British Journal of Pharmacology (2008) 153, 1242–1250
 0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Evidence for the participation of calcium in non-genomic relaxations induced by androgenic steroids in rat vas deferens

SSL Lafayette[1], I Vladimirova[2], L Garcez-do-Carmo, PT Monteforte, A Caricati Neto and A Jurkiewicz

Department of Pharmacology, Federal University of São Paulo, São Paulo, Brazil

Background and purpose: Androgens cause non-genomic relaxation in several smooth muscle preparations. However, such an effect has not been investigated in rat vas deferens yet. Our purpose was to study the effect of testosterone and derivatives in this tissue.
Experimental approach: The influence of androgens was tested on contraction and translocation of intracellular Ca^{2+} induced by KCl in rat vas deferens *in vitro*.
Key results: The testosterone derivative 5α-dihydrotestosterone produced a rapid and reversible concentration-dependent relaxation of KCl-induced contractions. Other androgens were also effective, showing the following rank order of potency: androsterone >5β-dihydrotestosterone >androstenedione >5α-dihydrotestosterone >testosterone. Calcium-induced contractions were also inhibited (about 45%) by 5α-dihydrotestosterone (30 μM). Moreover 5α-dihydrotestosterone blocked the increase of intracellular Ca^{2+} induced by KCl, measured by the fluorescent dye fura-2. Relaxation to 5α-dihydrotestosterone was resistant to the K^+ channel antagonists glibenclamide, 4-aminopyridine and charybdotoxin. It was not affected by removal of epithelium or by L-NNA (300 μM), an inhibitor of nitric oxide biosynthesis, nor by selective inhibitors of soluble guanylate cyclase, ODQ or LY 83583, indicating that nitrergic or cGMP mediated mechanisms were not involved. The androgen-induced relaxation was also not blocked by the protein synthesis inhibitor cycloheximide (300 μM) or by the classical androgen receptor flutamide (up to 100 μM), corroborating that the effect is non-genomic.
Conclusions and implications: Testosterone derivatives caused relaxation of the rat vas deferens, that did not involve epithelial tissue, K^+ channels, or nitric oxide-dependent mechanisms, but was related to a partial blockade of Ca^{2+} influx.
British Journal of Pharmacology (2008) **153,** 1242–1250; doi:10.1038/bjp.2008.18; published online 11 February 2008

Keywords: androgen; dihydrotestosterone; non-genomic action; rat vas deferens; calcium

Abbreviations: 5α-DHT, 5α-dihydrotestosterone; 5β-DHT, 5β-dihydrotestosterone; DMSO, dimethylsulphoxide; L–NNA, N^{ω}-nitro-L–arginine; LY 83583, 6-anilino-5,8-quinolinedione; ODQ, 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one

Introduction

It has been well established that male sex steroid hormones exert their classical biological effects by binding to nuclear receptors that modulate specific gene transcription and subsequent protein synthesis. These are relatively slow phenomena known as genomic effects (Beato and Klug, 2000). In addition, several reports have demonstrated other effects of steroids with a rapid onset and short duration in some isolated cells and tissues, including brain (McEwen, 1991; Brann *et al*., 1995), smooth muscles from cardiovascular (Yue *et al*., 1995; Costarella *et al*., 1996; Jones *et al*., 2004), gastrointestinal (Diaz *et al*., 2004), respiratory (Kouloumenta *et al*., 2006) and genitourinary systems (Perusquía *et al*., 2005) and other cell types (Lösel *et al*., 2005). This rapid action is assumed to be non-genomic, being initiated at the plasma membrane and supported by the existence of steroidal membrane receptors in several tissues (Blackmore *et al*., 1991; Farach-Carson and Davis, 2003; Lösel *et al*., 2005). However, the mechanism of action of these non-genomic effects of steroids is still not totally understood (Farach-Carson and Davis, 2003; Lösel *et al*., 2003).

In smooth muscle, the mechanism for non-genomic steroid-induced relaxations has been studied by a number

Correspondence: Professor A Jurkiewicz, Department of Pharmacology, Federal University of São Paulo, Rua 3 de maio 100, São Paulo 04044-020, Brazil.
E-mail: aron.farm@epm.br
[1]Current address: Federal University of Pernambuco, Recife, Brazil.
[2]Current address: Bogomoletz Institute of Physiology, Kiev, Ukraine.
Received 28 August 2007; revised 30 November 2007; accepted 18 December 2007; published online 11 February 2008

of authors (Jones *et al*., 2003). For instance, this effect may involve alterations of the function of cation channels, such as K^+ channels (Yue *et al*., 1995; Valverde *et al*., 1999; Tep-Areenan *et al*., 2002; Diaz *et al*., 2004) and Ca^{2+} channels (Jiang *et al*., 1992; Nakajima *et al*., 1995; Perusquía *et al*., 1996, 2005; Perusquía and Villalón, 1999). In addition, the acute non-genomic effects of steroids on vascular smooth muscles have been shown to be dependent, in some cases, on the presence of endothelial cells, probably due to the release of endothelial-derived relaxing factors (Costarella *et al*., 1996). Alternatively, the steroids can affect cyclic nucleotide signalling pathways (Mügge *et al*., 1993; White *et al*., 1995; Rodriguez *et al*., 1996; Deenadayalu *et al*., 2001).

It is known that the effects of male hormonally active steroids are the sum of the action of testosterone with other steroids, such as the 5-α-reduced dihydrotestosterone. This is the case of the rat vas deferens, an androgen-sensitive organ, which requires male sex hormones for growth and for structural and functional maintenance. Several publications have shown the genomic influence of sex hormones on vas deferens mostly *in vivo* (Martins and Valle, 1939; Calixto and Rae, 1981; Calixto *et al*., 1983). For instance, we have previously demonstrated that after deprivation of endogenous androgens by castration, the calcium channel density is drastically reduced in vas deferens, which is restored to control values if the rats are chronically treated with testosterone (Castillo *et al*., 1992; Lafayette, 1997). In conclusion, these previous chronic studies can be related to a genomic effect of the steroid, whereas a direct, non-genomic acute effect of testosterone or derivatives on vas deferens contractility has not yet been demonstrated in this organ.

The aim of this experiment was to study the acute inhibitory effect of androgens in the rat vas deferens. Therefore, we studied the effect of some sex hormones, such as testosterone (17β-hydroxy-4-androsten-3-one), 5α-dihydrotestosterone (5α-DHT) (17β-hydroxy-5α-androstan-3-one), 5β-dihydrotestosterone (5β-DHT) (17β-hydroxy-5β-androstan-3-one), androsterone (3α-hydroxy-5α-androstan-17-one) and androstenedione (4-androstene-3-17-dione), in tissues contracted with KCl. The initial tests were carried out with 5α-DHT, an endogenous 5α-reduced metabolite of testosterone. We analysed the role of epithelium, nitric oxide, cyclic GMP and K^+ channels on 5α-DHT effects. We have also examined the role of calcium translocation by making measurements of Ca^{2+}-induced contractions and changes on intracellular Ca^{2+} concentration.

Materials and methods

Preparation of the rat vas deferens

All animal procedures and experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals (US National Academy of Science). The experiments were performed on vasa deferentia of 3- to 4-month-old male Wistar rats weighing 250–300 g. The animals were killed under ether anaesthesia. The whole vas deferens was removed, cleaned of surrounding tissues and washed in a nutrient solution with the following composition (mM): 136.0 NaCl, 5.6 KCl, 1.8 $CaCl_2$, 0.36 NaH_2PO_4, 15.0 $NaHCO_3$ and 5.5 dextrose, pH 7.4. The organ was then placed in a temperature-controlled organ bath containing 10 ml of nutrient solution, continuously bubbled with air and maintained at 30 °C, as previously described (Picarelli *et al*., 1962; Jurkiewicz *et al*., 1975). The organs were equilibrated for 30 min under a resting tension of 1 g before use. After this period, isotonic contractions were recorded on kymographs, with tangential levers, giving a sixfold amplification.

Effect of 5α-DHT on rat vas deferens

After the equilibration period, the vas deferens was usually contracted three times with a single concentration of KCl (80 mM) at intervals of about 30 min between two successive doses. The KCl responses were recorded during 10 min and the organs were washed out three times about every 10 min between successive responses. When similar contractions were obtained, cumulative concentrations of steroids, mainly 5α-DHT (1 μM–1 mM) were applied during the sustained phase of contraction of KCl, to measure the corresponding relaxation. In some experiments, to evaluate the participation, or not, of a genomic mechanism on the effect of 5α-DHT, the protein synthesis inhibitor cycloheximide (300 μM) or a non-steroidal antagonist of the classical androgen receptor, flutamide (2-methyl-*n*-[nitro-3(trifluoromethyl)-phenyl] propanamide, 10–100 μM), was added to the organ bath 30 min before adding KCl and maintained during the exposure to 5α-DHT. In other experiments, the epithelium was removed as previously described (Caricati-Neto *et al*., 1995; Okpalaugo *et al*., 2002) before placing the vas deferens in the organ bath. As a rule, instead of 5α-DHT, the corresponding solvent dimethylsulphoxide (DMSO 0.05–1.5%) was used as a control.

In another group of experiments, 5α-DHT (30 μM) or control solvent DMSO (0.3%) was incubated 20 min before and maintained during the performance of a full cumulative concentration–response curve of KCl (1–300 mM). After washout, the procedure was repeated by adding one of the agents listed below, 10 min before the addition of 5α-DHT, to check their influence on the effect of the steroid upon KCl: (a) N^{ω}-nitro-L-arginine (L–NNA, 300 μM), which inhibits nitric oxide (NO) production through inhibition of NO synthase, (b) the selective inhibitors of soluble guanylate cyclase, ODQ (1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one) or LY 83583 (6-anilino-5,8-quinolinedione) and (c) the K^+ channel inhibitors charybdotoxin (0.1 μM), 4-aminopyridine (10 μM) and glibenclamide (30 μM), to examine the role, if any, of K^+ channels.

To test the possibility of a Ca^{2+} channel-blocking effect of 5α-DHT, the vasa deferentia were depolarized with 80 mM KCl in the absence of Ca^{2+} and presence of EGTA (1 μM), followed by a single concentration of $CaCl_2$ (10 mM), to evoke a contraction. This process was repeated until a reproducible response was obtained. At this point, 5α-DHT (30 μM) or control DMSO (0.3%) was added 20 min before the next addition of Ca^{2+}.

Intracellular Ca^{2+} measurement with fura-2

A strip (about 1.0 cm × 0.1 cm) of prostatic segment of rat vas deferens was mounted in a small quartz cuvette filled with

2.5 ml of a Krebs solution with the following composition (mM): NaCl 119, KCl 5.4, $CaCl_2$ 2.5, KH_2PO_4 0.6, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.7, bubbled with a mixture of 95% O_2 and 5% CO_2, at 37 °C. One end of the strip was connected to a strain gauge transducer to monitor the isometric tension under a resting tension of 1.0 g. Simultaneous registration of isometric tension and calcium-induced fluorescence were recorded. Fluorescence measurements were performed in a PTI System (Photon Technology International Inc., Monmouth Junction, NJ, USA) with excitation wavelengths at 340 and 380 nm and emission at 505 nm. At the beginning of experiments, the ratio 340/380 was measured before and after drug applications. After 15 min washing, fura-2AM (8 μM) and the non-cytotoxic detergent Pluronic F-127 (0.08%) were added for 5–6 h at room temperature. Then the strip was washed with fresh Krebs solution at 37 °C. KCl-induced contraction was recorded for 5 min in the absence or presence of 5α-DHT (30 μM) or DMSO (0.3%) incubated 20 min before KCl addition. At the end of the experiments, the minimum ratio (R_{min}) was obtained by addition of Mn^{2+} (2 mM) followed by EGTA (4 mM) and the maximal ratio (R_{max}) was obtained by addition of digitonin (0.1 mM).

Androgen potency

To compare the potency of 5α-DHT, the relaxations by testosterone, 5β-DHT, androsterone and androstenedione were evaluated on KCl-sustained contraction.

Data presentation and statistical analysis

The responses were measured in millimetres and expressed as percentages of a control or standard response, to be analysed by the Graph Pad Prism computer program. The following variables were derived: E_{max}, the maximal relaxation induced by the steroid of the KCl-induced contraction and EC_{50}, the concentration of the steroid inducing 50% of the E_{max} relaxation. The parameters obtained were expressed as means ± s.e.mean and the number of experiments (*n*) was at least six in each case, unless otherwise stated. Statistical analysis was carried out by using the Student's *t*-test, and significance was accepted at $P<0.05$.

Drugs

The following drugs and reagents were used: 5α-DHT (17β-hydroxy-5α-androstan-3-one), 5β-DHT, androsterone, androstenedione L–NNA, LY 83583, glibenclamide, 4-aminopyridine, charybdotoxin, flutamide (2-methyl-*n*-[nitro-3(trifluoromethyl)-phenyl]propanamide) and cycloheximide from Sigma Chemical Co., St Louis, MO, USA. Isradipine from RBI, Natick, MA, USA, and (+)-[^{3}H]isradipine (86 Ci mmol^{-1}) from New England Nuclear, Boston, MA, USA. ODQ, from Tocris, Bristol, UK, fura-2/AM from Molecular Probes, Oregon, USA and DMSO from Synth, SP, Brazil. All chemicals were reagent grade. The stock solution of 5α-DHT was dissolved in DMSO and added to the organ bath with a final DMSO concentration varying from 0.05 to 1.5%.

Results

Effect of 5α-DHT on KCl-induced tonic contraction

To check the inhibitory effect of 5α-DHT, two main groups of experiments were carried out as shown in Figure 1. In the first group (Figures 1a and b), a tonus (sustained contraction) was induced by a single dose of KCl (80 mM), and thereafter cumulative concentrations of androgen were added, to obtain concentration–response curves for relaxation. In relation to this group, Figure 1a shows a typical recording of an experiment in which 5α-DHT caused a concentration-dependent relaxation of KCl-induced contraction. The relaxation was observed within a few seconds after the addition of androgen, reaching an equilibrium for each concentration within about 6 min and attaining a maximum when usually the last of seven cumulative concentrations was added, after about 40 min. The corresponding concentration–response curve for 5α-DHT-induced relaxation over a range from 10 to 300 μM, yielded a maximal inhibition of KCl contraction (E_{max}) of 84.3 ± 2.7% (Figure 1b). The mean concentration of 5α-DHT producing a half of the E_{max} relaxation (EC_{50} ± s.e.mean) was 62.0 ± 10.2 μM. This effect was completely reversible after washout.

In the second group of experiments (Figure 1c), a single dose of androgen (30 μM) was initially added, followed, 20 min later, by a cumulative concentration–response curve for KCl, to check for the antagonism, by comparing this curve with that of control KCl. In relation to this group, it was observed that 5α-DHT did not induce any noticeable effect when added alone to the organ bath. However, it produced a significant inhibition on KCl concentration–response curve with a decrease of about 37% of the maximal response as shown in Figure 1c.

Effect of 5α-DHT on Ca^{2+} contractile response

To evaluate the possible relation with a Ca^{2+}-dependent process, 5α-DHT was assayed on Ca^{2+}-induced contractions in a Ca^{2+}-free, K^+-depolarization medium. Figure 2a shows that Ca^{2+} (10 mM) contractions were significantly reduced by about 45% after pre-incubation for 20 min with 5α-DHT (30 μM), indicating that a decrease of calcium translocation was involved in the steroid relaxation.

Effect of 5α-DHT on calcium intracellular translocation

Figures 2b1 and b2 show a typical record from strips of rat vas deferens in which real-time KCl-induced contraction and increase of intracellular calcium concentration, $[Ca^{2+}]_i$, were measured simultaneously in the same tissues. It is clear that both the contraction and increase of $[Ca^{2+}]_i$ were strikingly reduced by 5α-DHT. This finding is an additional demonstration that a reduction of calcium translocation is involved in the effect of the steroid. The effects were reversed after washout (not shown).

Effect of 5α-DHT in epithelium-free vas deferens

To assess if the androgen-induced relaxation was due to the release of inhibitory agents from epithelium, experiments

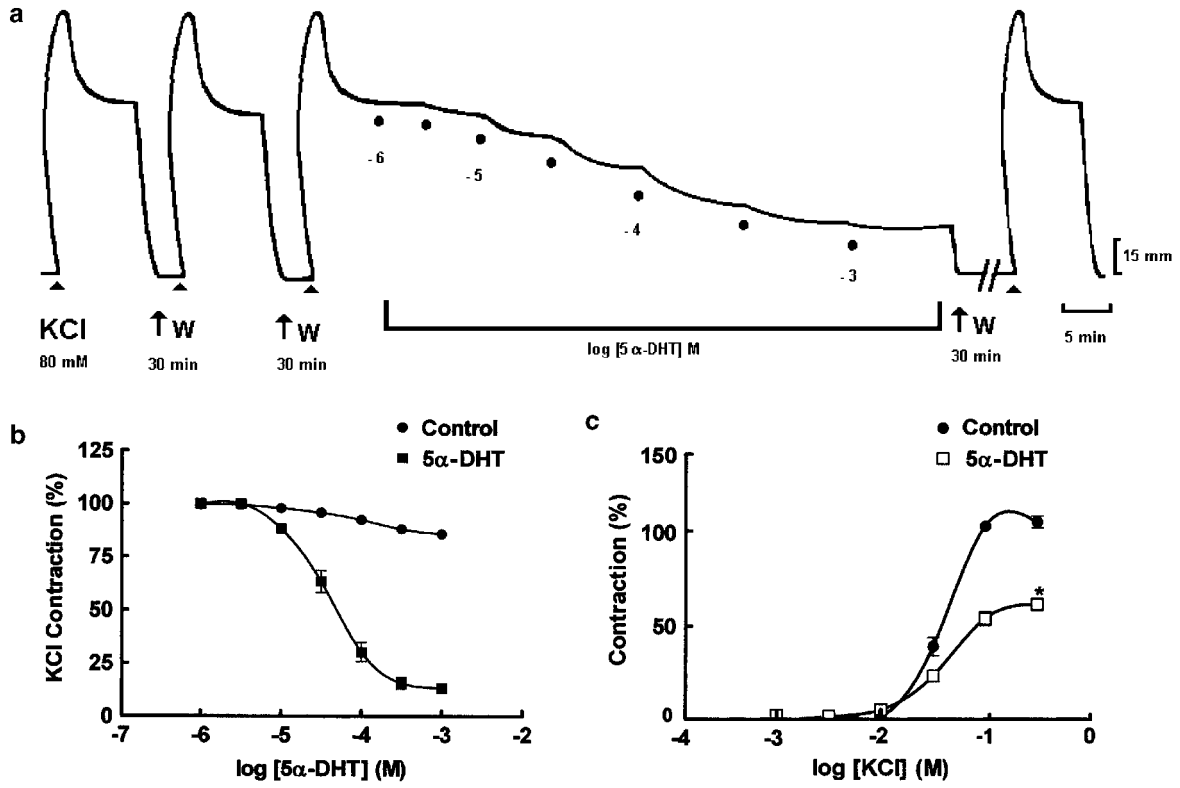

Figure 1 Relaxing effect of 5α-dihydrotestosterone (5α-DHT) on rat vas deferens. (**a**) Schematic tracing of a typical experiment. The tissue was pre-contracted with KCl (80 mM added at triangles) followed by adding 5α-DHT (at dots) to obtain the corresponding cumulative concentrations (–log values shown). Arrows indicate recording interruption (30 min) for organ washout (W); (**b**) mean concentration–response curves of 5αDHT or dimethylsulphoxide (DMSO) (control) on KCl contraction, (**c**) effect of pre-incubation of 30 μM of 5α-DHT on KCl concentration–response curves. 5αDHT or DMSO, 0.3% (control) was added 20 min before KCl. Data are expressed as percentage of the maximum effect of the initial curve for KCl (mean ± s.e.mean, $n = 6$). *Maximal effects (with 5α-DHT) are significantly different ($P < 0.05$) from the corresponding controls.

were carried out using the vas deferens after removing the epithelium shortly before, as previously described (Caricati-Neto *et al*., 1995; Okpalaugo *et al*., 2002). The inhibitory concentration–response curves of 5α-DHT show that no significant differences were observed between the curves for organs with and without epithelium (Figure 3a).

Effect of L–NNA, ODQ or LY 83583 on 5α-DHT-induced relaxation

To evaluate a possible relaxation through the release of NO (Moncada *et al*., 1991) by 5α-DHT, L–NNA (300 μM), a blocker of NO synthase, was added before this hormone. Figure 3b shows that L–NNA was totally ineffective. Another mechanism that might be involved in the 5α-DHT-induced relaxation is an activation of guanylate cyclase, leading to the formation of the relaxing nucleotide cGMP (Carvajol *et al*., 2000). To check this possibility, we pretreated the tissues with ODQ (10 μM), a specific inhibitor of guanylate cyclase, and found that the pretreatment did not influence the effects of 5α-DHT (Figure 3c). Similarly, another blocker of guanylate cyclase, LY 83583 (10 μM), was also unable to prevent the relaxation (data not shown, $n = 6$). These results indicated that NO or cGMP was not involved in these relaxations.

Effect of K^+ channel blockers on 5α-DHT-induced relaxation

To check the involvement of K^+ channels on 5α-DHT relaxation, experiments were carried out by incubating each of three inhibitors of K^+ channels, namely charybdotoxin, 4-aminopyridine or glibenclamide, before 5α-DHT. Figure 4 shows that these agents did not significantly modify the inhibitory effects of 5α-DHT, indicating that potassium channels were not involved.

Influence of cycloheximide and flutamide on the effect of 5α-DHT

To rule out the possibility that the effects of 5α-DHT might be related to gene transcription, the inhibitor of protein synthesis, cycloheximide, and the specific inhibitor of the classical androgen receptor, flutamide, were assayed on two separate groups of experiments. Cycloheximide (300 μM, Figure 5), or flutamide (10–100 μM, not shown), did not modify the relaxation curves to 5α-DHT.

Effect of other androgens on KCl tonic contraction

A group of experiments were undertaken to assess if, besides 5α-DHT, other related hormones, such as testosterone, 5β-DHT, androsterone and androstenedione would also elicit inhibitory effects upon KCl tonic contraction. Table 1 shows values of EC_{50} and maximal inhibitory effect for these

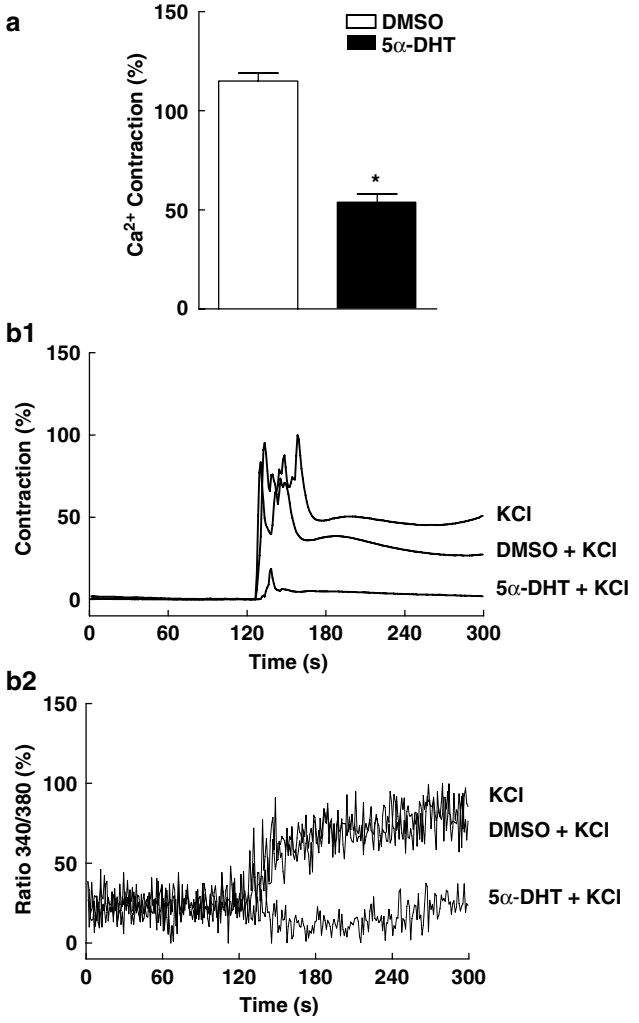

Figure 2 (**a**) Bar graph showing the inhibitory effect of 5α-dihydrotestosterone (5α-DHT; 30 μM) on contractions induced by Ca^{2+} (10 mM) in rat vas deferens depolarized by high KCl (80 mM) in a Ca^{2+}-free + EGTA (1 μM) medium. 5α-DHT was added 20 min before Ca^{2+}. *Significant difference in relation to control, in which dimethylsulphoxide (DMSO) (0.3%) was used instead of 5α-DHT ($n=6$). The effect of Ca^{2+} alone (not shown) was taken as 100%. (**b**) Typical experiment showing simultaneous measurement of the effect of 5α-DHT (30 μM, 20 min) on contraction (**b_1**) and fluorescence (**b_2**) induced by KCl (80 mM) in the prostatic portions of rat vas deferens. Fluorescence ratio was measured by alternating dual excitation wavelengths between 340 and 380 nm and was used to evaluate changes in $[Ca^{2+}]_i$. (**b_1**) Data are expressed as percentage of the maximal contraction with KCl. Although the DMSO (0.3%) control had a small inhibitory effect, it was much smaller than that caused by 5α-DHT. (**b_2**) Note that an overlap occurred between the tracings showing the Ca^{2+} signal after KCl and after KCl + DMSO.

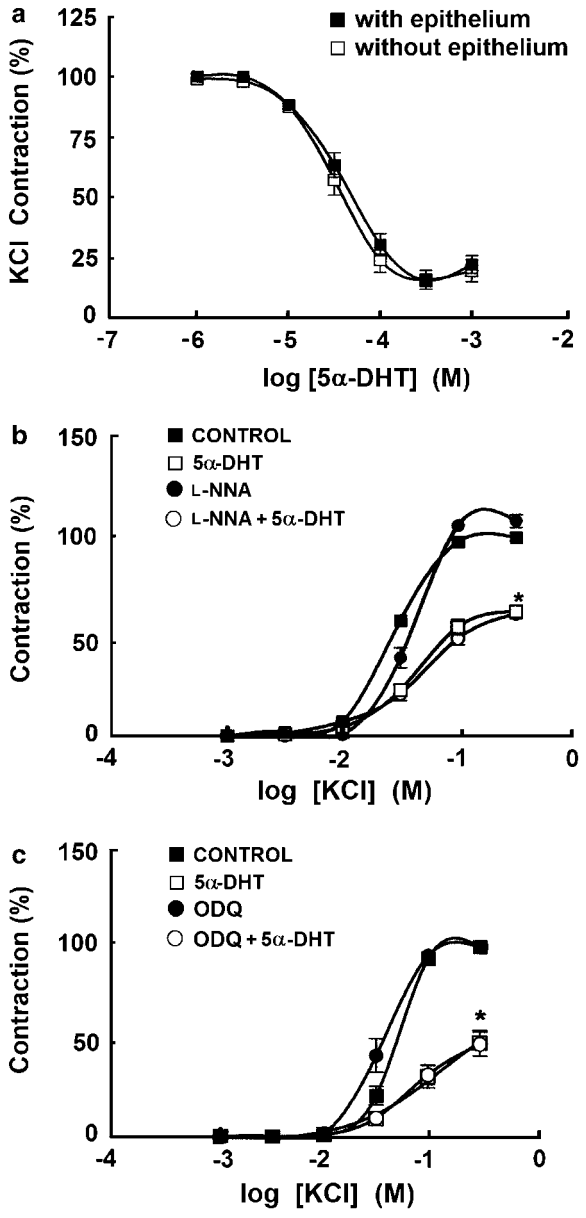

Figure 3 (**a**) Mean concentration–response curves of 5α-dihydrotestosterone (5α-DHT) on KCl tonic contraction, on vas deferens with and without epithelium, obtained from experiments similar to that shown in Figure 1a. (**b**) Effect of pre-incubation of 30 μM of 5α-DHT on KCl concentration–response curves in the absence or presence of N^{ω}-nitro-L-arginine (L–NNA) (300 μM) or (**c**) 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) (10 μM). (**b**, **c**), 5α-DHT was added 20 min before KCl, and L–NNA or ODQ was added 10 min before 5α-DHT. The solvent dimethylsulphoxide (DMSO) (0.3%) had no significant effect (not shown). Data are expressed as percentage of the maximal contraction with KCl. Points represent mean ± s.e.mean, $n=8$–10. *Significantly different ($P<0.05$) from the corresponding controls. Note that significant differences were not detected between maximal effects when either L–NNA or ODQ was added.

agents. The respective potency ratios show that the most potent relaxant androgens were androsterone and 5β-DHT, and that lower rank orders of potency were observed for the effects of androstenedione, 5α-DHT and testosterone.

Discussion and conclusions

We have shown that the endogenous testosterone metabolite, 5α-DHT and related androgens produced a rapid dose-dependent and reversible inhibitory effect on contractions induced in rat vas deferens, under various experimental conditions. These observations are indicative of a non-genomic effect, which is most probably due to a blockade of calcium influx through voltage-dependent Ca^{2+}

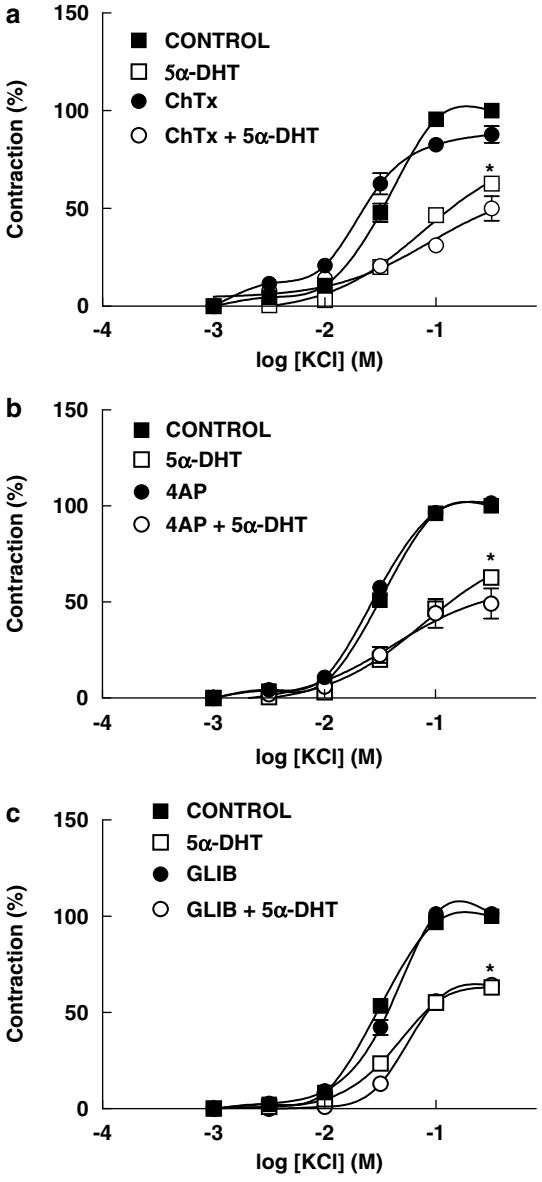

Figure 4 Effect of pre-incubation with 30 μM of 5α-dihydrotestosterone (5α-DHT) on KCl concentration–response curves in the absence or presence of K^+ channel antagonists: (**a**) charybdotoxin (ChTx 0.1 μM), (**b**) 4-aminopyridine (4AP 10 μM) and (**c**) glibenclamide (GLIB 30 μM). 5α-DHT was added 20 min before KCl and K^+ channel antagonists were added 10 min before 5α-DHT. The solvent dimethylsulphoxide (DMSO) (0.3%) had no significant effect (not shown). Data are expressed as percentage of the maximal contraction with KCl. Points represent mean ± s.e.mean, $n=8$. *Significantly different ($P<0.05$) from the corresponding controls. Note that there were no significant differences between maximal effects when the K^+ channel blockers were added to 5αDHT.

channels, as discussed below. Participation of epithelium-relaxing factors, NO, cGMP or potassium channels could not be demonstrated. To our knowledge, the occurrence of this type of non-genomic effect has not been previously described for the rat vas deferens.

The most potent relaxation was induced by androsterone and 5β-DHT as shown in Table 1. This result agrees with that of Perusquía *et al*. (2005) showing that both steroids are more

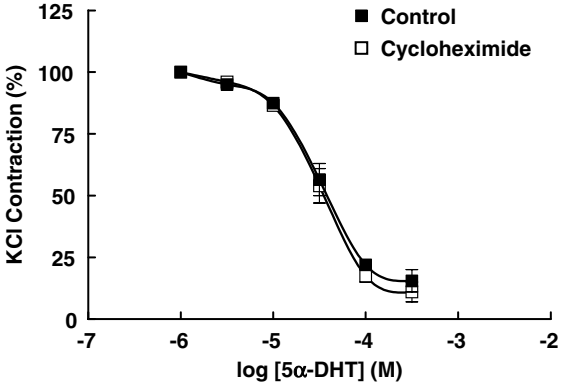

Figure 5 Mean concentration–response curves of 5α-dihydrotestosterone (5α-DHT) or dimethylsulphoxide (DMSO) (control) on KCl contraction, obtained from experiments similar to that shown in Figure 1a, except that cycloheximide (300 μM) was added to the tissue, 20 min before KCl addition. Data are expressed as percentage of KCl contraction (mean ± s.e.mean, $n=6$).

Table 1 EC_{50} (concentration for half E_{max}) and maximal inhibitory effect (E_{max}) of androgens on KCl (80 mM) contraction in rat vas deferens

	EC_{50} (μM)	*Rank order of potency*[a]	*E_{max} (%)*
5α-DHT	70 ± 5	4	88 ± 2
Testosterone	94 ± 19	5	82 ± 6
5β-DHT	46 ± 9[b]	2	96 ± 1
Androsterone	26 ± 4[b]	1	98 ± 1
Androstenedione	56 ± 15	3	76 ± 4

[a]Measured from EC_{50} values.
[b]$P<0.05$ vs 5α-dihydrotestosterone (5α-DHT).
Values are means ± s.e.mean, ($n=8$) in each group.

potent than testosterone and 5α-DHT in human myometrium *in vitro*.

The contraction induced by Ca^{2+} in depolarized rat vas deferens was highly sensitive to the inhibitory effect of 5α-DHT. Similar results were obtained with 5β-DHT in rat aorta by Perusquía and Villalón (1999). In rat vas deferens, the KCl-induced increase of intracellular calcium is mainly caused by membrane depolarization and Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channels (Castillo *et al*., 1992; Jurkiewicz *et al*., 1994). Thus, a decrease in Ca^{2+}-induced contraction after 5α-DHT may reflect a decline of Ca^{2+} influx through these channels. This conclusion agrees with our present experiment in which 5α-DHT caused a decrease of tension and Ca^{2+}-induced fura-2 fluorescence, measured simultaneously (Figure 2), as shown for other agents (Ribeiro *et al*., 2003). Similarly, Jiang *et al*. (1992) observed that 17β-estradiol has a negative inotropic effect on guinea-pig cardiac myocytes by inhibiting inward Ca^{2+} currents and so reducing fura-2 fluorescence. Thus, it remains to be determined whether or not, in rat vas deferens, 5α-DHT binds to a modulatory site on the L-type Ca^{2+} channels, as shown for certain steroids on GABA/benzodiazepine receptor Cl^- channel complex (Gee *et al*., 1987; McEwen, 1991). However, as it has been recently clearly shown through patch-clamp experiments that testosterone

inhibits L-type calcium channels in isolated smooth muscle cells (Scragg *et al*., 2004, 2007), it is quite probable that 5α-DHT induces a similar blockade.

Our results show that 5α-DHT relaxation of KCl-induced contraction does not depend on NO or epithelium, because epithelium removal and L–NNA, an antagonist of nitric oxide synthase, did not prevent the relaxation. Although previous studies have shown that nitric oxide synthase is present in rat vas deferens (Ceccatelli *et al*., 1994), the role of nitric oxide in the contractile mechanisms in this tissue is still unclear (Sunano, 1983; Vladimirova *et al*., 1994; Ventura and Burnstock, 1997). Our results agree with the finding that in human coronary artery, steroid-induced vasodilatation is an epithelium-independent effect (Mügge *et al*., 1993). On the other hand, an epithelium-dependent mechanism was observed in rat aorta (Costarella *et al*., 1996; Honda *et al*., 1999), canine coronary artery (Chou *et al*., 1996; Node *et al*., 1997) and airway smooth muscle (Kouloumenta *et al*., 2006), which can be attributed to the release of NO.

The involvement of some intracellular messengers in the non-genomic effect of steroids has been suggested in some tissues (Mügge *et al*., 1993; Ogata *et al*., 1996; Rodriguez *et al*., 1996; Rubín *et al*., 1999). In the present study, the finding that the relaxation by 5α-DHT was not influenced by ODQ or LY 83583, inhibitors of the biosynthesis of cGMP, indicates that at least this messenger was not involved here.

The K^+ channel antagonists glibenclamide, 4-aminopyridine and charybdotoxin did not inhibit the steroid effect here, suggesting that at least these K^+ channels, blocked by these drugs, were not involved in the inhibitory action of 5α-DHT in this tissue. The lack of effect of K^+ channel antagonists in our experiments cannot be ascribed to an absence of the corresponding types of channel, as large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}), ATP-sensitive K^+ channels (K_{ATP}) and voltage-dependent K^+ channels (K_V) have been described in the rat vas deferens (Grana *et al*., 1991; Huang, 1995; Harhun *et al*., 2003). A correlation between K^+ channels and non-genomic effects of steroids has been shown in many smooth muscles, such as rat aorta, mesenteric and coronary arteries (White *et al*., 1995; Yue *et al*., 1995; Valverde *et al*., 1999; Deenadayalu *et al*., 2001; Tep-Areenan *et al*., 2002) or duodenum (Diaz *et al*., 2004), although some studies did not clearly relate the non-genomic vasodilatation with these channels (Nakajima *et al*., 1995; Ogata *et al*., 1996).

The effects presented here are clearly non-genomic, since they occurred within minutes. For instance, it is known that androgens, estrogens or progesterones produce a rapid non-genomic relaxation of many vascular tissues (White *et al*., 1995; Yue *et al*., 1995; Costarella *et al*., 1996; Jones *et al*., 2004), as well of other smooth muscles (Kubli-Garfias *et al*., 1987; Diaz *et al*., 2004; Perusquía *et al*., 2005). In contrast to our results, classical genomic responses usually require hours or days to appear, because steroid molecules have to cross the cell membrane to interact with specific intracellular receptors, thereby influencing the transcription of genes into mRNA, followed by translation into protein molecules. As a consequence, the genomic effect is known to be reduced by steroid receptor antagonists, such as flutamide, as well as by inhibitors of translation, such as cycloheximide (Beato and Klug, 2000; Lösel *et al*., 2003). Therefore, the finding that our present effects were neither blocked by flutamide nor by cycloheximide strongly corroborated the assumption of a non-genomic effect.

As in our experiments, a number of previous publications showed that acute steroid effects in smooth muscle occur at the micromolar concentration range, which is higher than the normal nanomolar concentrations in circulatory system (Heaton, 2003; Lösel *et al*., 2003). For instance, concentrations from 10 to 100 μm of testosterone have been used in rabbit aortic rings (Yue *et al*., 1995), from 3 to 100 μM in human uterus strips (Perusquía *et al*., 2005), whereas a half-maximal relaxation was induced at a concentration of 50 μM in rat aorta (Costarella *et al*., 1996). Therefore, one could argue that these '*in vitro*' concentrations are non-specific or non-physiologic. However, even if the doses used here are higher than the doses in the so-called physiological conditions, this effect is worth being studied, as androgens, as for example testosterone, have been used in men for the treatment of varying degrees of pathophysiological conditions, such as osteoporosis, hypogonadism, loss of muscle strength and deterioration of mood (Heaton, 2003). In this case, it is known that i.m. injections of testosterone can lead to supraphysiological levels for several days after injection (Dobs *et al*., 1999). Furthermore, a word of caution seems to be necessary when comparing concentrations used '*in vivo*' and '*in vitro*', as drug concentrations in organ baths are most probably higher than that in the vicinity of receptors, given that the agent has to cross different tissue layers to reach the smooth muscle biophase (Busatto and Jurkiewicz, 1985), whereas '*in vivo*', receptors are reached directly through the blood circulation.

From the clinical standpoint, a beneficial role of non-genomic effects of testosterone has been described in vascular smooth muscle of patients with coronary arterial disease, whose symptoms of angina due to myocardial ischaemia were attenuated by vasodilatation after an infusion of physiological concentrations of testosterone (Scragg *et al*., 2004, 2007). Although the present studies in vas deferens provide stimulating pharmacological data about non-genomic effects of androgenic steroids, information is still lacking about the possibility of a therapeutic use, as for instance on the treatment of genitourinary tract dysfunctions.

In summary, it was shown here that testosterone derivatives have an acute relaxing effect, which is clearly non-genomic, and unrelated to epithelium-released agents, K^+ channels, cGMP or to a nitrergic system, but is compatible with a reduction of Ca^{2+} influx through cell membrane Ca^{2+} channels, in rat vas deferens.

Acknowledgements

We thank Haydee Reuter for technical assistance.

Conflict of interest

The authors state no conflict of interest.

References

Beato M, Klug J (2000). Steroid hormone receptors: an update. *Hum Reprod Update* **6**: 225–236.

Blackmore PF, Neulen J, Lattanzio F, Beebe SJ (1991). Cell surface-binding sites for progesterone mediate calcium uptake in human sperm. *J Biol Chem* **266**: 18655–18659.

Brann DW, Hendry LB, Mahesh VB (1995). Emerging diversites in the mechanism of action of steroid hormones. *J Steroid Biochem Mol Biol* **52**: 113–133.

Busatto PA, Jurkiewicz A (1985). Simultaneous measurement of contractile effects in the circular and longitudinal smooth muscle of the rat vas deferens by drugs perfused externally or via the lumen. *Br J Pharmacol* **85**: 737–746.

Calixto JB, Aucélio JG, Jurkiewicz A (1983). Influence of castration of the neonatal rat on the contractile effects of barium chloride and adrenaline in isolated vas deferens: the role of calcium. *Biol Reprod* **29**: 1057–1062.

Calixto JB, Rae GA (1981). Influence of castration of the neonatal rat on the pharmacological reactivity of the isolated vas deferens. *Biol Reprod* **25**: 481–486.

Caricati-Neto A, Pupo AS, Wanderley AG, Nuñez-Vergara M, Koh IHJ, Jurkiewicz NH *et al*. (1995). Role of the epithelium in the release of contractile agents from the rat vas deferens by clonidine. *Ann NY Acad Sci* **783**: 436–439.

Carvajol JA, Germain AM, Huidobro-Toro JP, Weiner CP (2000). Molecular mechanism of cGMP-mediated smooth muscle relaxation. *J Cell Physiol* **184**: 409–420.

Castillo CJF, Lafayette S, Caricati-Neto A, Sette M, Jurkiewicz NH, Garcia AG *et al*. (1992). Low dihydropyridine receptor density in vasa deferentia of castrated rats. *Br J Pharmacol* **105**: 257–258.

Ceccatelli S, Lunderg JM, Zhang X, Aman K, Hökfelt T (1994). Immunohistochemical demonstration of nitric oxide synthase in the peripheral autonomic nervous system. *Brain Res* **656**: 381–395.

Chou TM, Sudhir K, Hutchison SJ, Ko E, Amidon TM, Collins P *et al*. (1996). Testosterone induces dilation of canine coronary conductance and resistance arteries *in vivo*. *Circulation* **94**: 2614–2619.

Costarella CE, Stallone JN, Rutacki GW, Whittier FC (1996). Testosterone causes direct relaxation of rat thoracic aorta. *J Exp Ther* **277**: 34–39.

Deenadayalu VP, White RE, Stallone JN, Gao X, Garcia AJ (2001). Testosterone relaxes coronary arteries by opening the large-conductance, calcium-activated potassium channel. *Am J Physiol Heart Circ Physiol* **281**: H1720–H1727.

Diaz M, Ramírez CM, Marin R, Marrero-Alonso J, Gómez T, Alonso R (2004). Acute relaxation of mouse duodenum by estrogens. Evidence for an estrogen receptor-independent modulation of muscle excitability. *Eur J Pharmacol* **501**: 161–178.

Dobs AS, Meille W, Arver S, Sanders SW, Caramelli KE, Mazer NA (1999). Pharmacokinetics, efficacy, and safety of a permeation-enhanced testosterone transdermal system in comparison with bi-weekly injections of testosterone enanthate for the treatment of hypogonadal men. *J Clin Endocrin Metab* **84**: 3469–3478.

Farach-Carson MC, Davis PJ (2003). Steroid hormone interaction with target cells: cross talk between membrane and nuclear pathways. *J Pharmacol Exp Ther* **307**: 839–845.

Gee KW, Chang WC, Brinton RE, McEwen BS (1987). GABA-dependent modulation of the Cl-ionophore by steroids in rat brain. *Eur J Pharmacol* **136**: 419–423.

Grana E, Barbiere A, Zonta F (1991). Inhibition by glibenclamide of the effects of cromakalim on responses of rat vas deferens to naphazoline. *Eur J Pharmacol* **202**: 93–96.

Harhun MI, Jurkiewicz A, Jurkiewicz NH, Kryshtal DO, Shuba MF, Vladimirova IA (2003). Voltage-gated potassium currents in rat vas deferens smooth muscle cells. *Pflugers Arch* **446**: 380–386.

Heaton JPW (2003). Hormone treatment and preventive strategies in the aging male: whom and when to treat? *Rev Urol* **5** (Suppl 1): S16–S21.

Honda H, Unemoto T, Kogo H (1999). Different mechanisms for testosterone-induced relaxation of aorta between normotensive and spontaneously hypertensive rats. *Hypertension* **34**: 1232–1236.

Huang Y (1995). $BaCl_2$- and 4-aminopyridine-evoked phasic contractions in rat vas deferens. *Br J Pharmacol* **115**: 845–851.

Jiang C, Poole-Wilson PA, Sarrel PM, Mochizuki S, Collins P, MacLeod KT (1992). Effect of 17β-estradiol on contraction, Ca^{2+} current and intracellular free Ca^{2+} in guinea-pig isolated cardiac myocytes. *Br J Pharmacol* **106**: 739–745.

Jones RD, English KM, Jones TH, Channer KS (2004). Testosterone-induced coronary vasodilation occurs via a non-genomic mechanism: evidence of a direct calcium antagonism action. *Clin Sci* **107**: 149–158.

Jones RD, Puig PJ, Jones TH, Channer KS (2003). The vasodilatory action of testosterone: a potassium channel opening or a calcium antagonistic action? *Br J Pharmacol* **138**: 733–744.

Jurkiewicz A, Lafayette SS, Nunes SH, Martini LC, Garcez-do-Carmo L, Wanderley A *et al*. (1994). Decreased density of binding sites for the Ca^{2+} channel antagonist [^{3}H]isradipine after denervation of rat vas deferens. *Eur J Pharmacol* **256**: 329–333.

Jurkiewicz A, Markus RP, Picarelli ZP (1975). Effect of full agonists following calcium deprivation in rat vas deferens. *Eur J Pharmacol* **31**: 292–304.

Kouloumenta V, Hatziefthimiou A, Paraskeva E, Gourgoulianis K, Molyvdas PA (2006). Non-genomic effect of testosterone on airway smooth muscle. *Br J Pharm* **149**: 1083–1091.

Kubli-Garfias C, Medina-Jiménez M, García-Yánez E, Vásquez-Alvarez A, Perusquía M, Gómez-García N *et al*. (1987). Relaxant action of androgens, progestins and corticosteroids on isolated ileum of the guinea pig. *Acta Physiol Pharmacol Latinoam* **37**: 357–364.

Lafayette SSL (1997). Papel modulador da inervação autonômica e do hormônio sexual masculino sobre os canais de cálcio voltagem-dependentes e sua importância no processo contrátil em ducto deferente de rato. Thesis, UNIFESP/EPM, São Paulo, Brazil.

Lösel RM, Breiter S, Seyerfet M, Wheling M, Falkenstein E (2005). Classic and non-classic progesterone receptors are both expressed in human spermatozoa. *Horm Metab Res* **37**: 10–14.

Lösel RM, Falkenstein E, Feuring M, Schultz A, Hanns-Christian T, Rossol-Haseroth K *et al*. (2003). Nongenomic steroid action: controversies, questions, and answers. *Physiol Rev* **83**: 965–1016.

Martins T, Valle JR (1939). Endocrine control of the motility of the male accessory genital organs. *Endocrinology* **25**: 80–90.

McEwen BS (1991). Non-genomic and genomic effects of steroids on neural activity. *Trends Pharmacol Sci* **2**: 41–47.

Moncada S, Palmer MJ, Higgs EA (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* **43**: 109–142.

Mügge A, Riedel M, Barton M, Kuhn M, Lichtlen PR (1993). Endothelium independent relaxation of human coronary arteries by 17β-estradiol *in vitro*. *Cardiovasc Res* **27**: 1939–1942.

Nakajima T, Kitazawa T, Hamada E, Hazama H, Omata M, Kurachi Y (1995). 17β-Estradiol inhibits the voltage-dependent L-type Ca^{2+} currents in aortic smooth muscle cells. *Eur J Pharmacol* **294**: 625–635.

Node K, Kitakaze M, Kosaka H, Minamino T, Sato H, Kuzuya T *et al*. (1997). Roles of NO and Ca^{2+}-activated K^+ channels in coronary vasodilation induced by 17β-estradiol in ischemic heart failure. *FASEB J* **11**: 793–799.

Ogata R, Inoue Y, Nakano H, Ito Y, Kitamura K (1996). Oestradiol-induced relaxation of rabbit basilar artery by inhibition of voltage-dependent Ca channels through GTP-binding protein. *Br J Pharmacol* **117**: 351–359.

Okpalaugo EO, Garcez-do-Carmo L, Jurkiewicz NH, Jurkiewicz A (2002). Contractile responses of the rat vas deferens after epithelium removal. *Life Sci* **70**: 2943–2951.

Perusquía M, Hernández R, Morales MA, Campos MG, Villalón CM (1996). Role of endothelium in the vasodilating effect of progestins and androgens on the rat thoracic aorta. *Gen Pharmacol* **27**: 181–185.

Perusquía M, Navarrete E, Jasso-Kamel J, Montano LM (2005). Androgens induce relaxation of contractile activity in pregnant human myometrium at term: a nongenomic action on L-type calcium channels. *Biol Reprod* **73**: 214–221.

Perusquía M, Villalón CM (1999). Possible role of Ca^{2+} channels in the vasodilating effect of 5-dihydrotestosterone in rat aorta. *Eur J Pharmacol* **371**: 169–178.

Picarelli ZP, Hypollito N, Valle JR (1962). Synergistic effect of 5-hydroxytryptamine on the response of rat seminal vesicle to adrenaline and noradrenaline. *Arch Int Pharmacodyn* **138**: 354–363.

Ribeiro RA, Garcez-do-Carmo L, Vladimirova I, Jurkiewicz NH, Jurkiewicz A (2003). Nantenine blocks muscle contraction and Ca^{2+} transient induced by noradrenaline and K^{+} in rat vas deferens. *Eur J Pharmacol* **470**: 37–43.

Rodriguez J, Garcia de Boto MJ, Hidalgo A (1996). Mechanism involved in the relaxant effect of estrogens on rat aorta strips. *Life Sci* **58**: 607–615.

Rubín JM, Hidalgo A, Bordallo C, Cantabrana B, Sánchez M (1999). Positive inotropism induced by androgen in isolated left atrium of rat: evidence for cAMP-dependent transcriptional mechanism. *Life Sci* **65**: 1035–1045.

Scragg JL, Dallas ML, Peers C (2007). Molecular requirements for L-type Ca^{2+} channel blockade by testosterone. *Cell Calcium* **42**: 11–15.

Scragg JL, Jones RD, Channer KS, Jones TH, Peers C (2004). Testosterone is a potent inhibitor of L-type $Ca(2^{+})$ channels. *Biochem Biophys Res Commun* **318**: 503–506.

Sunano S (1983). Potentiating effects of high concentration of sodium nitroprusside on the contraction of guinea-pig vas deferens. *Jpn J Phamacol* **33**: 489–491.

Tep-Areenan P, Kendall DA, Randall MD (2002). Testosterone-induced vasorelaxation in the rat mesenteric arterial bed is mediated predominantly via potassium channels. *Br J Pharmacol* **135**: 735–740.

Valverde MA, Rojas P, Amigo J, Cosmeli D, Orio P, Bahamonde MI *et al*. (1999). Acute activation of maxi-K channels (hSLo) by estradiol binding to the β subunit. *Science* **285**: 1929–1931.

Ventura S, Burnstock G (1997). Evidence against nitrergic neuromodulation in rat vas deferens. *Eur J Pharmacol* **334**: 87–93.

Vladimirova I, Jurkiewicz NH, Jurkiewicz A (1994). Evidence for participation of nitric oxide in excitatory neurotransmission in rat vas deferens. *Life Sci* **55**: 1123–1128.

White RE, Darkow DJ, Lang JLF (1995). Estrogen relaxes coronary arteries by opening BKCa channels through a cGMP-dependent mechanism. *Circ Res* **77**: 936–942.

Yue P, Chatterjee K, Beale C, Poole-Wilson PA, Collins P (1995). Testosterone relaxes rabbit coronary arteries and aorta. *Circulation* **91**: 1154–1160.