Expression and functional activity of phosphodiesterase type 5 in human and rabbit vas deferens

R.Mancina¹*, S.Filippi²*, M.Marini³, A.Morelli¹, L.Vignozzi¹, A.Salonia⁵, F.Montorsi⁵, N.Mondaini⁴, G.B.Vannelli⁵, S.Donati¹, F.Lotti¹, G.Forti¹ and M.Maggi¹,⁶

¹Andrology Unit, Department of Clinical Physiopathology, ²Interdepartmental Laboratory of Functional and Cellular Pharmacology of Reproduction, Department of Pharmacology and Clinical Physiopathology, ³Department of Anatomy, Histology and Forensic Medicine, ⁴Department of Urology, University of Florence, ⁵Department of Urology, University Vita-Salute San Raffaele, Scientific Institute H. San Raffaele, 20132 Milan, Italy and ⁶To whom correspondence should be addressed at: Andrology Unit, Department of Clinical Physiopathology, University of Florence, V.le G. Pieraccini, 6, 50139 Florence, Italy. E-mail: m.maggi@dfc.unifi.it

The molecular mechanisms underlying the regulation of vas deferens (VD) motility and semen emission are still poorly understood. We now report evidence on VD expression of phosphodiesterase type 5 (PDE5), which regulates nitric oxide (NO)-induced relaxation and cGMP breakdown in smooth muscle cells. In human VD, the PDE5 abundance was relatively high (>3 x 10⁶ molecules/µg total RNA), although 10-fold lower than in corpora cavernosa (CC). Also cGMP metabolising activity was higher in CC than in VD. However, both tissues share the same sensitivity to a broad panel of cGMP-related PDE inhibitors: sildenafil, tadalaflal, diprydamole, zaprinast, vinpocetine, EHNA and cilostamide. Based on the rank order of potency of these PDE inhibitors, we found that the cGMP metabolizing activity in human VD mostly corresponds to PDE5. PDE5 was immunolocalized in all the muscular layers of human and rabbit VD and was found to be negatively involved in regulating NO-induced relaxation. In addition, by using a rabbit model of hypogonadotropic hypogonadism, we found that the PDE5 gene expression and activity are androgen-dependent in VD, as previously demonstrated in CC. In fact, the sensitivity to a NO-donor (NCX4040), its enhancement by PDE5 inhibitors and the PDE5-related cGMP breakdown were all affected by androgen manipulation. Our results provide a hypothesis explaining the beneficial effects of PDE inhibitors in patients with rapid ejaculation.

Key words: PDE5/rapid ejaculation/sildenafil/tadalafil/vas deferens

Introduction

Ejaculation comprises a set of neuromuscular events that, during the ultimate part of the sexual response cycle, enable the semen to progress from the cauda epididymis and the vas deferens (VD) to the urethral meatus. The external propulsion of the semen is an essential, although often neglected, component of mammalian reproduction. The ejaculatory process occurs in at least two distinct phases: emission—contraction of the smooth muscle along the entire male genital tract (MGT) and deposition of the seminal fluid into the posterior urethra, and expulsion—subsequent bulbospongious muscle activation and anterograde expulsion of the semen. The latter phase is under parasympathetic and somatic controls from the perineal branch of the pudendal nerve, originating from S2 to S4. In contrast, the emission phase is under sympathetic control from the emission centre located in the thoracic lumbar cord. At the time of ejaculation, both noradrenaline, released by short sympathetic neurons in the pelvic plexus, and adrenaline, released by the adrenal medulla after activation of the greater splanchic nerve, primarily regulate the contractility of seminal vesicles, prostate and VD (Kihara et al., 1997). Besides catecholamines, a role for nitric oxide (NO) in the control of the ejaculatory process was also recently suggested, following the observation that mice lacking the gene for endothelial NO synthase show ejaculatory abnormalities characterized by an increased propensity to ejaculate on a reduced stimulus (Kriegsfeld et al., 1999). In contrast, mice genetically deficient in the subtype P2X1 of purinergic receptors are infertile because of ejaculatory failure, this being associated with a decrease in neurogenic VD contraction (Mulryan et al., 2000). A lower breeding efficiency due to a decreased ejaculatory activity has been also described in mice with targeted deletion of the heme oxygenase-2 gene, the enzyme that produces carbon monoxide, a putative non-adrenergic, non-cholinergic (NANC) transmitter regulating bulbospongious firing (Burnett et al., 1998; Wickelgren, 2003). Hence, a complex cohort of purinergic, peptidergic and gaseous transmitters contained in the NANC nerves plays an ancillary, but relevant, role in controlling MGT motility and, therefore, also the ejaculatory latency. The cyclic nucleotides, cAMP and cGMP, act as second messengers for most of the NANC activity in the MGT and their intracellular levels are tightly tuned in both the rate of synthesis and degradation by different families of cyclases and phosphodiesterases (PDE) isoenzymes. Among PDEs, some isoforms are specific for cAMP (PDE4, PDE7 and PDE8), some others for cGMP (PDE5, PDE6 and PDE9) and, finally, some retain mixed activity (PDE1–3, PDE10 and PDE11, for a review see Lin et al., 2003). Numerous pharmaceutical compounds that selectively inhibit the catalytic activities of PDE have been developed, but only PDE5 inhibitors (PDE5i) reached
clinical application for erectile dysfunction (ED) and, later on, for pulmonary hypertension (Corbin and Francis, 2002; Manganiello, 2003). Recently, the PDE5i sildenafil has been suggested as a new treatment for one of the most common ejaculatory dysfunction, rapid ejaculation (RE), based on very encouraging results from a double-blind study (Abdel-Hamid et al., 2001). In this study, sildenafil was even more potent than serotoninergic drugs in delaying ejaculation and producing sexual satisfaction (Abdel-Hamid et al., 2001). Furthermore, in two independent studies the addition of sildenafil to the serotoninergic drug paroxetine, in otherwise paroxetine non-responder RE patients, significantly increased the therapeutic success and intercourse satisfaction (Salonia et al., 2002; Chen et al., 2003). Although it is possible that the beneficial effect of PDE5i in RE relies on facilitating erection in otherwise less aroused subjects (Goldmeier and Lamba, 2001), it is still possible that they act specifically with their target (i.e. PDE5) present in the musculature of the MGT. We have, in fact, recently reported a high abundance of PDE5 transcripts not only in the penis, but also in the other androgen-dependent muscular tissues, as VD (Morelli et al., 2004, 2002a,b). In this study, we confirm this preliminary observation in a larger series of human VD. In addition, we report for the first time, the immunolocalization, the biochemical and pharmacological characterization of PDE5 in human and rabbit VD. Finally, we found that also in VD PDE5 gene expression and activity is androgen-dependent, as previously reported in penile corpora cavernosa (CC, Morelli et al., 2004).

Materials and methods

Chemicals

1H-[1,2,4]oxadiazolo[4,3-c]quinoxalin-1-one (ODQ), erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), zaprinast, dipridamole, cistamidine and 8-bromo-cGMP (8-Br-cGMP) were obtained from Tocris (Bristol, UK). Vinpocetine, calmodulin, cGMP, 5’GMP and guanine were purchased from Sigma (St Louis, MO). KCl and thin-layer chromatography plates (20 × 20) silica gel 60 F254 were supplied by Merck (Darmstadt, Germany). [8-3H]cGMP (15.1 Ci/mmol) was purchased from Amer sham (Buckinghamshire, UK). InstaGel Plus was from Packard (Wellesley, MA, USA). Testosterone (T) enanthate was supplied by Schering AG (Berlin, Germany). Triptorelin enamine was supplied by Ipsen (Milan, Italy). Sildenafil was a gift from Dr C.Stief (Hanover Medical School, Germany). NCX4040 was synthesized at the NicOx Research Institute (Milan, Italy). The polyclonal anti-PDE5 antibody was a kind gift from Prof. M.Gior gi (Department of Basic and Applied Biology, University of L’Aquila, L’Aquila, Italy). Stock solutions of sildenafil, cistamidine and vinpocetine were made in ethanol; stock solutions of tadafalil, zaprinast, dipridamole, ODQ and NCX4040 were made in dimethylsulphoxide; the other substances were dissolved daily in double distilled water and further dilutions to the final concentrations were made in buffer solution. Preliminary experiments indicated that the concentrations of dimethylsulphoxide and ethanol used modified neither the vascular response nor the relaxation induced by the different agents.

Collection of human and rabbit tissues

Human VD samples were obtained from patients undergoing pelvic surgery for benign (n = 1; age 27 years; post-traumatic testicular swelling) and malignant diseases (n = 8; age range 60.2 ± 3.51 years; radical retropubic prostatectomy with pelvic lymphadenectomy for a clinically localized prostate cancer). Radical retropubic prostatectomy with pelvic lymphadenectomy was performed in all the patients according to the surgical technique described by Walsh (2002) with minor modifications (Da Pozzo et al., 1995). In each case, radical prostatectomy was performed with a spinal anesthesia according to our previous experiences (Salonia et al., 2004). From each patient, two segments of VD (2 cm in length) were obtained: the first one, named proximal, was taken from the part of the VD closest to the inguinal channel; the second segment, named distal, was obtained from the closest part to the prostate. Corpus cavernosum (CC) samples were obtained from patients undergoing penile prosthesis implantation (n = 3; age range 40–67 years). None of the patients took any medication including hormonal treatment for at least 6 months before surgery and their plasma T levels were in the normal range. Human mammary gland samples were collected from premenopausal women undergoing surgery for benign diseases. All tissue samples were collected after the approval of the Hospital Committee for Investigation in Humans and with the patients’ informed consent.

Rabbit VD were obtained from New Zealand White rabbits weighing approximately 3 kg. Immediately after removal, human and rabbit tissue samples were shock frozen in liquid nitrogen and stored at −80°C until RNA/protein preparation. For immunohistochemistry preparations, human and rabbit tissues were immediately fixed in Bouin’s solution and embedded in paraffin. For in vitro contractility studies, the tissue preparations were immediately placed and maintained in cold modified Krebs solution until use. All the animal experiments were performed in accordance to Decreto Legge 116/92 and approved by IACUC (Institutional Animal Care and Use Committee) of the University of Florence.

Isolation of RNA and cDNA synthesis

Total RNA was extracted from the frozen tissues using TRIZOL reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s instructions. RNA concentration and quality were measured by spectrophotometric analysis at 260 and 280 nm. RNA integrity was assessed by electrophoresis in agarose gel. For each sample, 400 ng of the total RNA was reverse transcribed to cDNA in 80 μl using TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA) under the following conditions: 10 min at 25°C, 30 min at 48°C, 5 min at 95°C.

RT–PCR and polycrylamide gel electrophoresis

Total RNA from rabbit VD was extracted and checked for quality and quantity as for human tissues (described above). Following the already published RT–PCR conditions (Morelli et al., 2004), 250 ng of total RNA were reverse transcribed for 30 min at 50°C, denatured for 2 min at 95°C and amplified for 22 cycles with the following steps: 45 s at 95°C, 1 min at 55°C, 1 min at 70°C. Primer design (sense primer: 5’-ACGGTATTTCCGTCTTCCTT-3’, exon 2; antisense primer: 5’-GTAAATGGCCACCGTTTC-3’, annealing to a sequence in exon 4) was based on homology to the human sequence as already published (Morelli et al., 2004). The amplified cDNA was run on a 8% non-denaturing polycrylamide gel and visualized under UV light after ethidium bromide staining. The integrity of total RNA was verified performing the RT–PCR for the rabbit housekeeping γ-nucleomucin actin (γ-ACT) gene using specific primers, covering a 328-bp region (sense: 5’-ACATGGA-GAAGATCTGGCAGAC-3’, nucleotide position 317–336; antisense: 5’-CATG-AGTGAGCTGCGGTAGT-3’, nucleotide position 626–645). RT–PCR conditions were the same as those used for PDES5 analysis.

Real-time RT–PCR (TagMan)

The quantitative assay was performed according to the fluorescent TaqMan methodology as already published (Morelli et al., 2004). Primers and probe for PDES5 mRNA were assay-on-demand gene expression products purchased from Applied Biosystems. PCR mixture (25 μl final vol) consisted of 1X final concentration of assay-on-demand mix, 1X final concentration of Universal PCR Master Mix (Applied Biosystems) and 25 ng of cDNA. Amplification and detection were performed with the ABI Prism 7700 Sequence Detection System with the following thermal cycler conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles at 95°C for 30 s and 60°C for 1 min. Each measurement was carried out in duplicate. Absolute quantification of the results was performed using an external standard curve prepared as previously described (Morelli et al., 2004). Known starting concentrations of the standard cDNA, ranging from 3.5 × 10¹ to 3.5 × 10⁵ copies, were used to construct an external calibration curve. Results are expressed as PDES5 mRNA molecules per microgram of total RNA.

Experimental hypogonadism and sex steroid replacement

New Zealand White male rabbits (n = 28) were divided into three groups and treated as previously described (Filippi et al., 2002a,b; Morelli et al., 2004; Vignozzi et al., 2004). Briefly, one group was kept intact (controls,
n = 8). Another group was treated with a single administration of 2.9 mg/kg of the long-acting GnRH analog triptorelin pamoate (n = 20). After 15 days a subset of triptorelin-treated rabbits were supplemented with a pharmacological dose of T enanthate (30 mg/kg weekly for 6 weeks, n = 10). After 2 months from triptorelin administration and after 1 week from the last sup-

**Measurement of T**

T plasma levels were measured with an Automated Chemiluminescence System (Bayer Diagnostics, East Walpole, MA) as previously described (Filippi et al., 2002a,b; Morelli et al., 2004; Vignozzi et al., 2004). Briefly, extraction was performed mixing samples with four volumes of diethyl-ester for 15 min, centrifuging for 5 min at 1030 g, and freezing the aqueous phase in dry ice. The organic phase was recovered, evaporated to dryness under a nitrogen stream and reconstituted in the assay buffer.

**Immunohistochemistry**

Immunohistochemical studies were performed, as previously described (Morelli et al., 2004), on the deparaffinized rehydrated sections of human and rabbit VD tissues. Briefly, the slides were stained for indirect immuno-

**PDE activity assay**

Human (VD and CC) and rabbit (VD) tissues were homogenized by Ultra-
turrax (5:1, vol/wt) in ice-cold buffer (20 mM HEPES, pH 7.2, 1 mM EDTA; 250 mM sucrose; 1 mM phenylmethylsulfonylfluoride). The homogenates were aliquoted and stored at −80°C until use. Protein concentration was determined by the Bradford method (Bradford, 1976). PDE activity for human and rabbit samples was carried out as described by Morelli et al. (2004). For IC50 determination, protein aliquots of 0.06 mg human and rabbit VD homogenates and 0.02 mg CC homogenate were incubated in a final volume of 200 μl for 5 min at 30°C, with 0.5 μM cold cGMP and 0.1 μM [3H]-cGMP in 40 mM MOPS (4-morpholinopropanesulfonic acid) buffer (pH 7.0), containing 1 mM EDTA, 0.8 mM EGTA, 5 mM Mg acetate, 0.2 mg/ml bovine serum albumin, with or without inhibitors. The inhibitors used to characterize PDE activity were tadalafil and sildenafil (selective PDE5i; from 10−11 to 10−5 M), zaprinast (selective PDE5, PDE6 and PDE9 inhibitor, from 10−9 to 10−4 M), ethylendiamine (selective PDE5, PDE6 and PDE10, from 10−9 to 10−4 M), and cilostamide (selective PDE3 inhibitor, from 10−9 to 10−4 M). For vinpocetine (selective inhibitor for PDE1, from 10−9 to 10−4 M), experiments were conducted as before, but without EDTA and EGTA and in the presence of 10 μM calcium and 20 mM calmodulin. Reac-
tions were terminated by incubation at 100°C for 1 min. Samples were then supplemented with cGMP, GMP and guanine as carriers (60 μl of a solution containing 3 mM cGMP, GMP and guanine) and centrifuged for 10 min at 10000 g. Aliquots of 60 μl of each sample were applied to 60 F254 silica-gel plates using absolute ethanol/H2O (70:30, vol:vol) as eluent to separate cGMP, GMP and guanine. Nucleotides and guanine are visible under UV light, and the corresponding lanes were identified comparing each ratio frontis with those of the standard molecules comigrated on the same plate. cGMP, GMP and guanine lanes were scraped, silica was extracted with 1 ml H2O, and the radioactivity was measured in InstaGel Plus by a β-counter instrument. The enzymatic activity was evaluated as the percentage of substrate into product conversion, as follows: conversion (%) = [products count/substrate + products counts] × 100.

**Studies of contractility**

Rabbit and human VD strips, isolated from the part of the VD closest to the inguinal channel, were vertically mounted under a stable resting tension in organ chambers containing 10 ml of modified Krebs solution at 37°C, gassed with 95% O2 and 5% CO2 at pH 7.4. The solution has the following millimolar composition: NaCl 118, KCl 4.7, KH2PO4 1.2, NaHCO3 25, MgCl2·6H2O 1.2, CaCl2 2.5, glucose 11.1 and disodium EDTA 0.01. The preparations were allowed to equilibrate for at least 120 min; during this period the bath medium was replaced every 15 min and tension was adjusted to a final value of 1000 mg. Changes in the isometric tension were recorded on a chart polygraph (Battaglia Rangoni, San Giorgio di Piano, Bologna, Italy). High potassium salt solution (KC1), made by the equimolar substitu-
tion of sodium with potassium, increased the tonic tension with the maxi-
mum effect obtained at 80 mM. Experiments were performed in strips precontracted with KCl (80 mM) able to produce a stable tension of about 1000 mg. This degree of contractile response was taken as 100% and the relaxant effect induced by different concentrations of the used drug (NO-donor, NCX4040) was referred to this value. Relaxant response to NCX4040 was measured after a 30 min pre-treatment with selected inhibitors (sildenafil, tadalafil and ODQ).

**Statistical analysis**

Results are expressed as mean ± SEM for n experiments. Statistical analysis was performed with Student’s t-test for paired or unpaired data, with analysis of variance followed by Fisher’s test in order to evaluate differences among human groups and P < 0.05 was taken as significant. Half-maximal response inhibiting concentration (IC50) values were calculated using the computer pro-
gram ALLFIT (De Lean et al., 1978), supplied by P.J.Munson, National Institutes of Health (Bethesda, MD). ALLFIT uses the constrained four-
parameter logistic model to analyse families of sigmoid curves, to obtain estimates of IC50 values and to compare them using an F-test and tests of randomness of the residuals around the fitted curves.

**Results**

Figure 1A shows absolute quantitation of PDE5 mRNA expression (real-time RT–PCR) in human VD (n = 7) as compared to human CC (n = 3), taken as a positive control. Results in mammary glands (n = 3), expressing a low abundance of the gene (Morelli et al., 2004), are also reported for comparison. Although PDE5 transcript abundance in both the distal (3.4 ± 1.2 × 104 molecules/μg total RNA) and the proximal (5.8 ± 2 × 106 molecules/μg total RNA) portion of the VD is one log unit lower than that of the penis (45 ± 13 × 106 molecules/μg total RNA, P < 0.001), we confirm its relatively high expression (Morelli et al., 2004), which does not differ in the two portions of the VD examined. To verify whether this PDE5 gene expression could correspond to a specific protein activity, we studied the ability of sildenafil (a selective PDE5i) to inhibit the conversion of cGMP into metabolites in human deferential extracts (n = 4), comparing data with those obtained in human penile extracts (n = 3). Results are summarized in Figure 1B and C. We found in both CC and VD that sildenafil dose-dependently inhibited cGMP degradation with the expected IC50 for PDE5. Interest-
ingly, sildenafil IC50 was not significantly different in the two tissues (IC50 = 1.7 ± 0.3 μM), although the maximum velocity was definitively higher in the penile than in the deferential extracts (0.26 ± 0.004 versus 0.078 ± 0.033 nmol/mg protein/min, P < 0.0001). To further characterize cGMP hydrolytic activity in human VD, we compared the inhibition curves of different families of PDE, simultaneously generated in human VD (Figure 2A) and CC (Figure 2B) preparations. The derived rank order of potency of PDE inhibitors, having different selectivity for the various PDE isozymes eventually acting in both the tissues, is graphically reported as pIC50–pIC50 plot in Figure 2C. As shown in the pIC50– pIC50 plot, the inhibitor pIC50 in VD and CC are closely related (r = 0.994) and lie within—or in close proximity to—the line of identity (y = 0.999x − 0.125), suggesting that both tissues express a similar pattern of PDE activity. In particular, two chemically dis-
tinct, but selective, PDE5i, as sildenafil and tadalafil, inhibited the majority of activity (67 ± 6%) either in human VD or in human.
CC, with virtually identical IC₅₀ (sildenafil 2.0 ± 1.1 nM and 2.3 ± 1 nM; tadalafil 2.4 ± 1.2 nM and 1.0 ± 0.4 nM, respectively). The IC₅₀ for dipyridamole in both VD (1 ± 0.4 μM) and CC (1.4 ± 0.5 μM) was compatible with its inhibitory activity for PDE5 (Soderling et al., 1998b), although this compound interacts, with a similar IC₅₀, with other members of the PDE superfamily, as PDE6 (Soderling et al., 1998b) and PDE10 (Fujishige et al., 1999), having cGMP as substrate. Also the zaprinast IC₅₀ was comparable to the presence of PDE5 (or PDE6, Soderling et al., 1998a) in both tissues (VD: 2.2 ± 0.8 μM; CC: 1.7 ± 0.6 μM), even if this compound, in the highest micromolar range, also inhibited PDE9-mediated cGMP hydrolysis (Soderling et al., 1998b). Interestingly, at variance with the selective PDE5i, sildenafil and tadalafil, dipyridamole and zaprinast almost completely blocked cGMP breakdown in both human VD and CC, suggesting their presence in these tissues of at least some cGMP-related PDE activity other than PDE5. The lack of inhibition at the expected (micromolar) concentrations for vinpocetine (PDE1 inhibitor) and EHNA (PDE2 inhibitor) ruled out a substantial contribution by these cAMP and cGMP unspecific PDE to cGMP catabolism in VD and CC. Finally, cilostamide, a compound that in nanomolar concentrations inhibits PDE3 (a cGMP-inhibited cAMP, cGMP unspecific PDE), affected cGMP breakdown in these human tissues only in the high micromolar range. Hence, the present pharmacological characterization of cGMP-related PDE activity strongly suggests that PDE5-like activity is present in VD, as well as in CC, and that it largely contributes to cGMP breakdown. To finally demonstrate the biological activity of PDE5 in human VD, we analyzed the effect of two selective PDE5i, tadalafil and sildenafil, on NO-induced VD relaxation. Human VD strips, derived from five different subjects, were incubated with rather selective PDE5i (sildenafil and tadalafil, both = 100 nM) or a vehicle, precontracted with KCl (80 mM) and then exposed to increasing concentrations of the NO-donor NCX4040 (Morelli et al., 2004). Results are reported in Figure 2D. Sildenafil and tadalafil induced a 23.3 ± 18 and 130 ± 98-fold increase, respectively, in NCX4040 relaxing potency (NCX4040 + vehicle IC₅₀ = 342 ± 227 μM; NCX4040 + sildenafil IC₅₀ = 14.6 ± 6 μM; NCX4040 + tadalafil IC₅₀ = 2.6 ± 0.9 μM), indicating, that in human VD, PDE5 physiologically hampers NO-related cGMP activity. Involvement of cGMP pathway in NCX4040-induced relaxation in human VD, was substantiated by using the guanylyl cyclase inhibitor, ODQ, that almost totally abolished this relaxing effect (Figure 2D). Addition of increasing concentrations of the cell permeant cGMP analog 8-Br-cGMP to ODQ-pretreated strips progressively rescued NCX4040-induced relaxation (Figure 2D). Hence, cGMP formation (ODQ) and degradation (PDE5) play a definitive role in controlling human VD relaxation.

The finding that PDE5 is involved in controlling smooth muscle relaxation in human VD suggests a possible localization of the enzyme in the muscular structure of the ductus. To verify this point we performed immunohistochemical studies in human VD (Figure 3), using previously characterized antibody (Giordano et al., 2001; Morelli et al., 2004). All the different muscular layers of the VD (Figure 3A and B), as well as endothelial and smooth

Figure 1. Expression and activity of PDE5 in human VD. A, absolute expression of PDE5 gene (molecules/μg total RNA, Real-time RT–PCR) in different portions of the human VD (n = 7), as compared to human corpus cavernosum (n = 3) and human mammary gland (n = 3). B and C, effect of increasing concentrations of the selective PDE5i sildenafil on the rate of conversion (Vi: nmol/mg protein × minute) of labelled cGMP to metabolites in human VD (B) and corpora cavernosa (C). * = P < 0.001.
muscle cells of the blood vessels, were intensely labelled, while luminal epithelial cells were definitely negative. Results obtained in human CC are also shown for comparison in Figure 3C and they are essentially similar to those already reported (Morelli et al., 2004), showing immuno-positivity in blood vessels (endothelium + muscle) and smooth muscle cells of the cavernae. The specificity of staining was demonstrated through the complete absence of labelling in VD sections obtained by omitting the antibody (Figure 3D).

We also analysed the immunodistribution of PDE5 in rabbit VD. It was similar to that observed in human VD, with an intense labelling in the muscular wall of the ductus and negativity of the epithelial layer (Figure 3E and F). Following our previous demonstration that PDE5 activity in rabbit CC is androgen-regulated (Morelli et al., 2004), we tested this effect also in the rabbit VD. We induced hypogonadotropic hypogonadism in adult rabbits through a single administration of a long-lasting GnRH analog (triptorelin). After 2 weeks, hypogonadal rabbits were replaced or not replaced with weekly administration of T (30 mg/kg). T values at the time of death (2 months after triptorelin administration and 1 week after the last administration of T) are reported in Table I. Results from functional studies are in Figure 4. In rabbit VD preparations, sildenafil inhibited cGMP conversion with an IC50 = 7.8 ± 2.6 nM (Figure 4A). Androgen ablation (triptorelin) or supplementation (triptorelin + T) did not significantly affect this parameter (P = 0.163). However, we observed a significant (P < 0.005 versus control) reduction in the cGMP conversion rate in hypogonadal rabbits when compared to controls, which was completely restored (P = 0.0001 versus hypogonadal) and even amplified (P < 0.001 versus control) in the T-replaced group (Figure 4A). This hypogonadism-induced alteration of sildenafil inhibition curves for cGMP conversion might indicate a direct effect of T on PDE5 expression, as previously reported in CC (Morelli et al., 2004). We therefore measured PDE5 mRNA expression (semi-quantitative RT–PCR) in VD extracts from control and hypogonadal rabbit, replaced or not with T, according to a previously described method (Morelli et al., 2004). We found that hypogonadism reduced PDE5 gene expression (P < 0.05), and that T replacement completely reversed it (Figure 4A, insets). In line with these observations are in vitro contractility studies (Figure 4B,
C and D). In deferential strips from untreated rabbits (Figure 4B), the NO-donor NCX4040 relaxed KCl-contracted preparations with an IC50, consistent with the values observed in human VD and previously reported in rabbit CC (Morelli et al., 2004). As for human strips, the inhibition of cGMP hydrolysis with the PDE5i sildenafil or tadalafil (100 nM) significantly ($P < 0.0001$) decreased the NCX4040 IC50 value and shifted its relaxation curve by at least one log unit to the left (Figure 4B and Table II). Blocking cGMP formation with ODQ, an inhibitor of NO-sensitive soluble guanylyl cyclase, completely abolished the relaxing effect of NCX4040, which, in turn, was progressively rescued by the addition of increasing concentrations of the cell permeant cGMP analog 8-Br-cGMP (Figure 4B). This indicates that, also in rabbit, a guanylyl cyclase-dependent cGMP pathway, sensitive to PDE5 inhibition, regulates VD tone. In hypogonadal rabbits, the relaxing potency of NCX4040 was significantly ($P < 0.01$) enhanced when compared to that of

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<th>Table I. Testosterone plasma levels in experimental rabbit groups</th>
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<td><strong>T</strong> (nmol/l)</td>
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<td>(a) Male control ($n = 4$)</td>
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<td>(b) Hypogonadal ($n = 7$)</td>
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<td>(c) Hypogonadal + T ($n = 7$)</td>
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Blood for T measurements was drawn 2 months after a single administration of the long-acting GnRH analog triptorelin pamoate (2.9 mg/kg, hypogonadal, groups b and c) and after 1 week from the last injection of T enanthate (30 mg/kg/week, group c). Triptorelin pamoate significantly reduced T plasma levels (group b). Weekly injection with T enanthate (group c) restored T plasma levels to values not significantly different from untreated rabbits (group a).

**$P < 0.01$ versus group a; *$P < 0.05$ versus group b. $n =$ number of animals.

Figure 3. Immunolocalisation of PDE5 in VD. A, transversal section of human VD (×50); note the intense positivity in all the muscular layers of the ductus (black arrow). Endothelial cells of the blood vessels were also labelled (arrowhead). Conversely, epithelial cells in the mucosa were definitively unstained (white arrow). B Higher magnification of A (×200). The inner longitudinal (I), the intermediate circular (C), and the outer longitudinal (O) muscular layers of the human VD were individually immunostained. C transversal section of human corpora cavernosa (×50), examined as positive control (Morelli et al., 2004). Black arrows show immunopositivity in cavernous smooth muscle cells; arrowheads show immunonegativity in endothelial cells of arteriolar wall and lacunar spaces. E tangential section of rabbit VD (×50). As in human VD, immunolabelling was essentially found in the robust smooth muscle layer (black arrows) and vascular endothelial cells (arrowhead), while the luminal epithelium was negative (white arrow). D and F Haematoxylin counterstained sections of human (D, ×50) and rabbit (F, ×50) VD (control sections), obtained by omitting the primary PDE5 antibody.

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Table II. Effect of PDE5i on NCX4040 pIC$_{50}$

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<thead>
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<th></th>
<th>Control</th>
<th>Hypogonadism</th>
<th>Hypogonadism + T</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCX4040</td>
<td>3.98 ± 0.07 (–)</td>
<td>4.30 ± 0.07$^a$(–)</td>
<td>4.12 ± 0.04 (–)</td>
</tr>
<tr>
<td>NCX4040 + Sildenafil (100 nM)</td>
<td>5.07 ± 0.09$^b$(12.31 ± 4.20)</td>
<td>4.50 ± 0.13 (1.60 ± 0.80)</td>
<td>5.52 ± 0.08$^c$(25.00 ± 6.30)</td>
</tr>
<tr>
<td>NCX4040 + Tadalafil (100 nM)</td>
<td>4.97 ± 0.09$^b$(9.66 ± 3.20)</td>
<td>4.45 ± 0.19 (1.40 ± 0.80)</td>
<td>5.49 ± 0.12$^e$(23.40 ± 7.70)</td>
</tr>
</tbody>
</table>

Inhibition constants expressed as pIC$_{50}$ (– log$_{10}$(IC$_{50}$)) ± SEM for NCX4040 alone or in the presence of two distinct PDE5i (100 nM) in VD strips from untreated rabbit (control) or rabbit treated for 2 months with a GnRH analog alone (hypogonadism) or in combination with weekly supplementation of testosterone (Hypogonadism + T). Values in brackets represent the pIC$_{50}$ ratio ± SEM of the respective PDE5i treated strips versus control, which numerically corre-
spond to the leftward shift in IC$_{50}$ values. Numbers in exponent represent statistical significance, as derived from simultaneous fitting of sigmoidal curves using the program ALLFIT:

$^a$P < 0.01 versus NCX4040 alone in Control.
$^b$P < 0.0001 versus NCX4040 alone in Control.
$^c$P < 0.0001 versus NCX4040 alone in Hypogonadism + T.
$^d$P < 0.001 versus NCX4040 + Sildenafil in Control.
$^e$P = 0.06 versus NCX4040 + Tadalafil in Control.
sildenafil (P < 0.001) and was borderline for tadalafil (P = 0.06). The effect of ODQ in impairing NCX4040 relaxation was not apparently sensitive to the different androgen milieu (Figure 4B–D). In addition, the different androgen milieu did not also affect the contractile responsiveness to KCl (80 mM, Table III).

**Table III.** Response to KCl (80 mM) in VD from experimental rabbit groups

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>n</th>
<th>Tension Mean ± SEM (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>1025 ± 113.6</td>
</tr>
<tr>
<td>Hypogonadal</td>
<td>10</td>
<td>873.2 ± 62</td>
</tr>
<tr>
<td>Hypogonadal + T</td>
<td>10</td>
<td>889.5 ± 170.3</td>
</tr>
</tbody>
</table>

Tension (mg) developed by VD strips in the different experimental conditions. n = number of preparations tested. Results were not statistically significantly different.

**Discussion**

This study represents the first demonstration that human VD expresses a cGMP-related phosphodiesterase activity that, at least in its greatest part, corresponds to PDE5. Expression of specific transcripts for PDE5 was demonstrated in both human and rabbit VD. As far as we know, this is the first demonstration of the expression of a PDE member, PDE5, in VD. It is interesting to note that selective PDE5i, as sildenafil and tadalafil, at the expected nanomolar concentrations, reduced almost 70% of the total cGMP metabolising activity of human VD. Other less selective PDE5i as dipyridamole and zaprinast almost completely abolished cGMP metabolism. Since, besides PDE5, dipyridamole and zaprinast interact, at the presently observed concentrations, also with PDE6 (Soderling et al., 1998b) and PDE10 (Fujishige et al., 1999) and with PDE6 (Soderling et al., 1998a) and PDE9 (Soderling et al., 1998b), respectively, it is possible that other cGMP-related PDEs are present in VD. Also cilostamide almost completely inhibits cGMP conversion to metabolites in human VD, but only in the high micromolar range, at which selectivity for PDE3a and PDE3b is lost. For instance, at 10 μM, cilostamide interacts also with PDE5 (Sudo et al., 2000). Both EHNA (PDE2 selective) and vinpocetine (PDE1 selective) inhibit conversion at concentrations far from that expected for selectivity. Hence, although it is possible that other cGMP directed PDE are present in VD (as PDE6, PDE9 and PDE10), demonstration, characterization and activity of these isoenzymes is beyond the aim of this study and needs further investigation. Nonetheless it is noteworthy that the rank order of potency of several PDE inhibitors in reducing cGMP hydrolysis activity was identical in VD and in CC, a tissue with a well-known enriched PDE5 expression (for a review see Lin et al., 2003). However, PDE5 expression and activity in human VD was significantly lower than in the penis (the present study and Morelli et al., 2004), but was sufficient to substantially hydrolyse cGMP and, thereafter, to negatively regulate NO-induced smooth muscle relaxation. As in the penis (Morelli et al., 2004), PDE5 was almost entirely immunolocalized in the robust smooth muscle layers that physiologically regulate VD contractile tone. Hence, this study allows for a better understanding of the clinical observation that in the human male, PDE5i increase ejaculatory latency (Abdel-Hamid et al., 2001; Salonia et al., 2002; Chen et al., 2003), being subsequently suggested to be a new therapeutic option for RE (Mitka, 2003; Mondaini et al., 2003; Abdel-Hamid, 2004). Among the ejaculatory abnormalities in humans, RE is the most common disorder, with a reported prevalence between 1 and 75% (Laumann et al., 1999). The American Psychiatric Association defines RE as persistent and recurrent ejaculation (not related to the use of any substance) in response to minimal sexual stimulation, immediately after penetration and before the desired moment, which causes interpersonal difficulties (American Psychiatric Association, 1994). In fact, this ejaculatory dysfunction is an important source of personal suffering and distress to the man and his partner, having profound effects on the psychosexual relationship of the couple. Although it is often referred to as an isolated sexual problem (Waldinger, 2002), in a recent series of more than 750 patients reporting ED, almost one third of the subjects complained of RE (Corona et al., 2004b).

Hence, the two disorders might share some pathogenetic mechanisms. In fact, in contrast to the pathogenesis of ED, the molecular events underlying RE have been scarcely investigated and often the symptom has been attributed to an underlying psychological cause (Schuster and Ohl, 2002). Studies in animal models indicate that the cGMP-generating pathway of NANC nerves might be involved in regulating not only penile erection but also the ejaculatory reflex. Accordingly, mice genetically lacking the gene for endothelial NO synthase (Kriegsfeld et al., 1999) or rat treated with NO synthase inhibitors (Hull et al., 1994; Bialy et al., 1996) ejaculate after a shorter time, while those treated with NO precursors showed the opposite trend (Hull et al., 1994). In this study we confirmed that a NO-donor, NCX4040, which fully relaxes CC preparations (Morelli et al., 2004), substantially decreases human and rabbit VD contractility in vitro. This NCX4040-induced VD relaxation is cGMP mediated, because an inhibitor of cGMP formation, as ODQ, completely prevented it. This is in keeping with previous observations in rats, using other cGMP enhancing agents (Schultz et al., 1977). In addition, we showed, for the first time, that selective PDE5i, as sildenafil and tadalafil, by decreasing cGMP degradation, significantly enhanced NCX4040-induced VD relaxation. This suggests that PDE5 physiologically restricts VD responsiveness to NO. A similar finding was previously observed by our group in CC and was determined to be androgen-mediated (Morelli et al., 2004). We now report that, indeed, even in VD androgen-deprivation reduces PDE5 expression and activity and limits responsiveness to PDE5i. In fact, in hypogonadal rabbits both the sildenafil-sensitive cGMP hydrolysing activity and the effect of sildenafil and tadalafil in enhancing NO-responsiveness were significantly blunted, being fully restored by T replacement. Accordingly, hypogonadal rabbits were more sensitive to NCX4040-induced relaxation, most probably because of a decreased PDE5-related cGMP hydrolysing activity, as previously observed in CC (Morelli et al., 2004). An androgen-regulation of VD expression and activity has not been previously reported. Interestingly, we could not find any difference in T plasma levels between patients with and without RE (Corona et al., 2004a,b). It is possible that estrogens, aromatase derived T metabolites, more than androgens, regulate VD motility, because mice lacking aromatase (Matsumoto et al., 2003) or the β subtype of estrogen receptor (Temple et al., 2003) showed prolonged ejaculatory latency. However, the effect of estrogens on PDE5 activity has not been investigated in the present study.

In conclusion, in this study we reported, for the first time, expression and activity of PDE5 in VD, which modulates NO responsiveness, in an androgen-dependent manner. Whether or not such an activity is related to the ameliorating effect of PDE5i in RE symptoms, should be further investigated. However, our finding suggests an attractive hypothesis, since RE is often present in patients complaining of ED (Corona et al., 2004b) and therefore eventually need PDE5i therapy. Hence, it is possible that the same class of compounds—PDE5i—might not only enhance penile erection by increasing cGMP levels in CC but also increase...
ejaculatory latency acting on PDE5 expressed in other androgen-regulated portions of the MGT, as VD, deeply involved in semen emission.

Acknowledgements
Rosa Mancina and Sandra Filippi were fellowship recipients of a special research grant from Eli Lilly (Sesto Fiorentino, Florence, Italy). The authors thank Prof. M.Rizzo (Department of Urology, University of Florence, Florence, Italy) and Prof. E.A.Jamini (Department of Experimental Medicine, University of L’Aquila, 67100, L’Aquila, Italy) for helpful suggestions during the course of the study; Prof. Mauro Giorgi (Department of Basic and Applied Biology, University of L’Aquila, L’Aquila, Italy) for kindly providing the polyclonal anti-PDE5 antibody. This study was supported by grants from the COFIN2002-MIUR (Progetti di Ricerca di Rilevanza Nazionale). Andrology unit belongs to the Centro di Ricerca, Trasferimento e Alta Formazione MCIDNENT of the University of Florence.

References
Submitted on November 9, 2004; accepted on December 16, 2004.

PDE5 in vas deferens

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