

Androgens Regulate Phosphodiesterase Type 5 Expression and Functional Activity in Corpora Cavernosa

ANNAMARIA MORELLI, SANDRA FILIPPI, ROSA MANCINA, MICHAELA LUCONI, LINDA VIGNOZZI, MIRCA MARINI, CLAUDIO ORLANDO, GABRIELLA BARBARA VANNELLI, ANTONIO AVERSA, ALESSANDRO NATALI, GIANNI FORTI, MAURO GIORGI, EMMANUELE A. JANNINI, FABRIZIO LEDDA, AND MARIO MAGGI

Andrology Unit (A.M., S.F., R.M., M.L., L.V., G.F., Ma.M.) and Clinical Biochemistry (C.O.), Department of Clinical Physiopathology, Department of Anatomy, Histology and Forensic Medicine (Mi.M., G.B.V.), Department of Urology (A.N.), Department of Pharmacology (F.L.), University of Florence, Florence 50139, Italy; Chair of Internal Medicine III (A.A.), Department of Physiopathology, University of Rome “La Sapienza,” Rome 00100, Italy; and Department of Experimental Medicine (M.G., E.A.J.), University of L’Aquila, 67100 L’Aquila, Italy

By real-time RT-PCR and Western blot analysis, we found that phosphodiesterase type 5 (PDE5) mRNA and protein abundance was several fold higher in human male than in female reproductive tracts. The highest mRNA level ($>1 \times 10^7$ molecules/ μg total RNA) was detected in human corpora cavernosa (CC), where PDE5 protein was immunolocalized in both muscular and endothelial compartment. The possible role of androgens in regulating PDE5 expression was studied using a previously established rabbit model of hypogonadotropic hypogonadism. In this model, hypogonadism reduced, and testosterone (T) supplementation restored, CC PDE5 gene and protein expression. In addition, T supplementation completely rescued and even enhanced cyclic GMP conversion to metabolites, without changing IC_{50} for sildenafil ($\text{IC}_{50} = 2.16 \pm 0.62$ nM). In control CC strips, sildenafil dose-dependently increased relaxation induced by electrical field stimulation, with $\text{EC}_{50} = 3.42 \pm 1.7$ nM. Hypogonadism reduced, and T

increased, sildenafil effect on electrical field stimulation, again without changing their relative EC_{50} values. CC sensitivity to the NO-donor NCX4040 was greater in hypogonadal rabbit strips than in control or T-treated counterparts. Moreover, sildenafil enhanced NCX4040 effect in eugonadal rabbit strips but not in hypogonadal ones. This suggests that androgens up-regulate PDE5 in rabbit penis. We also measured PDE5 gene expression and metabolic activity in human CC from male-to-female transsexual individuals, chronically treated with estrogens and cyproterone acetate. Comparing the observed values vs. eugonadal controls, PDE5 mRNA, protein, and functional activity were significantly reduced. In conclusion, we demonstrated, for the first time, that androgens positively regulate PDE5, thus providing a possible explanation about the highest abundance of this enzyme in male genital tract. (*Endocrinology* 145: 2253–2263, 2004)

PHOSPHODIESTERASES (PDEs) are a large superfamily of metallophosphohydrolases critically involved in regulation of cellular cAMP and/or cyclic GMP (cGMP) levels by many stimuli (1). During the last decade, numerous pharmaceutical compounds that selectively inhibit the catalytic activities of PDEs have been developed for the treatment of various diseases (2–4), but only PDE type 5 (PDE5) inhibitors reached clinical application (5). PDE5 is a cGMP-binding enzyme that specifically hydrolyzes cGMP to 5'-GMP. This enzyme was first purified from rat lung (6), and its enzyme activity has been found in many other tissues. Human PDE5 gene has been identified by three independent groups (7–9), and an alternative splicing of this gene results in four transcript variants, encoding distinct isoforms. It has been found that two alternate promoters regulate transcrip-

tion of three PDE5 isoforms, A1, A2, and A3 (10). A clear-cut physiological significance for these isoforms has not been demonstrated. By regulation of intracellular concentrations of cGMP, PDE5 is important for relaxation of vascular smooth muscle cells, including those of the penis. Up to now, how PDE5 inhibitors act specifically on penile blood flow and less well in the general circulation has not been completely understood. In addition, it is unclear why the enhancement of intracellular cGMP concentration does not generate major unwanted biological effects in tissues other than corpora cavernosa (CC). One possibility is that sexual stimulation, which is necessary for PDE5 inhibitors effectiveness, causes a specific release of NO in the penis, which would produce a large increase in cGMP synthesis mainly in this tissue (11). An alternative possibility is that PDE5 is not equally distributed in human tissues. Moreover, the presence of a consensus sequence for the androgen receptor in the 5'-flanking region of the PDE5 promoter (12) suggests that androgens could regulate PDE5 expression. To date, no absolute quantitative comparison of the levels of PDE5 transcript in any tissue of any species has been reported. This study was designed for this purpose and, in particular, to

Abbreviations: ACh, Acetylcholine; AoD, assay-on-demand; CC, corpora cavernosa; cGMP, cyclic GMP; EFS, electrical field stimulation; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PDE, phosphodiesterase; PDE5, PDE type 5; Phe, phenylephrine; T, testosterone.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

quantitate PDE5 abundance in human reproductive as well as in nonreproductive tissues and to investigate whether androgens regulate PDE5 expression using a previously established rabbit model of hypogonadotropic hypogonadism (13, 14).

Materials and Methods

Chemicals

Phenylephrine (Phe), HCl, acetylcholine (ACh), guanethidine, atropine, indomethacin, cGMP, 5'GMP, and guanine were purchased from Sigma (St. Louis, MO). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was obtained from Tocris (Bristol, UK). KCl and thin-layer chromatography plates (20 × 20) silica gel 60 F254 were supplied by Merck (Darmstadt, Germany). Testosterone (T) enanthate was supplied by Schering AG (Berlin, Germany). Triptorelin pamoate was supplied by Ipsen (Milan, Italy). Sildenafil was a gift from Dr. Stief (Hannover Medical School, Hannover, Germany). InstaGel Plus was from Packard (Meriden, CT). [8-³H]cGMP (15.1 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). NCX4040 was synthesized at Research Institute NicOx (Milan, Italy). Stock solutions of sildenafil and indomethacin were made in ethanol; stock solutions of ODQ and NCX4040 were made in dimethylsulfoxide (DMSO); the other substances were dissolved daily in double-distilled water, and further dilutions to the final concentrations were made in Krebs' solution. Control experiments showed that the used concentrations of dimethylsulfoxide modified neither the vasoconstrictor response to Phe nor the relaxation induced by the different agents.

Collecting of human and rabbit tissues

Gene expression analysis was performed in commercially available RNAs from human male tissues (Stratagene, La Jolla, CA; and Ambion Inc., Austin, TX), including brain, liver, stomach, skeletal muscle, and heart. The remaining RNAs were derived from male (CC, testis, epididymis, vas deferens, prostate) and female (uterus, breast, ovary, placenta) human tissues collected during surgery for benign diseases. Female tissues were all from premenopausal women. In detail, CC samples were obtained from patients undergoing penile prosthesis implantation (n = 4; age range, 40–67 yr), correction of Peyronie's disease (n = 1; age, 52 yr), or congenital curvature of the penis (n = 1; age, 24 yr). This group of patients did not take any medication or hormone treatment for at least 6 months before surgery. All tissue samples were collected after the approval of the Hospital Committee for Investigation in Humans and with informed patient's consent. In addition, CC were also obtained from five transsexual men (age range, 21–35) undergoing surgery for sexual reassignment. All transsexual individuals were treated for at least 2 yr with estrogens and the antiandrogen cyproterone acetate (100 mg daily). As expected, their T plasma levels, as measured 1–4 d before surgery, were in the hypogonadal range (T = 2.66 ± 0.22 nM). Informed consent to the donation of the excised organs for scientific aims was obtained from all enrolled subjects. The study protocol was approved by the Ethical Committee of the Surgery Institution (Department of Plastic Surgery "Azienda Ospedaliera S. Camillo-Forlanini," Rome, Italy).

Rabbit CC and uterus were obtained from New Zealand White rabbits weighing approximately 3 kg, as previously described (13, 14). CC were carefully dissected free from the tunica albuginea, whereas uterine horns were excised and separated from fat deposit and mesenteric attachments. 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, tissue preparations were immediately placed and maintained in cold Krebs solution until use. Atropine (1 μM) was present in the bathing solutions until the beginning and throughout the experiments for rabbit uterus. All the animal experiments were performed in accordance to D.L. 116/92 and approved by the Institutional Animal Care and Use Committee of the University of Florence.

Experimental hypogonadism and sex steroid replacement

New Zealand White male rabbits (n = 21) were divided into three groups and treated as previously described (13, 14). Briefly, one group was kept intact (controls, n = 7). Another group was treated with a single administration of 2.9 mg/kg of the long-acting GnRH analog triptorelin pamoate (n = 14). After 15 d, a subset of triptorelin-treated rabbits were supplemented with a pharmacological dose of T enanthate (30 mg/kg weekly for 6 wk, n = 7). After 2 months from triptorelin administration and after 1 wk from the last supplementation of T, rabbits were killed, and blood was drawn from the heart for T measurements.

Measurement of T

T plasma levels were measured with an Automated Chemiluminescence System (Bayer Diagnostics, East Walpole, MA) as previously described (13, 14). Briefly, extraction was performed mixing samples with 4 vol diethyl-ester for 15 min, centrifuging for 5 min at 2000 rpm, 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.

Isolation of RNA and cDNA synthesis

Total RNA was extracted from 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 total RNA was reverse transcribed to cDNA in 80 μl using TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA) at the following conditions: 10 min at 25 C; 30 min at 48 C, 5 min at 95 C.

Real-time RT-PCR (TaqMan)

The assay was performed according to the fluorescent TaqMan methodology as already published (15, 16). Primers and probe for PDE5 mRNA were assay-on-demand (AoD) gene expression products selected at <http://store.appliedbiosystems.com/> web site from Applied Biosystems. The assay was designed for the detection and quantitation of specific human mRNA PDE5 sequence (assay ID: Hs00153649_m1), but it cannot discriminate the four PDE5 transcript variants (NCBI accession numbers: NM_033437, NM_033431, NM_033430, NM_001083). PCR mixture (25 μl final vol) consisted of 1× final concentration of AoD mix, 1× final concentration of Universal PCR Master Mix (Applied Biosystems), 25 ng 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, and 40 cycles at 95 C for 30 sec and 60 C for 1 min. Each measurement was carried out in duplicate.

Absolute quantitation of the results was performed using an external standard curve. For preparation of PDE5 cDNA standard, we amplified a 461-bp fragment by RT-PCR (primer sense: 5'-ACCGCTATTCCCT-GTTCCTT-3', annealing to a sequence in exon 2; primer antisense: 5'-AAGGTCAAGCAGCACCTGAT-3', annealing to a sequence at 5–6 exons junction), using RNA from brain tissue as template. The RT-PCR product, which includes the sequence targeted by PDE5 AoD system, was cloned by using TOPO TA Cloning kit (Invitrogen Corporation), according to the manufacturer's instructions. RNA sense transcript was then prepared by *in vitro* transcription (Ribomax kit from Promega, Madison, WI), accurately quantitated by spectrophotometric analysis and finally reverse transcribed to cDNA according to Applied Biosystems protocol (described above). Known starting concentrations of standard cDNA, ranging from 3.5 × 10⁶ to 3.5 × 10¹ copies, were used to construct an external calibration curve. Results are expressed as PDE5 mRNA molecules per microgram of total RNA.

RT-PCR and PAGE

Total RNA from rabbit CC and uterus was extracted and checked for quality and quantity as for human tissues (described above). Total RNA (250 ng) was 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 sec at 95 C, 1 min at 55 C, 1 min at 70 C. Because no rabbit PDE5 sequence is

deposited in the GenBank at NCBI, primer design was based on homology to the human sequence (sense primer: 5'-ACC GCT ATT CCC TGT TCC TT-3', exon 2; antisense primer: 5'-GTA AAT GTC CCA CCG TTT CC-3' annealing to a sequence in exon 4). The amplified cDNA was run on an 8% nondenaturing polyacrylamide gel and visualized on UV light after ethidium bromide staining. To confirm the amplification specificity, the product of expected 340 bp was sequenced using the ABI-Prism 310 automatic sequencer (Applied Biosystems). Sequencing was performed according to the Applied Biosystems protocol and showed an homology (rabbit *vs.* human) of 97% (data not shown). The integrity of total RNA was verified performing the RT-PCR for the rabbit housekeeping γ -nonmuscle actin gene (NCBI accession no. X60733). The γ -nonmuscle actin-specific primers, covering a 328-bp region, were: 5'-ACA TGG AGA AGA TCT GGC AC-3' (sense, nucleotide position 317–336); 5'-CAT GAG GTA GTC GGT CAG GT-3' (antisense, nucleotide position 626–645). RT-PCR conditions were the same of those used for PDE5 analysis.

SDS-PAGE and Western blot analysis

Protein analysis was performed as previously described (14). Tissue samples were homogenized, and 30 μ g of protein extracts was separated on 10% denaturing SDS-polyacrylamide gel. After protein transfer to nitrocellulose membranes, the PDE5 protein was revealed by the previously characterized (17, 18) anti-PDE5 primary antibody (1:1000 in 10% blocking solution-0.1% Tween 20, 20 mM Tris, and 150 mM NaCl) followed by peroxidase-conjugated secondary IgG (1:3000). Reacted proteins were revealed by BM enhanced-chemiluminescence system (Roche Diagnostics, Milan, Italy).

Immunohistochemistry

Immunohistochemical studies were performed, as previously described (19), on deparaffinized rehydrated sections of the following tissues: human and rabbit CC and human urethra. Briefly, the slides were stained for indirect immunoperoxidase technique using polyclonal anti-PDE5 (1:100 vol/vol) (17) as primary antibody. Controls were performed as previously described (18, 19).

PDE activity assay

Human (CC) and rabbit (CC and uterus) 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 phenylmethylsulfonyl fluoride). The homogenates were aliquoted and stored at -80°C until use. Protein concentration was determined by the Bradford method (20). PDE activity for rabbit and human samples was carried out as described by Moreland *et al.* (21), with slight modifications in particular on the cGMP-GMP-guanine separation method. For IC_{50} determination, protein aliquots of 0.02 mg were incubated for 5 min at 30°C , with 0.5 μM cold cGMP and 0.1 μM [^3H]-cGMP in 40 mM MOPS (4-morpholinopropane sulfonic acid) buffer (pH 7.0), containing 1 mM EDTA, 0.8 mM EGTA, 5 mM Mg acetate, 0.2 mg/ml BSA, with or without sildenafil (from 10^{-11} to 10^{-6} M), in a final vol of 200 μl . Reactions 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 $1000 \times g$. Aliquots of 60 μl of each sample were applied to 60 F₂₅₄ silica-gel plates, using absolute ethanol/H₂O (70:30, vol:vol) as eluant 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 radioactivity was measured in InstaGel Plus by a β -counter instrument. The enzymatic activity was evaluated as percentage of substrate into product conversion as follows: conversion (%) = [products count/(substrate + products counts)] \times 100. The total recovery of cGMP, GMP, and guanine was 95–100%.

Studies of contractility

Rabbit CC and uterus strips were vertically mounted under a stable resting tension (1.8 g for CC and 1 g for uterus) in organ chambers

containing 10 ml Krebs solution at 37°C , gassed with 95% O₂-5% CO₂ at pH 7.4. The solution had the following mM composition: NaCl, 118; KCl, 4.7; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 25; CaCl₂, 2.5; glucose, 10. The preparations were allowed to equilibrate for at least 90 min; during this period, the bath medium was replaced every 15 min. Changes in isometric tension were recorded on a chart polygraph (Battaglia Rangoni, San Giorgio di Piano, Bologna, Italy). The presence of functional endothelium in CC was assessed by testing the vasodilator effect of ACh; preparations in which ACh (3 μM) reduced the tone by less than 40% were not used for the study. High potassium salt solution (KCl), made by equimolar substitution of sodium by potassium, increased the tonic tension, with the maximum effect obtained at 80 mM. Experiments were carried out in strips precontracted with Phe at selected concentration (100 μM for CC and 0.1–1 μM for uterus) 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 drugs (sildenafil and NCX4040) was referred to this value. The relaxant effect of sildenafil was evaluated in response to electrical field stimulation (EFS) (22, 23). EFS was performed using two platinum plates, parallel to the preparations, connected to a pulse generator at 10 V, 0.5 msec, 5 Hz, for 30 sec. Trains of field pulses were given at 15 min intervals in Phe-precontracted strips after pretreatment with 3 μM guanethidine, 1 μM atropine, and 3 μM indomethacin. The drug influence on recorded relaxant response was tested by administering sildenafil, at increasing concentrations, 7 min before each train. Relaxant response to a NO-donor, NCX4040, was measured after a 30-min pretreatment with selected inhibitors (ODQ and sildenafil).

Statistical analysis

Results are expressed as mean \pm SEM for *n* experiments. Statistical analysis was performed with Student's *t* test for paired or unpaired data, with ANOVA followed by Fisher's test to evaluate differences between groups, and $P < 0.05$ was taken as significant. EC_{50} and IC_{50} values were calculated using the computer program ALLFIT (24), supplied by P. J. Munson, National Institutes of Health (Bethesda, MD).

Results

Expression of PDE5 mRNA was quantitatively assayed in a large panel of human tissues using an originally designed real-time RT-PCR method. Absolute quantitative assay was performed constructing an external calibration curve with known starting molecules of a standard cDNA template, ranging from 3.5×10^6 to 3.5×10^1 copies. As shown in Fig. 1A, the specific transcript for PDE5 was detected in all tissues, although with a rather peculiar pattern of expression. Penile CC showed the highest abundance of PDE5 transcript ($2.3 \pm 0.6 \times 10^7$ copies/ μg total RNA). A rather elevated PDE5 gene expression was also detected in other portions of the male genital tract (testis, epididymis, vas deferens, and prostate), although lower than in CC. We also found that overall PDE5 mRNA abundance is at least 1 to 2 log units higher in male than in female reproductive tissues, such as placenta, breast, uterus, and ovary. Among the other male nonreproductive tissues, skeletal muscle, lung, stomach, and endocrine glands (thyroid and adrenal) showed PDE5 gene expression abundance comparable with that observed in male genital tract tissues other than CC. We also performed Western blot analysis of PDE5 protein in different human tissues (Fig. 1B). Overall, PDE5 protein expression largely mirrors mRNA quantitative distribution, as detected by Taq-Man analysis. The expected major band of approximately 98 kDa (17, 18) was detected in the entire male reproductive tract and in several nonreproductive tissues. The most intense labeling was found in CC tissue. Only a faint, almost undetectable, signal was observed in uterus, breast, and pla-

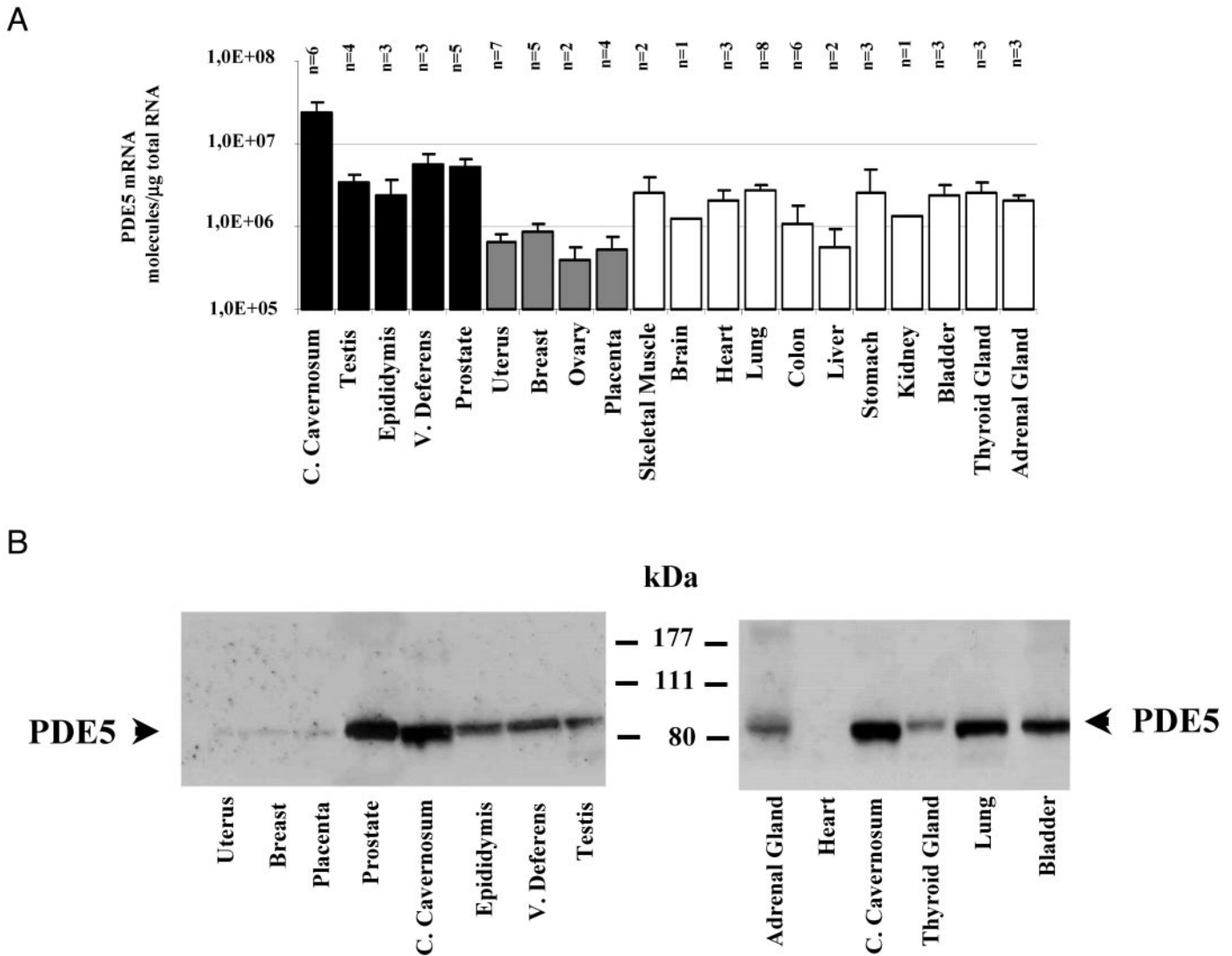


FIG. 1. A, Quantitative tissue distribution of human PDE5 mRNA as detected by real-time RT-PCR. Data are expressed, in a logarithmic scale, as PDE5 mRNA molecules per microgram of total RNA \pm SEM for *n* samples, where *n* is more than 1. Quantitation was obtained according to the absolute standard curve method as described in *Materials and Methods*. *Black columns*, Male reproductive tissues; *gray columns*, female reproductive tissues; *white columns*, other male nonreproductive tissues. B, Western blot analysis of PDE5 in human tissues. Thirty micrograms of proteins were separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and probed with anti-PDE5 antibody. A specific major band of expected size (\sim 98 kDa) is evident in all lanes.

centa. Immunohistochemical studies were performed to investigate PDE5 localization in transversal sections of human CC. As expected, a distinct specific immunoreactivity for PDE5 was detected in the smooth muscle cells of the lacunar and arteriolar wall (Fig. 2, A–D). Interestingly, a strong PDE5 positivity was also observed in the endothelial compartment of the penile lacunar and vascular spaces. Moreover, epithelium of human penile urethra showed immunopositivity for PDE5 (Fig. 2E). The specificity of staining was demonstrated through the complete absence of labeling obtained by omitting primary antibody in control sections (Fig. 2F). Moreover, the antibody immunospecificity was previously confirmed by Giordano *et al.* (18), preadsorbing the anti PDE5 antibody with GST peptide used for immunization of animals.

To test the hypothesis that androgens regulate PDE5 expression, we analyzed mRNA (RT-PCR) and protein (West-

ern blot, immunohistochemistry) expression of PDE5 in CC derived from a previously established rabbit model of hypogonadotropic hypogonadism (13, 14). Briefly, adult rabbits were treated once with triptorelin (2.9 mg/kg) and, after 2 wk, supplemented weekly with vehicle or T (30 mg/kg). T values at the time of death (2 months after triptorelin administration and 1 wk after the last administration of T) are reported in Table 1. Rabbit uterus was also analyzed for comparison. As shown in Fig. 3, both PDE5 mRNA and protein levels were significantly decreased in hypogonadal rabbits, whereas T administration restored PDE5 expression levels to those of the control. Uterus showed a low basal expression of both PDE5 gene and protein. Immunohistochemical localization of PDE5 was performed in transversal section of CC from all rabbit experimental groups. An intense immunopositivity was detected in control animals (Fig. 4A),

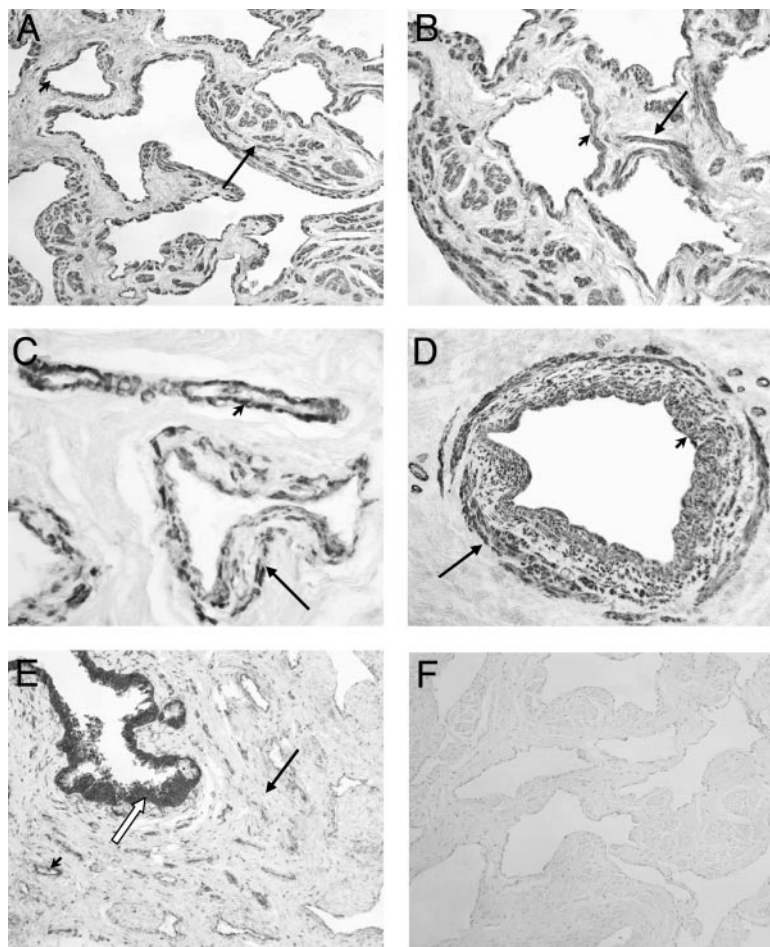


FIG. 2. Localization of cells immunoreactive with the anti-PDE5 antibody in transversal sections of human penile tissue. A–D, An intense immunopositivity for PDE5 in the smooth muscle (black arrows) and endothelial (arrowheads) cells of the arteriolar wall and lacunar spaces of CC (A, magnification, $\times 50$; B, magnification, $\times 80$; C, magnification of small vessels and capillaries, $\times 200$; D, magnification of arteriolar vessel, $\times 150$). E, Transversal section of human penile urethra: an intense positivity is evident in epithelium (white arrow, magnification, $\times 50$). F, Control section obtained omitting the primary PDE5 antibody and counterstained with hematoxylin.

TABLE 1. Testosterone plasma levels in experimental rabbit groups

	T (nmol/liter)
a) Male control (n = 7)	14.4 \pm 2.8
b) Triptorelin-treated (n = 7)	2.76 \pm 0.53 ^a
c) Triptorelin-treated + T (n = 7)	19.1 \pm 4.7 ^b
d) Female control (n = 5)	1.30 \pm 0.30

Blood for T measurements was drawn after 2 months from a single administration of the long-acting GnRH analog triptorelin pamoate (2.9 mg/kg, groups b and c) and after 1 wk from the last injection of T enanthate (30 mg/kg/wk; 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). n, Number of animals.

^a $P < 0.01$ vs. group a.

^b $P < 0.05$ vs. group b.

which was prevented by omitting the primary antibody (Fig. 4B). In hypogonadal rabbits, PDE5 labeling was largely decreased (Fig. 4C), but restored after T supplementation (Fig. 4D).

To further investigate the effect of androgens on penile PDE5, we evaluated the effect of androgens on cGMP hydrolyzing activity in the same experimental model. Measurements were performed either in the presence or in the absence of sildenafil (100 nM) and resulted in a linear function dependent on time and protein content, in a range between 2–30 min and 0.01–0.2 mg/ml, respectively (data not

shown). By using a short incubation time (5 min), a substantial amount of total cGMP hydrolyzing activity was inhibited by 100 nM sildenafil (% inhibition = 77.24 \pm 3.8, n = 6). We therefore performed all the subsequent experiments in this experimental condition. In CC from control rabbits, sildenafil inhibited cGMP hydrolysis with the expected IC₅₀ (IC₅₀ = 2.16 \pm 0.62 nM, Fig. 5). ALLFIT analysis of sigmoid inhibition curves for sildenafil obtained in control and hypogonadal rabbits, treated or not with T, indicated that androgen manipulation did not significantly affect IC₅₀ values for sildenafil, whereas it affected the cGMP hydrolysis rate (Fig. 5). In fact, chronic treatment with triptorelin significantly reduced the percentage of cGMP conversion to metabolites ($P < 0.005$), whereas T supplementation not only restored, but also increased, the percentage of conversion over the control value ($P < 0.01$). In uterine homogenates, representing the negative control, the total cGMP hydrolyzing activity was relatively low and almost unaffected by sildenafil (Fig. 5).

To evaluate the influence of hypogonadism and androgen supplementation on PDE5 functional activity in rabbit CC, we tested the effect of sildenafil on NO-induced CC relaxation. Table 2 reports the net contractile force generated by KCl (80 mM) in preparations from the different experimental groups. No statistically significant difference was found among groups. In the first series of experiments, we evalu-

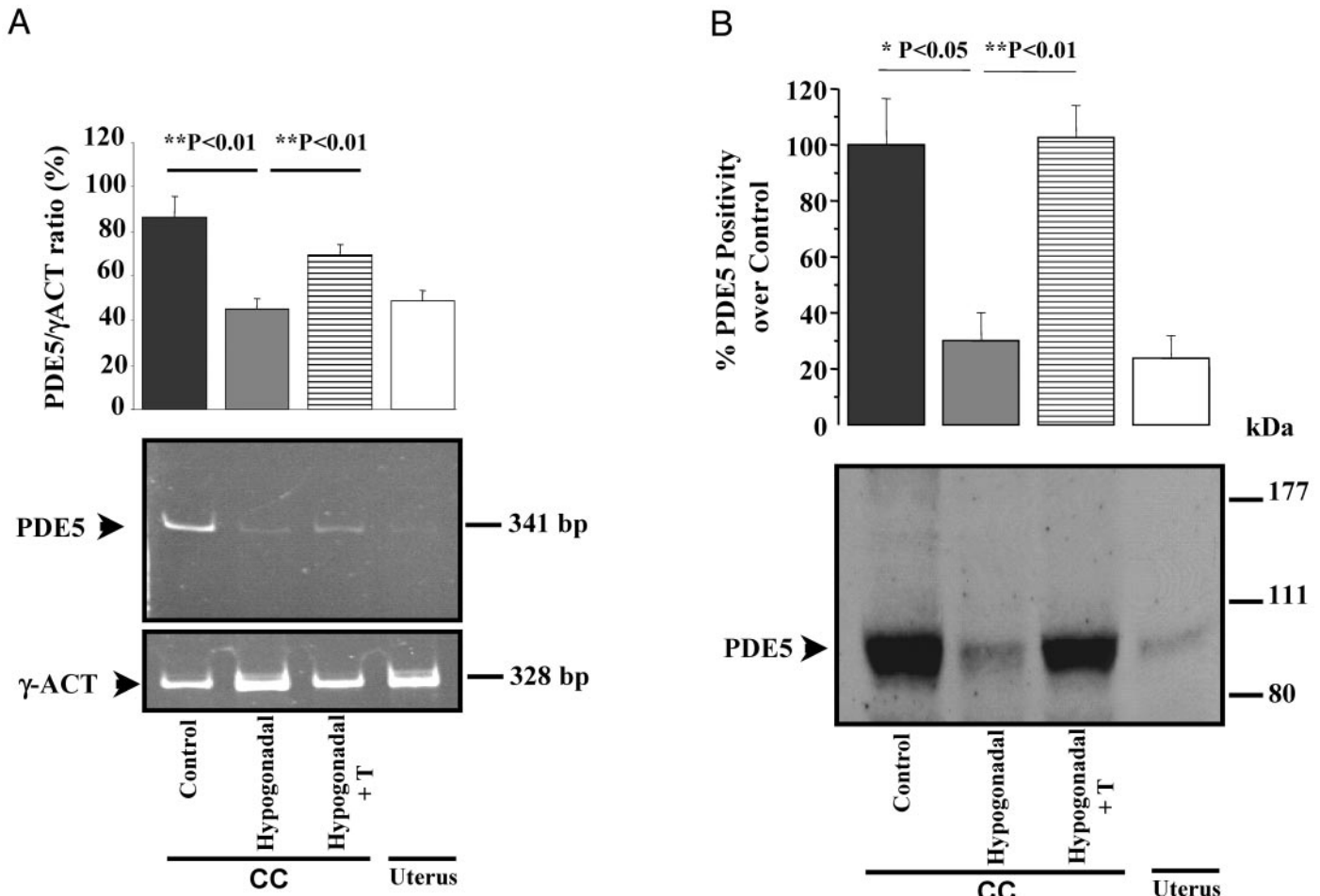


FIG. 3. PDE5 mRNA and protein expression obtained by RT-PCR/polyacrylamide gel electrophoresis (A) and Western blot analysis (B), respectively, in rabbit CC and uterus. A, Densitometric evaluation of PDE5 mRNA expression over the γ -actin housekeeping gene as obtained from band intensity of RT-PCR products. B, Protein expression level is calculated in percentage of band intensity over the control taken as 100%. *Black column*, Control; *gray column*, hypogonadal; *hatched column*, hypogonadal plus T; *white column*, uterus. *P* values (hypogonadal vs. control and hypogonadal plus T vs. hypogonadal) are also indicated. Data are mean \pm SEM, $n = 3$.

ated the effect of increasing concentration of sildenafil on CC relaxation induced by EFS trains (10 V, 0.5 msec, 5 Hz, 30 sec) given at 15-min intervals to CC strips pretreated with guanethidine and indomethacine and precontracted with Phe, as previously described (22, 23). Figure 6A shows the typical relaxant response of CC to EFS and how it is affected by sildenafil. Sildenafil increased EFS-induced relaxation in a dose-dependent fashion, with $EC_{50} = 3.42 \pm 1.7$ nM (Fig. 6B). Similar EC_{50} values were also observed in rabbits treated with triptorelin alone and with triptorelin plus T (Fig. 6B). However, hypogonadism significantly reduced ($P < 0.05$), and T replacement enhanced ($P < 0.0001$) the sildenafil-stimulated, EFS-induced maximal relaxation observed in control CC. To further investigate whether the observed androgen modulation of CC responsiveness to EFS was related to a downstream effect to cGMP formation, we compared the IC_{50} values of a NO donor (NCX4040) (25) in the different experimental groups. Experiments were also performed in the presence of inhibitors of cGMP formation (ODQ, 1 μ M) or degradation (sildenafil, 100 nM), according to a previously described protocol (23). In control rabbits (Fig. 7A), NCX4040

relaxed CC preparations, with $IC_{50} = 62.6 \pm 14$ μ M. As expected (23), this IC_{50} value of NCX4040 was shifted to the left by sildenafil ($IC_{50} = 1.35 \pm 0.32$ μ M, $P < 0.0001$) and to the right by ODQ ($IC_{50} = 4.26 \pm 3.66$ μ M, $P < 0.0001$). In hypogonadal rabbits (Fig. 7B), NCX4040 was more potent in relaxing CC preparations than in control rabbits ($IC_{50} = 19.6 \pm 3.9$ μ M, $P < 0.0001$), but, unlike the control group ($P < 0.0001$), sildenafil did not further shift the curve to the left. In T-replaced hypogonadal rabbits (Fig. 7C), the relative potency of NCX4040 ($IC_{50} = 122.7 \pm 29$ μ M) was significantly reduced when compared with the untreated hypogonadal rabbits ($P < 0.0001$) and even slightly reduced when compared with the control group ($P = 0.064$). However, T replacement significantly restored the sildenafil-induced enhanced sensitivity to NCX4040 ($IC_{50} = 2.9 \pm 0.7$ μ M, $P < 0.0001$ vs. without sildenafil, Fig. 7C), as previously observed in the control group but not in the hypogonadal one ($P < 0.0001$ vs. sildenafil in hypogonadism, Fig. 7B). In uterine strips (Fig. 7D), the relative potency of NCX4040 ($IC_{50} = 32 \pm 15$ μ M) and the lack of enhancement by sildenafil ($IC_{50} = 15.1 \pm 5.4$ μ M) was in keeping with the results in CC from

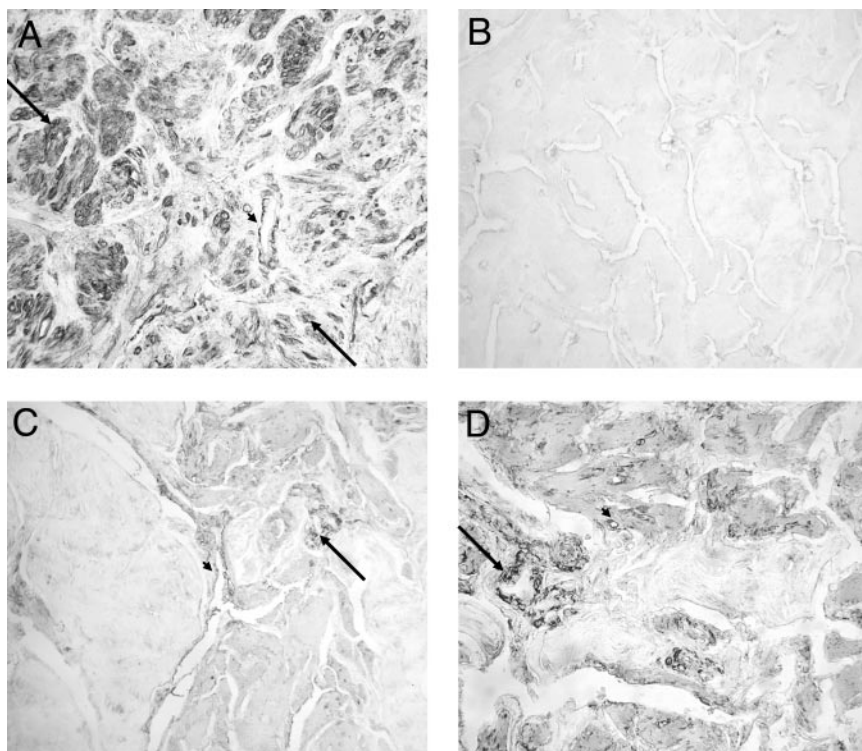


FIG. 4. Immunolocalization of PDE5 in transversal sections of rabbit CC (magnification, $\times 80$). A (intact control), An intense immunopositivity for PDE5 in the smooth muscle (black arrows) and endothelial (arrowheads) cells of the vessels and lacunar spaces of CC. B, Control section obtained omitting the primary PDE5 antibody and counterstained with hematoxylin. C, Treatment with triptorelin induced a sustained decreasing positivity in the smooth muscle (black arrows) and endothelial (arrowheads) cells. D, Treatment with triptorelin plus T restored the reactivity for PDE5 in smooth muscle (black arrows) and endothelial (arrowheads) cells.

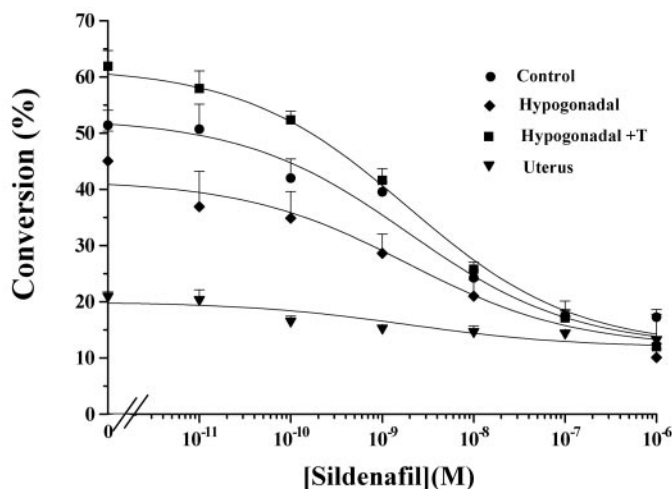


FIG. 5. Sildenafil-induced inhibition curves of cGMP-hydrolyzing activity in CC of control (closed circle, $n = 3$), hypogonadal (closed diamonds, $n = 4$), and hypogonadal T-replaced (closed squares, $n = 3$) rabbits and in rabbit uterus (closed triangles, $n = 3$). Aliquots of tissue homogenates (0.1 mg protein/ml) were incubated with $0.5 \mu\text{M}$ cGMP and $0.1 \mu\text{M}$ [^3H]cGMP in the absence or presence of sildenafil (10^{-11} – 10^{-6} M). Ordinate, cGMP hydrolyzing activity expressed as conversion percentage. Conversion (%) = [products count/(substrate+products counts)] $\times 100$. Abscissa, Sildenafil concentration. Inhibition curves obtained in three different experiments were fitted simultaneously with the program ALLFIT using the four-parameter logistic equation.

hypogonadal rabbits (Fig 7B). Neither hypogonadism nor T replacement affected the ODQ-induced decreased responsiveness to NCX4040 observed in control CC and uterine strips (Fig. 7, A and D).

Finally, to verify whether hypogonadism reduced PDE5

TABLE 2. Response to KCl (80 mM) in corpora cavernosa from experimental rabbit groups

Experimental group	N	Tension Mean \pm SEM (mg)
Control	15	717 \pm 105.7
Hypogonadal	8	859 \pm 103.3
Hypogonadal + testosterone	8	953 \pm 181.2

N, Number of preparations tested. Differences in tension were not statistically significant.

expression and activity also in humans, we performed experiments in CC derived from transsexual individuals undergoing phallectomy for sexual reassignment. In these transsexual individuals, presurgical pharmacological treatment with estrogens and cyproterone acetate induced a hypogonadal state, which resulted in a substantially decreased T plasma levels at the time of phallectomy (total T = 2.66 ± 0.22 nM; normal range, 10.6–37 nM). Interestingly, in this group of individuals, PDE5 mRNA and protein in CC were significantly ($P < 0.05$) less expressed than in the eugonadal control group (Fig. 8, left and right insets, respectively). Also the conversion rate of cGMP to metabolites was significantly reduced ($P < 0.0001$), whereas the IC_{50} for sildenafil was unchanged and comparable with that observed in penile rabbit extracts ($\text{IC}_{50} = 2.55 \pm 1.20$ nM, Fig. 8).

Discussion

It is generally assumed that PDE5 inhibitors have pharmacological specificity, with temporally and spatially limited activity on erectile function, just because they amplify local, erotic-induced NO production and cGMP formation (11). This study reports the first evidence that their relative specificity could be due to a physiological predominance of

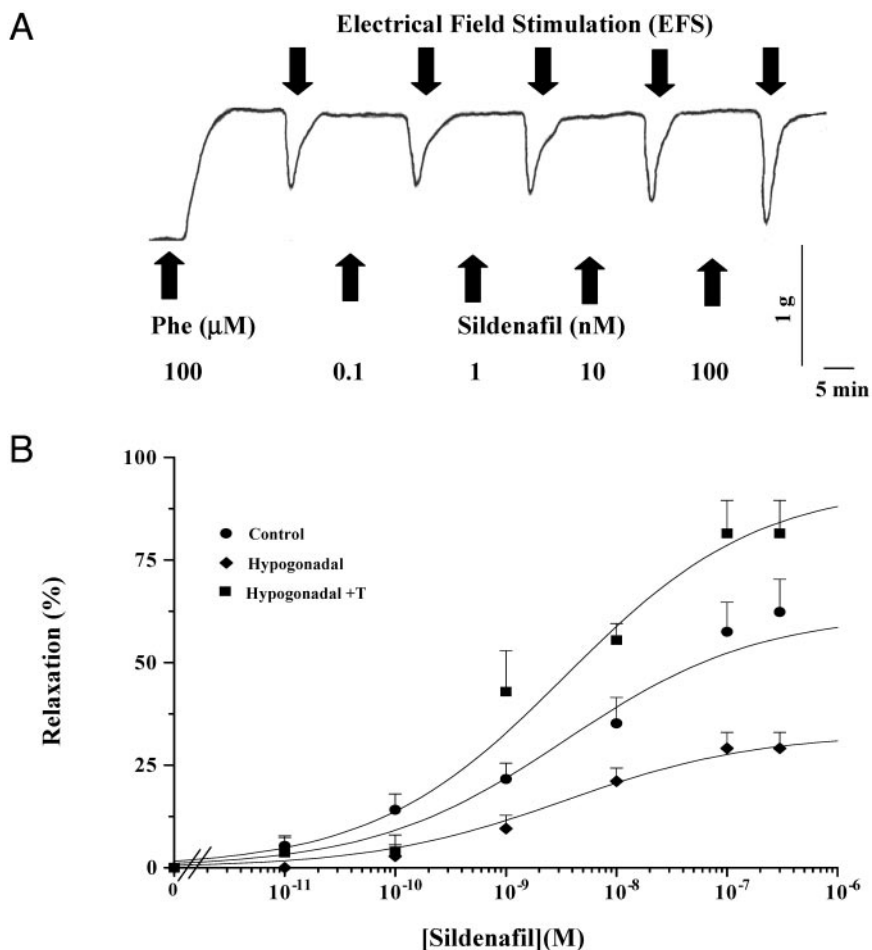


FIG. 6. Effect of triptorelin-induced hypogonadism and T replacement on responsiveness to EFS (10 V, 0.5 msec, 5 Hz, for 30 sec) in rabbit CC preparations precontracted by Phe. A, Typical experiment showing the effect of increasing concentrations of sildenafil on the relaxant response induced by EFS. B, Effect of increasing concentrations of sildenafil on the response induced by EFS in rabbit CC preparations precontracted by Phe, control (closed circles, $n = 7$), and hypogonadal rabbits supplemented with (closed squares, $n = 5$) or without (closed diamonds, $n = 5$) weekly administration of T. Ordinate, Relaxation, expressed as percentage over the control; abscissa, concentration of sildenafil.

the pharmacological target, *i.e.* PDE5, in penile organ and that this predominance is androgen-dependent. In fact, we report, for the first time, that PDE5 gene expression in human CC is 10-fold more abundant than in other male reproductive tissues and 10- to 100-fold higher than in other male non-reproductive tissues. A nonhomogeneous tissue distribution of PDE5 transcripts was suspected by previous studies based on RT-PCR (10, 26, 27) or Northern analysis (7–9, 26–28), and it has always been claimed, but never conclusively demonstrated by absolute quantitative comparison, that the penis expresses more PDE5 than any other human tissues (5, 26, 27, 29). Our study substantiated this view by using an originally designed quantitative real-time RT-PCR, supported by Western blot and immunohistochemical analysis. Based on our findings, it could be hypothesized that PDE5 penile overexpression reflects a physiological condition in which a rapid hydrolysis of the pro-erectile nucleotide cGMP allows penile smooth muscle cells to reside in the contracted state for the majority of time. Only specific signals, such as those occurring during sexual activity, can reverse this tonic inhibition, allowing penile erection, which represents a relatively exceptional event compared with the contracted condition.

Although the molecular mechanism underlying the high expression of PDE5 transcript in CC remains to be completely clarified, our findings about the relative predominance of PDE5 transcript in male *vs.* female genital tract indicate that

sex steroids might play a role. To investigate a possible androgen modulation of PDE5 expression and activity, we performed a series of experiments in rabbits using a previously established model of hypogonadotropic hypogonadism (13, 14). RT-PCR, Western blot, immunohistochemistry, cGMP hydrolyzing activity, and *in vitro* contractility analyses, taken together, strongly indicate that androgens up-regulate PDE5 expression in the penis. First, penile sensitivity to a NO-donor was greater in hypogonadal rabbits than in eugonadal (control or T-supplemented hypogonadal) animals and not further enhanced by sildenafil. This hypersensitivity to NO was also observed in rabbit uterus, indicating that it could be due to a lower expression of PDE5 in the hypogonadal penis and in the uterus. In fact, PDE5 expression was relatively low in rabbit uterus (as also observed in the human uterus by real-time RT-PCR), and PDE5 functional activity was almost undetectable in this tissue. In rabbit penis, PDE5 gene and protein expression was significantly reduced after androgen deprivation, and it was completely restored by T administration. Furthermore, in rabbit CC, hypogonadism significantly blunted PDE5 hydrolytic activity and functional responsiveness to sildenafil, both of them being completely rescued and even enhanced by T supplementation. Although the lower sildenafil-induced relaxation in castrated animals could be simply ascribed to a reduced contractile muscular compartment of the

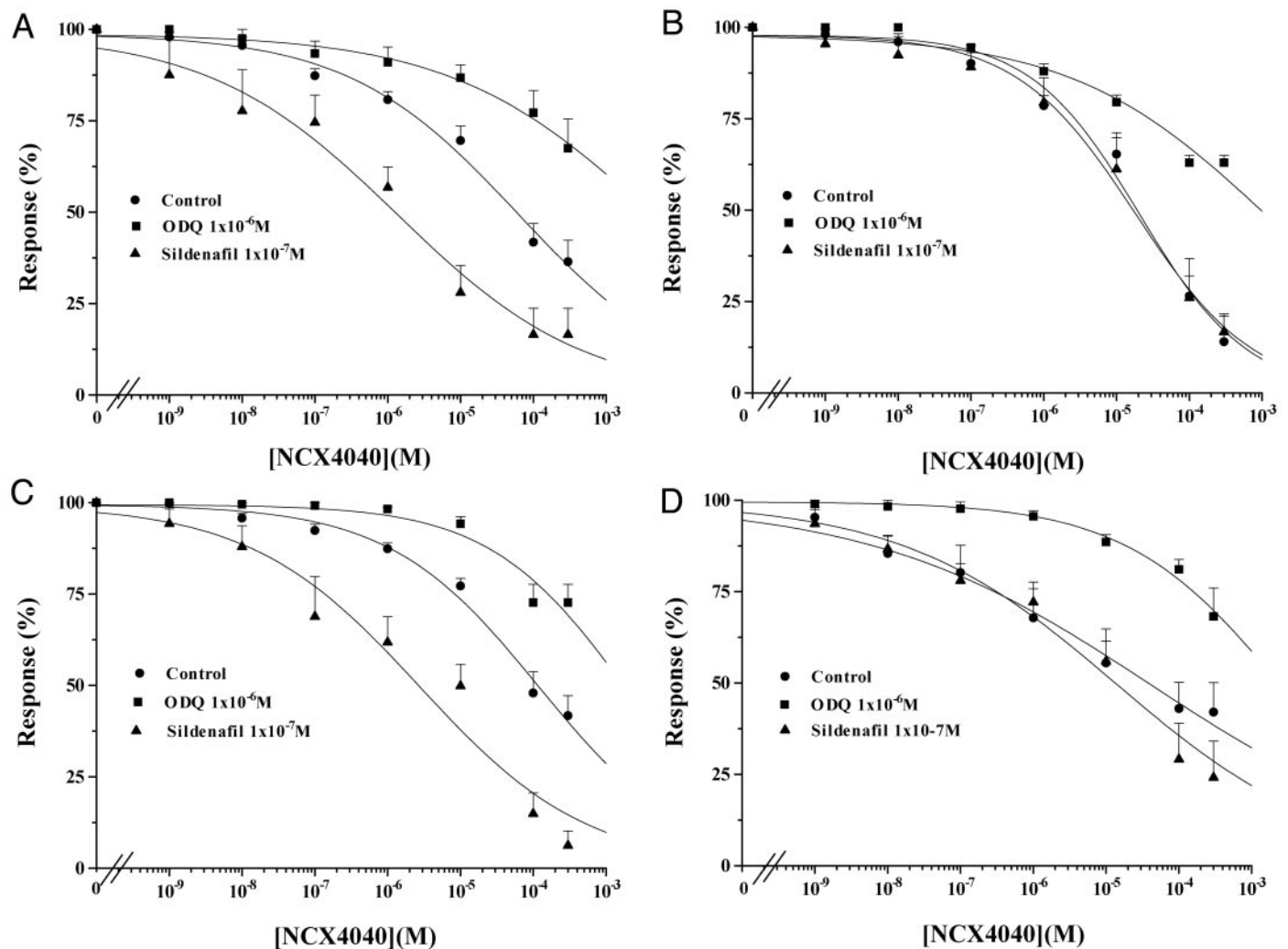
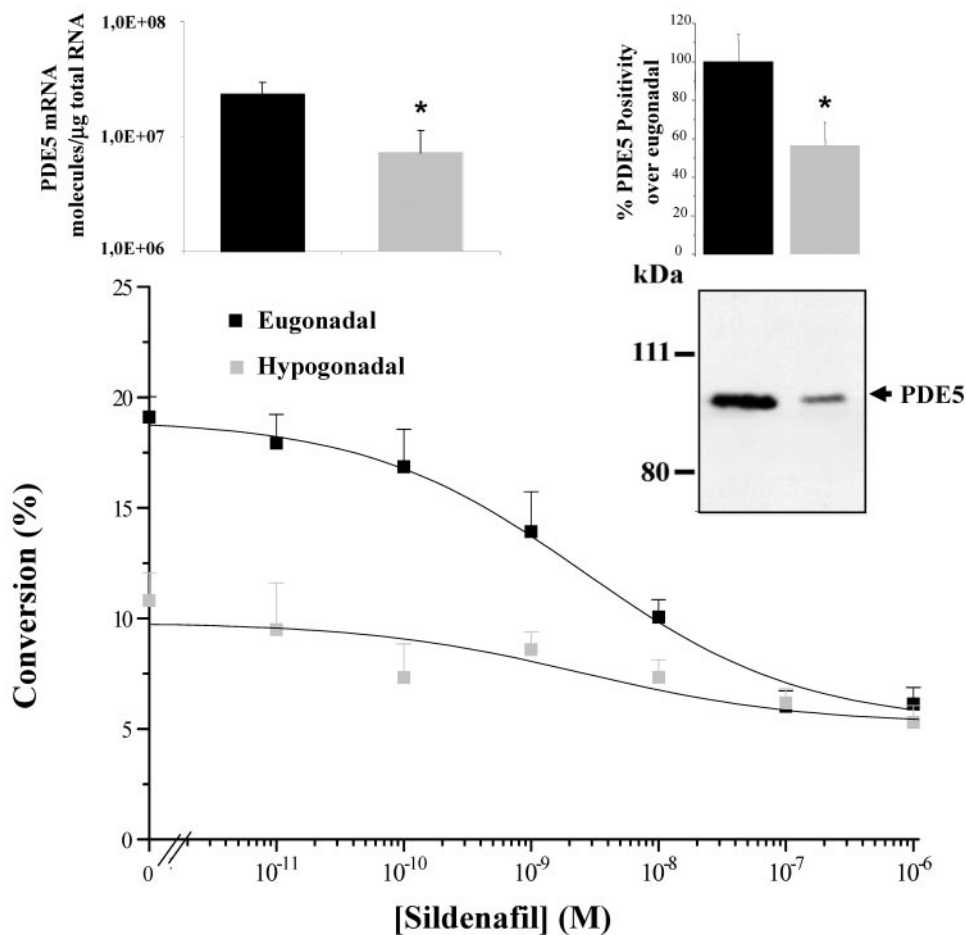


FIG. 7. Effect of triptorelin-induced hypogonadism and T replacement on responsiveness to a NO-donor (NCX4040) in rabbit CC preparations precontracted by Phe. Concentration-relaxant response curves induced by increasing concentrations of NCX4040 alone (closed circles) and in the presence of either 1 μ M ODQ (closed squares) or 100 nM sildenafil (closed triangles) in untreated (A) and hypogonadal rabbit with (C) or without (B) weekly administration of T. D, Concentration-relaxant response curves for NCX4040 in untreated rabbit uterine strips precontracted by Phe. Ordinate, Increase in tone induced by Phe before addition of NCX4040 was taken as 100% response, and the effect of the drug was evaluated as a percentage of this response. Abscissa, Concentrations of NCX4040. Drugs were added to preparations 30 min before obtaining the curve for NCX4040. Each point represents means \pm SEM of at least four experiments for each group.

penis (30, 31), our results suggest a different underlying mechanism. In fact, by immunohistochemistry, we found an almost-absent PDE5 staining in the smooth muscle cells of hypogonadal CC. In addition, the relaxing ability of NCX4040 was even greater in androgen-deprived, than in androgen-supplemented, penile tissues. Last, the net contractile responsiveness to KCl did not change among groups. This was most probably because, in our rabbit model, the decrease in total T was not profound enough to induce a massive penile fibrosis. Hence, we deduced that androgens positively regulate expression and functional activity of PDE5 (and therefore, responsiveness to sildenafil) in rabbit penis. Preliminary evidence indicates the same androgen effect also in human penis. In fact, in CC from male-to-female transsexual individuals, chronic exposure to estrogens and to the antiandrogen cyproterone acetate, leading to an overt hypogonadism (T < 3 nM), significantly reduced PDE5

mRNA and protein expression as well as cGMP hydrolysis. Our findings are in keeping with previous observations showing that responsiveness to PDE5 inhibitors was reduced in hypogonadal rabbits (31) and humans (32) and restored by T administration (33, 34). Although this view should be substantiated by larger clinical studies, it implies that T is necessary for a full PDE5-inhibitor responsiveness. Hence, T is important not only for allowing cGMP formation, through a positive modulation of NO synthase (35–37), but also for increasing cGMP degradation. This androgen-induced two-step regulation of NO activity and cGMP formation in CC might be relevant to timely synchronize penile erections to sexual acts, which are clearly androgen-dependent. In fact, it has long been established that T is absolutely required for maintaining sexual desire and interest, sexual thoughts, and fantasies, and therefore for allowing a normal sexual life and intercourse frequency (38–40). However, this antithetic, dual

FIG. 8. Inhibition curves of cGMP hydrolyzing activity by sildenafil (10^{-11} – 10^{-6} M) in human CC from eugonadal (black squares, $n = 3$) and hypogonadal (gray squares, $n = 4$) individuals. Ordinate, cGMP hydrolyzing activity expressed as conversion percentage. Conversion (%) = [products count/(substrate+products counts)] \times 100. Abscissa: Sildenafil concentration. Inhibition curves obtained in two different experiments were fitted simultaneously with the program ALLFIT using the four-parameter logistic equation. *Left histogram*, Absolute quantitation of PDE5 transcript in human eugonadal (black column, $n = 6$) and hypogonadal (gray column, $n = 5$) CC, as detected by real-time RT-PCR (data are expressed as means of PDE5 mRNA molecules per microgram of total RNA \pm SEM). *Right inset*, PDE5 protein expression as detected by Western blot analysis. Means \pm SEM of the percentage of PDE5 positivity in human hypogonadal (gray column, $n = 5$) compared with eugonadal (black column, $n = 3$) CC taken as 100%. Quantification of PDE5 band intensities was made directly on the film by image scanning analysis. Statistically significant difference in both mRNA and protein expression was found in hypogonadal *vs.* eugonadal samples; *, $P < 0.05$.



control of androgens on cGMP formation and degradation might justify why penile erection can also occur in the absence of androgens. In the absence of androgens, a lower NO formation (35–37) might be counterbalanced by a lower cGMP degradation (present study). In fact, penile erections have been described in infants and children and even during fetal life (41). In a study on castrated men (42), Kinsey was the first to discount the importance of T for maintaining erectile function. Later on, further studies substantiated the notion that hypogonadal men, when adequately stimulated (visual erotic stimulations), have a normal erectile activity (43–45). In particular, Kwan *et al.* (44), found that video sexual stimulated erection was comparable in eugonadal and hypogonadal men, and that, in the latter group, the time achieved to reach detumescence was substantially longer. This finding is probably due to low PDE5 expression levels in the penis of hypogonadal individuals.

In conclusion, the PDE5 enrichment in human penile tissue likely underlies the selectivity of the NO-enhancing effect of PDE5 inhibitors in the penis and the relative absence of serious systemic side effects. In addition, it can justify the rather unsuccessful use of various NO donors, even when injected locally, in the treatment of erectile dysfunction (46, 47). Moreover, the androgen requirement for PDE5 expression can explain both its tissue distribution and the lower responsiveness of hypogonadal subjects to PDE5 inhibitors.

Acknowledgments

The authors thank Nicola Mondaini (Department of Urology, University of Florence, Florence, Italy), Emanuele Belgrano and Giovanni Liguori (Department of Urology, University of Trieste, Trieste, Italy), and Aldo Felici (Department of Plastic Surgery "Azienda Ospedaliera S. Camillo-Forlanini," Rome, Italy) for providing surgical human penile samples.

Received December 15, 2003. Accepted January 26, 2004.

Address all correspondence and requests for reprints to: Mario Maggi, M.D., Andrology Unit, Department of Clinical Physiopathology, University of Florence, V.le G. Pieraccini, 6, 50139 Florence, Italy. E-mail: m.maggi@dfc.unifi.it.

This work was supported by grants from COFIN2002-MIUR (Progetti di Ricerca di rilevanza nazionale) and from NicOx (Sophia Antipolis Cedex, France).

A.M. and S.F. contributed equally to this work.

References

- Francis SH, Turko IV, Corbin JD 2001 Cyclic nucleotide phosphodiesterases: relating structure and function. *Prog Nucleic Acid Res Mol Biol* 65:1–52
- Schudt C, Gantner F, Tenors H, Hatzelmann A 1999 Therapeutic potential of selective PDE inhibitors in asthma. *Pulm Pharmacol Ther* 12:123–129
- Movsesian MA 1999 β -Adrenergic receptor agonists and cyclic nucleotide phosphodiesterase inhibitors: shifting the focus from inotropy to cyclic adenosine monophosphate. *J Am Coll Cardiol* 34:318–324
- Lin CS, Xin ZC, Lin G, Lue TF 2003 Phosphodiesterases as therapeutic targets. *Urology* 61:685–691
- Mangiello V 2003 Cyclic nucleotide phosphodiesterase 5 and sildenafil: promises realized. *Mol Pharmacol* 63:1209–1211

6. Francis SH, Corbin JD 1988 Purification of cGMP-binding protein phosphodiesterase from rat lung. *Methods Enzymol* 159:722–729
7. Loughney K, Hill TR, Florio VA, Uher L, Rosman GJ, Wolda SL, Jones BA, Howard ML, McAllister-Lucas LM, Sonnenburg WK, Francis SH, Corbin JD, Beavo JA, Ferguson K 1998 Isolation and characterization of cDNAs encoding PDE5A, a human cGMP-binding, cGMP-specific 3',5'-cyclic nucleotide phosphodiesterase. *Gene* 216:139–147
8. Stacey P, Rulten S, Dapling A, Phillips SC 1998 Molecular cloning and expression of human cGMP-binding cGMP-specific phosphodiesterase (PDE5). *Biochem Biophys Res Commun* 247:249–254
9. Yanaka N, Kotera J, Ohtsuka A, Akatsuka H, Imai Y, Michibata H, Fujishige K, Kawai E, Takebayashi S, Okumura K, Omori K 1998 Expression, structure and chromosomal localization of the human cGMP-binding cGMP-specific phosphodiesterase PDE5A gene. *Eur J Biochem* 255:391–399
10. Lin CS, Chow S, Lau A, Tu R, Lue TF 2002 Human PDE5A gene encodes three PDE5 isoforms from two alternate promoters. *Int J Impot Res* 14:15–24
11. Corbin JD, Francis SH 1999 Cyclic GMP phosphodiesterase-5: target of sildenafil. *J Biol Chem* 274:13729–13732
12. Lin CS, Chow S, Lau A, Tu R, Lue TF 2001 Identification and regulation of human PDE5A gene promoter. *Biochem Biophys Res Commun* 280:684–692
13. Filippi S, Luconi M, Granchi S, Natali A, Tozzi P, Forti G, Ledda F, Maggi M 2002 Endothelium-dependency of yohimbine-induced corpus cavernosum relaxation. *Int J Impot Res* 14:295–307
14. Filippi S, Luconi M, Granchi S, Vignozzi L, Bettuzzi S, Tozzi P, Ledda F, Forti G, Maggi M 2002 Estrogens, but not androgens, regulate expression and functional activity of oxytocin receptor in rabbit epididymis. *Endocrinology* 143:4271–4280
15. Pinzani P, Orlando C, Raggi CC, Distante V, Valanzano R, Tricarico C, Maggi M, Serio M, Pazzagli M 2001 Type-2 somatostatin receptor mRNA levels in breast and colon cancer determined by a quantitative RT-PCR assay based on dual label fluorogenic probe and the TaqMan technology. *Regul Pept* 99:79–86
16. Casini Raggi C, Calabro A, Renzi D, Briganti V, Cianchi F, Messerini L, Valanzano R, Cameron Smith M, Cortesini C, Tonelli F, Serio M, Maggi M, Orlando C 2002 Quantitative evaluation of somatostatin receptor subtype 2 expression in sporadic colorectal tumor and in the corresponding normal mucosa. *Clin Cancer Res* 8:419–427
17. D'Amati G, di Gioia CR, Bologna M, Giordano D, Giorgi M, Dolci S, Jannini EA 2002 Type 5 phosphodiesterase expression in the human vagina. *Urology* 60:191–195
18. Giordano D, De Stefano ME, Citro G, Modica A, Giorgi M 2001 Expression of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in mouse tissues and cell lines using an antibody against the enzyme amino-terminal domain. *Biochim Biophys Acta* 1539:16–27
19. Filippi S, Vannelli GB, Granchi S, Luconi M, Crescioli C, Mancina R, Natali A, Brocchi S, Vignozzi L, Bencini E, Noci I, Ledda F, Forti G, Maggi M 2002 Identification, localization and functional activity of oxytocin receptors in epididymis. *Mol Cell Endocrinol* 193:89–100
20. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
21. Moreland RB, Goldstein I, Traish A 1998 Sildenafil, a novel inhibitor of phosphodiesterase type 5 in human corpus cavernosum smooth muscle cells. *Life Sci* 62:PL309–PL318
22. Maggi M, Filippi S, Ledda F, Magini A, Forti G 2000 Erectile dysfunction: from biochemical pharmacology to advances in medical therapy. *Eur J Endocrinol* 143:143–154
23. Filippi S, Crescioli C, Vannelli GB, Fazzini A, Natali A, Riffaud JP, Maggi M, Ledda F 2003 Effects of NCX 4050, a new NO donor, in rabbit and human corpus cavernosum. *Int J Androl* 26:101–108
24. De Lean A, Munson PJ, Rodbard D 1978 Simultaneous analysis of families of sigmoidal curves: applications to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol* 235:E97–E102
25. Shukla N, Angelini GD, Ascione R, Talpahewa S, Capoun R, Jeremy JY 2003 Nitric oxide donating aspirins: novel drugs for the treatment of saphenous vein graft failure. *Ann Thorac Surg* 2003 75:1437–1442
26. Lin CS, Lin G, Lue TF 2003 Isolation of two isoforms of phosphodiesterase 5 from rat penis. *Int J Impot Res* 15:129–136
27. Kuthe A, Magert H, Uckert S, Forssmann WG, Stief CG, Jonas U 2000 Gene expression of the phosphodiesterases 3A and 5A in human corpus cavernosum penis. *Eur Urol* 2000 38:108–114
28. Kotera J, Fujishige K, Imai Y, Kawai E, Michibata H, Akatsuka H, Yanaka N, Omori K 1999 Genomic origin and transcriptional regulation of two variants of cGMP-binding cGMP-specific phosphodiesterases. *Eur J Biochem* 262:866–872
29. Corbin JD, Francis SH 2002 Pharmacology of phosphodiesterase-5 inhibitors. *Int J Clin Pract* 56:453–459
30. Traish AM, Park K, Dhir V, Kim NN, Moreland RB, Goldstein I 1999 Effects of castration and androgen replacement on erectile function in a rabbit model. *Endocrinology* 140:1861–1868
31. Traish AM, Munarriz R, O'Connell L, Choi S, Kim SW, Kim NN, Huang Y-H, Goldstein I 2003 Effects of medical or surgical castration on erectile function in an animal model. *J Androl* 24:381–387
32. Guay AT, Perez JB, Jacobson J, Newton RA 2001 Efficacy and safety of sildenafil citrate for treatment of erectile dysfunction in a population with associated organic risk factors. *J Androl* 22:793–797
33. Aversa A, Isidori AM, Spera G, Lenzi A, Fabbri A 2003 Androgens improve cavernous vasodilation and response to sildenafil in patients with erectile dysfunction. *Clin Endocrinol* 58:632–638
34. Yassin A, Diede HE, Saad F, Traish A 2003 Combination therapy of tadalafil and testosterone in hypogonadal non-responders. *Int J Impot Res* 5:6:527
35. Penson DF, Ng C, Cai L, Rajfer J, Gonzalez-Cadavid NF 1996 Androgen and pituitary control of penile nitric oxide synthase and erectile function in the rat. *Biol Reprod* 55:567–574
36. Mills TM, Lewis RW 1999 The role of androgens in the erectile response: A 1999 Perspective. *Mol Urol* 3:75–86
37. Mills TM, Dai Y, Stopper VS, Lewis RW 1999 Androgenic maintenance of the erectile response in the rat. *Steroids* 64:605–609
38. Wang C, Swerdloff RS, Iranmanesh A, Dobs A, Snyder PJ, Cunningham G, Matsumoto AM, Weber T, Berman N, THE TESTOSTERONE GEL STUDY GROUP 2000 Transdermal testosterone gel improves sexual function, mood, muscle strength, and body composition parameters in hypogonadal men. *J Clin Endocrinol Metab* 85:2839–2853
39. Zitzmann M, Nieschlag E 2001 Testosterone levels in healthy men and the relation to behavioural and physical characteristics: facts and constructs. *Eur J Endocrinol* 144:183–197
40. Corona G, Mannucci E, Petrone L, Gionmi R, Mansani R, Fei L, Forti G, Maggi M, Psycho-biological correlates of hypoactive sexual desire in patients with erectile dysfunction. *Int J Impot Res*, in press
41. Shirozu H, Koyanagi T, Takashima T, Horimoto N, Akazawa K, Nakano H 1995 Penile tumescence in the human fetus at term—a preliminary report. *Early Hum Dev* 41:159–166
42. Kinsey AC, Pomeroy WB, Martin CE 1948 Sexual behaviour in the human male. Philadelphia: WB Saunders; 218–262
43. Bancroft J, Tennent G, Loucas K, Cass J 1974 The control of deviant sexual behaviour by drugs. I. Behavioural changes following oestrogens and anti-androgens. *Br J Psychiatry* 125:310–315
44. Bancroft J, Wu FC 1983 Changes in erectile responsiveness during androgen replacement therapy. *Arch Sex Behav* 12:59–66
45. Kwan M, Greenleaf WJ, Mann J, Crapo L, Davidson JM 1983 The nature of androgen action on male sexuality: a combined laboratory-self-report study on hypogonadal men. *J Clin Endocrinol Metab* 57:557–562
46. Wegner HE, Knispel HH, Klan R, Miller K 1995 Efficacy of linsidomine chlorhydrate, a direct nitric oxide donor, in the treatment of human erectile dysfunction: results of a double-blind cross over trial. *Int J Impot Res* 7:233–237
47. Martinez-Pineiro L, Cortes R, Cuervo E, Lopez-Tello J, Cisneros J, Martinez-Pineiro JA 1998 Prospective comparative study with intracavernous sodium nitroprusside and prostaglandin E1 in patients with erectile dysfunction. *Eur Urol* 34:350–354