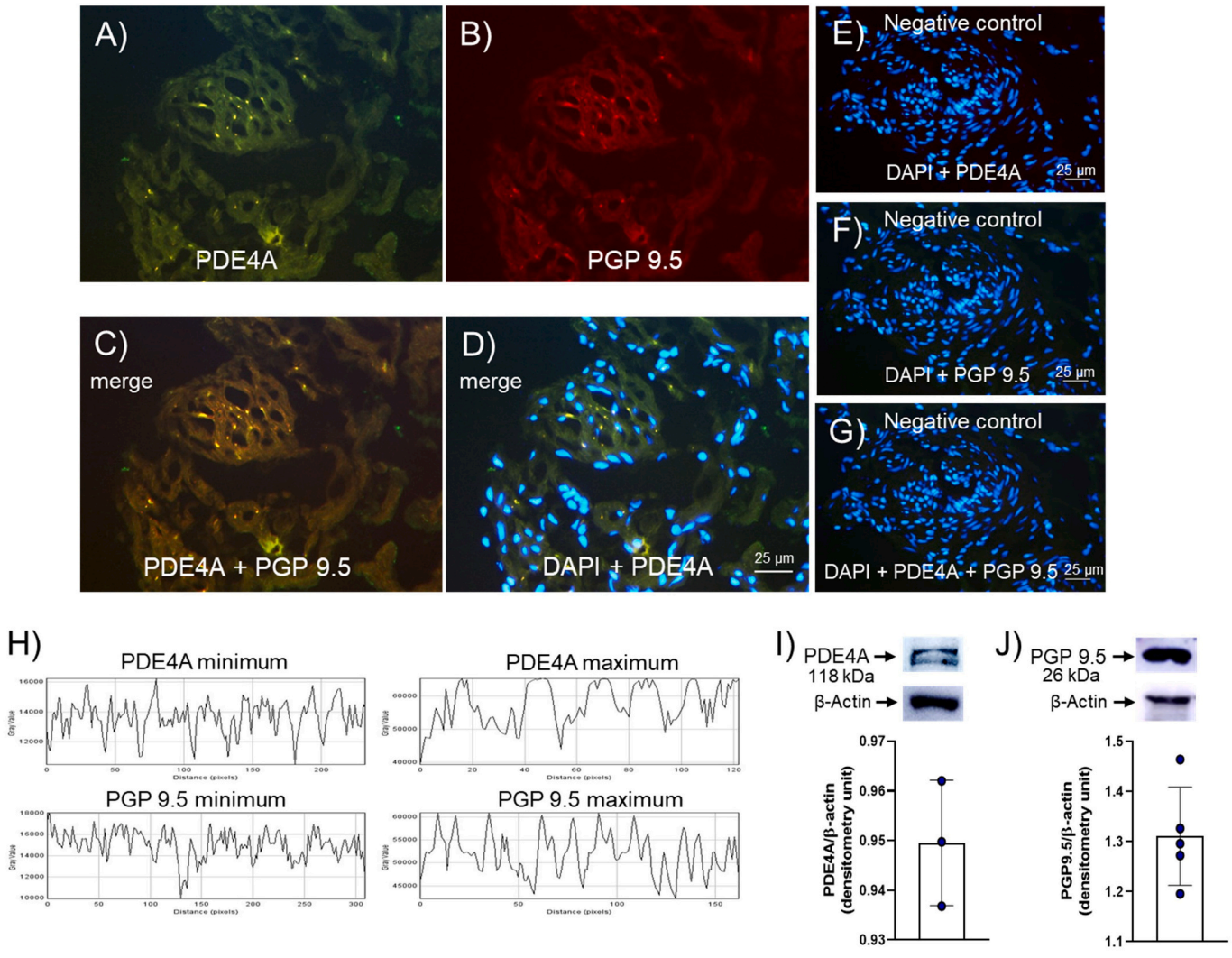

Fig. 1. PDE4A expression within nerve cells of the corpus cavernous (CC) smooth muscle fibers. Immunofluorescence labeling in rat CC (A-D). PDE4A protein expression in rat CC sections (green areas) (A). Protein gene product 9.5 (PGP9.5) expression exhibiting cavernous tissue nerves in rat CC sections (red areas) (B). Same fields (A, B, E and F). Merged immunofluorescence labeling for PDE4A and PGP 9.5 in rat CC sections showing PDE4A expression within nerve cells running parallel to CC smooth muscle fibers (yellow areas) (C). DAPI immunofluorescence staining labeling cell nuclei (blue areas) (D). Scale bars (25 μm). Negative controls exhibited a lack of immunoreactivity when rat CC sections were incubated without the primary antibody (E, F and G) (n = 5). Minimum and maximum fluorescence intensity for PDE4A and PGP 9.5 reveal a predominant colocalization of PDE4A and PGP 9.5 (H). Western blot of rat CC smooth muscle homogenates exhibiting bands at 118 kDa and 26 kDa, which correspond to the expected PDE4A (I) and PGP 9.5 (J), respectively, molecular weights (n = 3–5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

terms of pD_2, where $pD_2 = -\log EC_{50}$ and EC_{50} is the agonist concentration needed to produce half-maximal response. pD_2 was estimated by computerized non-linear regression analysis (GraphPad Prism 6.01, San Diego, CA). Relaxations are represented as a percentage of the contraction induced by 3 μM PhE. The results are stated as the mean ± SD of *n* (number of rats). Differences between groups were analyzed using paired Student's *t*-test or one-way analysis of variance (ANOVA) when appropriate. Statistical significance was considered when $P < 0.05$. Data were analyzed using a standard software (GraphPad Prism 6.01, San Diego, CA).

3. Results

3.1. Expression of PDE4A isoenzyme

PDE4A expression in rat CC samples (n = 6) was analyzed by double immunofluorescence using a PDE4A selective antibody along with a pan-neuronal marker protein gene product (PGP) 9.5. PDE4A-IR was detected mainly co-localized with PGP 9.5, within the nerve cells of the cavernous smooth muscle fibers (Fig. 1A–D). Likewise, the quantification analysis of the co-localization among PDE4A isoform and the PGP 9.5, demonstrated values between 70 and 75% (Fig. 1H). In the absence of the primary antibody, the samples did not exhibit IR (Fig. 1E–G). Additionally, western blot assays revealed the presence of a blot at 118 kDa and 26 kDa, corresponding to the molecular weights for PDE4A (Fig. 1I) and PGP 9.5 (Fig. 1J), respectively, indicating that PDE4A and PGP 9.5 are expressed in the whole CC sample.

3.2. Effect of NOS and CSE blockade and roflumilast on H_2S production

The H_2S levels produced by rat CC samples (7.83 ± 0.51 nM/mg.min^{-1}) were reduced as consequence of NOS and CSE blockade with, respectively, L-NOARG (100 μM) and PPG (1 mM) and recovered, in a dose-dependent manner, by increasing concentrations of selective PDE4 inhibitor roflumilast (10–100 μM) (Table 1) (Fig. 2).

3.3. Functional studies

Rat crura CC strips were normalized under a force of 0.35 ± 0.05 mN. PhE (3 μM) induces a sustained smooth muscle tone of 3.9 ± 0.1 mN (n = 23).

3.3.1. Relaxations to roflumilast and tadalafil

Roflumilast (0.1 nM–30 μM) exerted stronger smooth muscle relaxations than that produced by the PDE5 inhibitor tadalafil (0.1 nM–30 μM) (pD_2 and Emax values of 7.8 ± 0.8 and 99.6 ± 0.2% and 6.9 ± 0.3* and 96.0 ± 4% for roflumilast and tadalafil, respectively, $^*P < 0.05$ vs control, paired *t*–test, n = 7) (Fig. 3).

3.3.2. Effects of a threshold roflumilast concentration on EFS- and isoproterenol-induced relaxations

Under NANC conditions, EFS induced frequency-dependent relaxations of rat CC strips contracted with PhE. These EFS-induced relaxations were potentiated by threshold (0.3 nM) roflumilast concentration (Table 2) (Fig. 4). Moreover, roflumilast also enhanced the isoproterenol responses (pD_2 and Emax values of 7.1 ± 0.4 and 14.3 ± 3% and 7.5 ± 0.3* and 11.8 ± 2.4%, in the absence or presence of roflumilast, respectively, $^*P < 0.05$ versus control value, paired *t*–test, n = 8) (Fig. 5).

Table 1
Effect of CSE and NO synthase blockade and roflumilast on endogenous H_2S production in rat corpus cavernosum samples.

	n	H_2S level (nM/mg.min^{-1})
Control	4	7.83 ± 0.51
PPG (1 mM)	6	3.31 ± 0.38***
L-NOARG (100 μM)	4	5.77 ± 0.35*
Roflumilast (10 μM)	5	11.45 ± 0.83**
Roflumilast (30 μM)	5	12.75 ± 0.64****
Roflumilast (100 μM)	4	15.20 ± 0.33****

Results are expressed as mean ± SD of *n* experiments.$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ versus control value (analysis of variance followed by Bonferroni method).

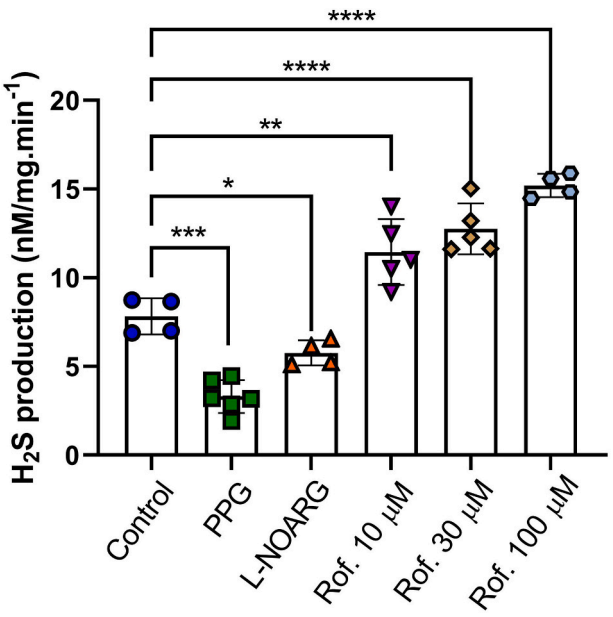

Fig. 2. Roflumilast enhances endogenous H_2S production in rat corpus cavernosum. Quantification of H_2S levels produced in control conditions (without any treatment), in presence of DL-propargylglycine (PPG, 1 mM), N^G-nitro-L-arginine (L-NOARG, 100 μM) inhibitors of CSE and NO synthase, respectively, and in presence of roflumilast (Rof. 10, 30 and 100 μM), a selective PDE4 inhibitor. Bars represent mean ± SD. of 4–6 rats. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs control (one-way ANOVA followed by Bonferroni post hoc test).

4. Discussion

The current study suggests that PDE4 is expressed in the nerve cells of the CC smooth muscle fibers and plays a pivotal role in regulating rat CC tone. PDE4 inhibition and consequent increases in cAMP levels produce a powerful smooth muscle relaxation in part due to its effects in facilitating gasotransmitters-mediated neurogenic response. This conclusion is supported by the following findings: (1) A marked PDE4A isoenzyme expression is mainly localized within the nerve cells of the cavernous smooth muscle fibers. (2) Roflumilast produced a strong CC relaxation. (3) An increase in endogenous H_2S production by roflumilast that was reduced by NO and H_2S synthesis enzyme blockade. (4) An augmented EFS- and isoproterenol-induced relaxations induced by pretreatment with a roflumilast threshold concentration.

In erectile tissue, a marked PDE5 expression has been described regulating endogenous cGMP levels linked to the capacity to reach a penile erection [9,16,28]. cGMP favors the release of NO from nitrergic neurons in rabbit CC, thus highlighting the key role of the NO/cGMP signaling pathway in nerve-mediated penile vasorelaxation [29]. In cavernous smooth muscle and vascular endothelium of human cavernous arteries a positive IR for PDE4 and a cAMP-specific PDE co-localized with PKA has also been detected together with PDE5. Therefore, the latter indicates an interaction between cAMP- and cGMP-dependent pathways in the regulation of penile smooth muscle tone [16,30,31]. In the present study, western blot analysis showed a marked PDE4A protein expression in the whole CC tissue and the double labeling

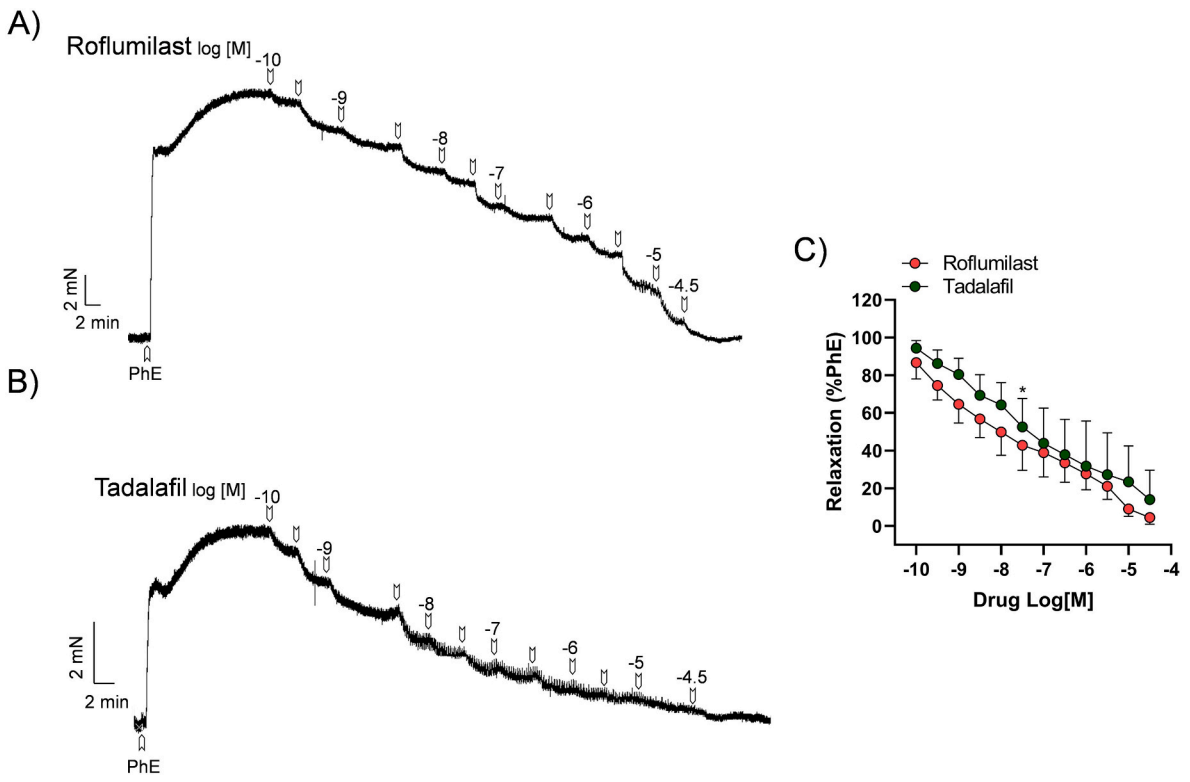

Fig. 3. Roflumilast induces a stronger rat CC relaxation compared to that produced by PDE5 inhibitor tadalafil. Isometric tension recordings displaying the relaxations produced by roflumilast (0.1 nM −30 μM) (A) and tadalafil (0.1 nM–30 μM) (B) on 3 μM phenylephrine (PhE)-contracted rat CC strips. The vertical bar displays force (mN) whereas the horizontal bar displays time (min). W: wash. Graph summarises concentration-dependent relaxation curves to tadalafil (0.1 nM–30 μM) (green circles) and roflumilast (0.1 nM–30 μM) (red circles) in rat CC samples (B). Results are expressed as mean ± SD of 7 rats. $^{*}P < 0.05$, vs pD_2 control (paired *t*–test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Effect of 0.3 nM roflumilast on relaxations to electrical field stimulation (EFS, 1 ms duration, 0.2–16 Hz, 20s trains, with constant current output adjusted to 75 mA) in rat corpus cavernosum strips.

	EFS (Hz)						
	0.2	0.5	1	2	4	8	16
Control	2 ± 1	2 ± 1	6 ± 4	12 ± 5	31 ± 11	46 ± 17	56 ± 14
Roflumilast	9 ± 5*	11 ± 4*	19 ± 11*	23 ± 17*	52 ± 12*	63 ± 12*	68 ± 13*

Results are expressed as inhibition percentage of 3 μM phenylephrine-induced precontraction and represent the mean ± SD of 7 experiments. $^{*}P < 0.05$ versus control, paired *t*–test, n = 7.

immunofluorescence revealed a positive IR for PDE4A mainly localized within the nerve cells of the cavernous smooth muscle fibers. This data unmasks that PDE4A expression is mainly (>70%) localized within intramural nerve fibers that there distributed along the entire penile smooth muscle. Indeed, this data agrees with previous reports where IR for PDE4 is described in both smooth muscle and endothelial cells of the CC tissue [16]. The abundant presence of PDE4A in nerves suggests a possible modulatory role of neuronal cAMP on the inhibitory gaseous neurotransmission of cavernous tissue.

In penile tissues, mRNA expression of NOS has been detected in parasympathetic neurons including a homogeneous population, as all appear to be able of forming NO. In contrast to the endothelium of penile vessels, the endothelial cells that coat the cavernous spaces may not be able of NO synthesis [6,32]. NO is known to play a key role in regulating nerve-dependent penile vasorelaxation. This is supported by the fact that the NOS inhibitors selectively block neurogenic erectile responses and that the neuronal isoform of NOS localized in the penile innervation [33–35]. In addition to NO, H_2S is another gaseous transmitter vasodilator that is involved in the neurogenic-dependent relaxation of human CC smooth muscle [36]. Endogenous H_2S production was significantly decreased due to low activities of H_2S synthesis enzymes, CSE and cystathionine β-synthase in a rat model of diabetes accompanied with erectile dysfunction [37]. Interestingly, an interplay between endogenous H_2S and NO has been reported to be implicated in erectile function [38–40]. H_2S exerts its pro-erectile effects by enhancing the NO pathway since H_2S levels are tightly related to the levels of NO enzyme activity and CC tissue cGMP. This indicates that, in CC tissues, H_2S has an important role in the erectile function modulating the NO enzyme activity, which brings out a synergic role of both gasotransmitters regulating erectile function [38–40]. In our study, endogenous H_2S production was decreased by the H_2S and NO synthesis inhibitors. Hence, this fact indicates that CSE-dependent H_2S generation also involves a NO synthetic pathway, pointing to a possible synergy between both gaseous neurotransmitters in CC tissue [38–40]. Nevertheless, H_2S production in CC samples was re-established and increased, in a concentration-dependent fashion, above control levels, in response to increasing roflumilast concentrations. These results imply that the PDE4A expressed in the nerve cells would be implicated in the production and/or release of inhibitory gasotransmitters from nerves. Moreover, selective PDE4 inhibition enhances endogenous H_2S production, thus providing indications that blockade of PDE4 results in a positive feedback mechanism on the local nerve-induced release of gasotransmitters. This fact, together with the potentiated roflumilast-induced neurogenic relaxations, suggests that selective PDE4 inhibition enhances smooth muscle relaxation via facilitatory effects on NO- and H_2S-dependent inhibitory neurotransmission in rat CC. These results agree with studies reported in pig and human bladder outlet, where

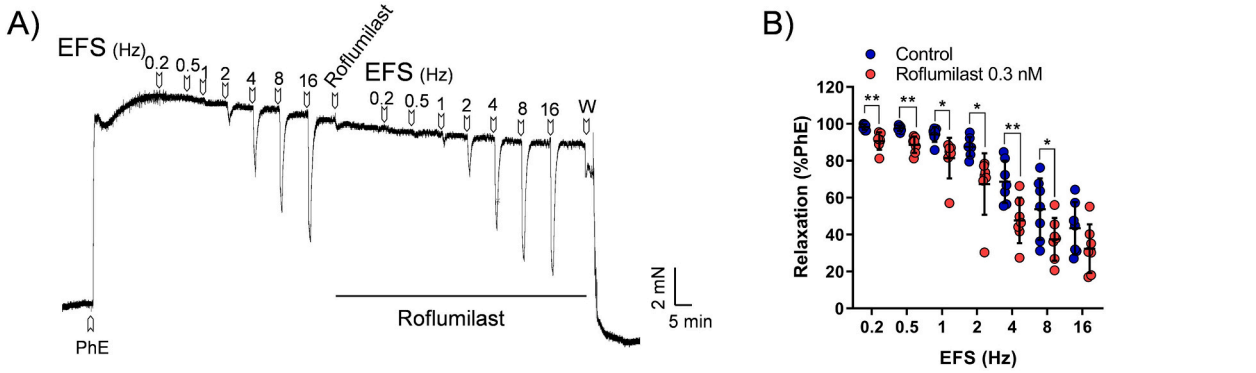

Fig. 4. Roflumilast enhances nerve-mediated relaxation in rat corpus cavernosum (CC). Original isometric force recordings exhibiting frequency-dependent relaxation in response to electrical field stimulation (EFS, 0.2–16 Hz,1 ms in duration, and 20 s trains) in untreated (control) and treated with roflumilast (0.3 nM). EFS was performed on rat CC strips precontracted with phenylephrine (PhE, 3 μM) and maintaining non-adrenergic and non-cholinergic conditions respectively with guanethidine (10 μM) and atropine (1 μM). The vertical bar displays force (mN) and the horizontal bar displays time (min). W: wash (A). Graph summarises frequency-dependent relaxation curves to EFS in untreated (control, blue circles) and treated with 0.3 nM roflumilast (red circles) rat CC strips (B). Bars represent the mean ± SD of 7 rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs control (one-way ANOVA followed by Bonferroni post hoc test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

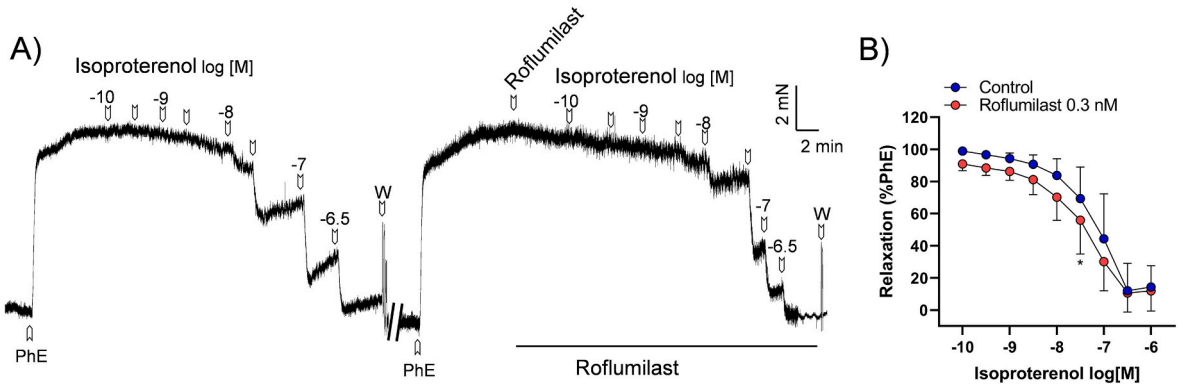

Fig. 5. Roflumilast potentiates β-adrenoceptor agonist relaxation in rat corpus cavernosum (CC). Original isometric force recordings exhibiting the relaxations produced by β-adrenoceptor agonist isoproterenol (0.1 nM–0.3 μM) in untreated (control) and treated with roflumilast (0.3 nM), on 3 μM phenylephrine (PhE)-precontracted rat CC strips. The vertical bar displays force (mN) and the horizontal bar displays time (min). W: wash (A). Graph summarises concentration-dependent relaxation curves to isoproterenol in untreated (control, blue circles) and treated with roflumilast (red circles) rat CC strips (B). Bars represent the mean ± SD of 8 rats. $^{*}P < 0.05$, vs pD_2 control (paired *t*–test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PDE4 isoenzyme is mostly located within nerve fibers, and selective PDE4 inhibitors exerted a positive feedback fashion on NO- and H_2S-mediated inhibitory neurotransmission [18]. However, our data contrast with those described in rabbit CC where PDE5 inhibition result in a negative effect on the release of NO from nerves [41]. In the current study, the fact that roflumilast promotes a potent CC smooth muscle relaxation is in accordance with that reported in the human bladder neck and urethra, where together with the powerful relaxant effect produced by selective PDE5 inhibitors sildenafil, vardenafil and tadalafil, a high relaxant efficacy for PDE4 inhibitors is also described [18,19].

In addition to PDE5 and PDE4 inhibitors, β-adrenoceptor agonists are also involved in CC relaxation [42,43]. 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 [43,44]. Even though the cAMP-PKA is the canonical signaling pathway mediating β-adrenergic relaxations, cAMP-independent mechanisms have also been described to be involved in the β-adrenergic relaxations which include direct activation of K^+ channels or the blockade of voltage-gated Ca^{2+} channels and α_1-adrenoceptors [45–47]. An interaction between PDE4 inhibitors and beta-adrenoceptor agonists has been reported in the bladder outlet region. Selective PDE4 inhibitors, such as rolipram and roflumilast potentiate β-adrenoceptor stimulation relaxation in pig and human bladder neck [18,48]. In the present study, threshold concentrations of roflumilast promoted a leftward shift of the relaxation concentration-dependent curve to the β-adrenoceptor agonist isoproterenol, suggesting that PDE4 inhibitors, besides causing a potent smooth muscle relaxation per se, increase the response promoted via β-adrenoceptor activation in the CC. Because erectile dysfunction, overactive bladder and bladder outlet obstruction can coexist and since CC, prostate, urethra and bladder share common pathways, drugs targeting simultaneously these organs may offer a synergic and valuable pharmacological tool for patients presenting these conditions [49–57]. In fact, roflumilast, an anti-inflammatory drug, has been used as an add-on pharmacological tool to produce long-effect bronchodilatation in asthma and COPD. Thus, this suggests the possible usefulness of roflumilast, alone or in combination with the PDE5 inhibitors in those groups of patients suffering from erectile dysfunction which show refractoriness to the first line treatment of this disease [24–26].

5. Conclusion

These results suggest that PDE4 is mainly localized in the nerve cells

of rat CC, where roflumilast induces a potent cavernous smooth muscle relaxation. Moreover, roflumilast enhances endogenous H_2S synthesis and nerve-mediated relaxation which indicates that selective PDE4 blockade promotes NO- and H_2S-mediated gaseous inhibitory neurotransmission.

CRediT authorship contribution statement

Conception and design: VSF, DP, BC and MH. Acquisition, analysis, and interpretation of the data: VSF, MELO, MPM, AAT, PR, JND, MVB, SB, DP, BC and MH. Drafting or revising and final approval of the manuscript: VSF, MELO, MPM, AAT, PR, JND, MVB, SB, DP, BC and MH.

Declaration of competing interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

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