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# Modulatory role of endogenous androgens on airway smooth muscle tone in isolated guinea-pig and bovine trachea; involvement of $\beta_2$-adrenoceptors, the polyamine system and external calcium

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ABSTRACT

Androgens relax several smooth muscles, including the airways. They also contract ileum and myocardium via nongenomic mechanisms. To find out whether androgens modulate airway smooth muscles in different species and further assess their mechanism of action, regarding the role of β-adrenoceptors, polyamines and extracellular $Ca^{2+}$, and the modulation of contraction, 5α-dihydrotestosterone, testosterone and 5β-dihydrotestosterone were used. A preliminary study was performed to evaluate the effect of 5α-dihydrotestosterone, a non-aromatisable derivate of testosterone, in isolated guinea-pig trachea and a more exhaustive characterisation was followed in bovine trachea, to also characterise the effect of testosterone and 5β-dihydrotestosterone. The androgens elicited a nongenomic epithelium-independent relaxation of the trachea which had been precontracted. In the bovine trachea, the order of potency was: testosterone>5α-dihydrotestosterone=5β-dihydrotestosterone. This effect was inversely proportional to the magnitude of carbachol-raised tone and was independent of $\beta_2$-adrenoceptors, since the β-blockers, propranolol and ICI-118,551, and $\beta_2$-adrenoceptor desensitisation did not modify 5α-dihydrotestosterone-elicited relaxation. 5α-Dihydrotestosterone was unable to displace the radiolabel, [$^3$H]dihydroalprenolol, from these receptors in the binding assay. Polyamine synthesis was not involved in this androgen effect, since an ornithine decarboxylase inhibitor, α-difluoromethylornithine, was ineffective. The androgens were more effective relaxing bovine trachea precontracted by KCl (80 mM), suggesting a calcium entry blockade, as reported for several smooth muscles. This mechanism might be involved in the observed 5α-dihydrotestosterone facilitation of salbutamol-relaxation. Androgens facilitated carbachol-elicited contraction independently of polyamine synthesis, contrary to what has been reported in the ileum. Therefore, androgens modulate tracheal smooth muscle tone which might be of importance in the regulation of airway reactivity.



## 1. Introduction

Androgens produce rapid nongenomic effects in most studied smooth muscles (Losel et al., 2002; Heinlein and Chang, 2002). Although contradictory results concerning the acute effects of androgens on vasomotor responses have been reported. Pharmacological concentrations of androgens mainly produce the relaxation of isolated tissues from different species (Yue et al., 1995; Costarella et al., 1996; Chou et al., 1996; Rosano et al., 1999; Tep-areenan et al., 2002), including humans (Malkin et al., 2006; Perusquia et al., 2007). On the other hand, acute exposure to testosterone, even at concentrations where testosterone has no effect, facilitated the effect of several vasoconstrictors, raising the possibility of androgens being detrimental to vasomotor function (Greenberg et al., 1974; Herman et al., 1997).

Spasmolytic effects of androgens have also been reported in several nonvascular smooth muscles, such as in the ileum (Kubli-Garfias et al., 1987) and the myometrium (Perusquia et al., 1990, 2005; Sanchez Aparicio et al., 1993), involving the blockade of $Ca^{2+}$ channels. On the other hand, it has also been reported that androgens potentiated ileal contractile activity by nongenomic mechanisms, which involved intracellular polyamine signaling and $Ca^{2+}$ sensitisation via Rho kinase activation (Gonzalez-Montelongo et al., 2006).

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Furthermore, they elicited an acute cardiotonic effect, in which polyamines are required for the androgen effect on the β-adrenoceptors (Bordallo et al., 2001; Velasco et al., 2008).

Recently, relaxation of rabbit tracheal smooth muscle by testosterone via epithelium and NO-mediation has been reported. This finding has been used to explain, in part, gender differences in airway diseases (Kouloumenta et al., 2006). However, this is a complex issue as suggested by studies on the effect of sex hormones on respiratory function (Caracta, 2003; Cevrioglu et al., 2004; Carey et al., 2007).

The aim of this study was to establish whether androgen-elicited relaxation occurs in different species and to see if $\beta_2$-adrenoceptors-, polyamines- and calcium-dependent mechanisms were involved in this response, as was reported in the androgen-elicited cardiotonic effect (Rubin et al., 1999; Bordallo et al., 2001; Velasco et al., 2002; Farrar and Rodnick, 2004). Furthermore, we studied the plausibility of androgenic modulation of the contraction of airway smooth muscle, as was described in vascular smooth muscle (Greenberg et al., 1974; Herman et al., 1997) and in the ileum (Gonzalez-Montelongo et al., 2006).

## 2. Materials and methods

### 2.1. Isolation of tracheal guinea-pig rings and bovine strips

Two-month-old male guinea-pigs, weighing 350–400 g, from Charley River (Barcelona, Spain) were killed by exsanguination under anesthesia with pentobarbital (i.p.) (Directive 2003/65/CE and Spain RD 1201/2005). The experimental protocol was approved by the Institutional Ethics Committee of the University of Oviedo. The trachea was removed and placed in a Petri dish in Krebs solution (mM composition: NaCl, 118; KCl, 4.75; $CaCl_2$, 2.5; $KH_2PO_4$, 1.19; $NaHCO_3$, 25; $MgSO_4$, 1.2 and glucose, 11) and cut into rings of approximately 2 mm in width.

Bovine tracheal preparations were chosen for performing biochemical characterisations, since the gross dimension of this smooth muscle provides the opportunity to perform parallel biochemical assays. Bovine tracheal smooth muscle strips were obtained from healthy male cattle of the "Asturiana de los Valles" calf breed (Asturias, Spain) and were aged between 10 and 13 months. After the slaughter period (<90 min), tissue samples were taken from the lower half of the tracheae, and immersed in Krebs solution that had been pre-gassed with a mixture of 95% $O_2$ and 5% $CO_2$, and kept at 4 °C during transportation. They were brought to the laboratory within 30 min. Thereafter, the tracheae were opened longitudinally by cutting through the midline of the cartilage rings. Then, 4–6 transversal adjacent and similar rectangular segments (approximately 20×3 mm and 233.93±5.78 mg in weight) were obtained according to the method previously described and validated (Hashjin et al., 1995). Surrounding connective tissue was removed and special care was taken to respect the integrity of the epithelium.

The tracheal preparations were mounted in a 6 ml organ bath, containing Krebs solution at 37 °C and bubbled with a 95% $O_2$ and 5% $CO_2$ mixture, and were subjected to 0.5 g and 2 g of tension, for guinea-pig rings and bovine strips respectively. Isometric responses were measured on a Uni-graph 50 (Letica) polygraph through isometric transducers UF1 (Pioden Controls LTD). Tissues were allowed to stabilise for at least 1 h for tracheal guinea-pig rings and 2 h for bovine strips, before the beginning of each experiment. During this period the buffer solution was renewed every 30 min. In some tracheal samples, the epithelial layer was gently removed with a cotton-tipped applicator for guinea-pigs rings and dissected from the smooth muscle of bovine strips. In these experiments, an intact strip from the same animal was always used as a control. Epithelium removal was confirmed by histological observations.

### 2.2. Experimental procedure

After the stabilisation period the preparations were contracted by an addition of carbachol (0.3 μM) to the organ baths. Then, the drug was washed out by removing and replacing the incubation solution. After a 90 min interval, the effect of carbachol was studied in a cumulative manner (3 nM to 30 μM) or the trachea was contracted with a single concentration of carbachol to study the concentration-dependent relaxation of 5α-dihydrotestosterone, testosterone and 5β-dihydrotestosterone (1 to 100 μM). To be able to compare the effect of these androgens, they were studied simultaneously in three different strips obtained from the same animal, and each experiment was repeated at least in strips from 5 different animals.

The mechanisms involved in androgen-elicited relaxation were studied in bovine tracheal strips by adding suitable drugs before 5α-dihydrotestosterone (100 μM) was administered. The times of incubation were 5–10 min for the β-adrenoceptor antagonists and 30 min for the remaining drugs. The assayed concentrations were: 10 μM for the antiandrogen flutamide (Sanchez Aparicio et al., 1993; Tep-areenan et al., 2002); 1 μM for the β-adrenergic antagonist propranolol; 0.5 μM for the $\beta_2$-adrenergic antagonist ICI-118,551 (Bilski et al., 1983); 10 μM for the phosphodiesterase inhibitor IBMX (Beavo et al., 1970); 10 mM for the ornithine decarboxylase inhibitor α-difluoromethylornithine (Metcalf et al., 1978; Bordallo et al., 2001). Only one concentration–response curve, or a single administration, was performed in each preparation. For this, a pair of strips obtained from the same animal was always used. One preparation was used as a control and the other one was exposed to the drug.

The role of the $\beta_2$-adrenoceptor on 5α-dihydrotestosterone-elicited relaxation was also studied after adrenoceptor desensitisation, by incubating the preparations for 2 h with 30 μM salbutamol in carbachol (0.3 μM)-elicited raised tone. Then, the drug was washed out and 45 min later the tissue was contracted with carbachol (0.3 μM). The desensitisation was evaluated by means of the absence of relaxation to subsequent addition of salbutamol (10 μM).

The androgen effect on β-adrenoceptor mediated relaxation was studied by adding 5α-dihydrotestosterone (100 μM) into the organ bath 30 min before performing the concentration–response curve to salbutamol (1 nM to 30 μM).

The effect of the androgens, testosterone, 5α- and 5β-dihydrotestosterone (100 μM), was studied simultaneously in preparations, obtained from the same trachea, with KCl (80 mM)-raised tone, using a modified Krebs solution with 43 mM NaCl and 80 mM KCl, instead of 118 mM NaCl and 4.75 mM KCl. The influence of external calcium in the androgen-elicited relaxation was studied, by adding cumulative concentrations of $CaCl_2$ (3 to 10 mM) to the organ bath.

Furthermore, the effect of 5α-dihydrotestosterone (100 μM) on cumulatively concentration-dependent contraction by carbachol (0.1, 0.3 and 3 μM) was studied. To this end, two carbachol-elicited contractions were performed in pairs of tracheal strips. One of the preparations was incubated with 5α-dihydrotestosterone (100 μM), 30 min before performing the second cumulative contraction by carbachol (0.1, 0.3 and 3 μM). The second strip was used as a control. The effect of intracellular polyamines on carbachol-elicited contractions and on 5α-dihydrotestosterone (100 μM)-elicited relaxation was studied by incubating the preparations with α-difluoromethylornithine (10 mM) 30 min before the addition of the androgen to the organ bath.

### 2.3. Determination of intracellular cAMP levels in isolated bovine tracheal strips

After a stable contraction due to carbachol (0.3 μM) (control) or acute exposure (15 min) to 5α-dihydrotestosterone (100 μM) and salbutamol (3 μM), either in the absence or the presence of an inhibitor of phosphodiesterases, IBMX (10 μM, for 30 min), the bovine tracheal strips were immediately removed, placed in liquid nitrogen and preserved at −80 °C until used. To determine cAMP levels, the tissues were homogenised using a Polytron in buffer containing 4 mM EDTA (to prevent enzymatic degradation of cAMP) and the proteins

were coagulated with perchloric acid (10–15%, at 4 °C). The extracts were centrifuged at 18,000 ×g for 15 min. Cyclic AMP in the supernatant (pH adjusted with $NaHCO_3$) was assayed by means of a [$^3$H]AMP radioassay kit following the manufacturer's protocol (GE HealthCare). Cyclic AMP levels are expressed as pmol mg protein$^{-1}$.

### 2.4. Binding assay in bovine tracheal membranes

The method was essentially performed, with modification, as described by Hartmann et al. (1995). Briefly, the smooth muscles were cut into small pieces and gently homogenised in ice-cold buffer (sucrose 0.2 M, Tris 200 mM, pH 7.4). The homogenate was centrifuged at 700 ×g (15 min) and the supernatant was spun at 10,000 ×g (15 min) and then at 40,000 ×g (30 min). The membrane pellet was resuspended in buffer (mM: Tris 50, $MgCl_2$ 10; pH 7.4) at a final protein concentration of 1 mg/ml. All processes were performed at 4 °C. Aliquots were frozen in liquid nitrogen and stored at −80 °C.

Binding assays were carried out in 500 µl, containing 100 µg of membrane protein and buffer, incubated at 30 °C for 20 min. Tracheal smooth muscle $\beta_2$-adrenoceptor densities (*Bmax*) were estimated in saturation experiments using [$^3$H]dihydroalprenolol and specific binding was defined as the portion displaceable by ICI-118,551 (0.5 µM). 5α-Dihydrotestosterone binding displacement experiments were performed using increasing concentrations, 0.1 to 100 µM, in the presence of [$^3$H]dihydroalprenolol (1 nM). The bound ligand was separated by rapid vacuum filtration through Whatman GF/C filters. Radioactivity was determined by liquid scintillation. All experiments were conducted in triplicate and repeated independently at least four times.

### 2.5. Drugs

The following drugs were used: 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), 5β-dihydrotestosterone (17β-hydroxy-5β-androstan-3-one), carbachol (carbamylcholine chloride), flutamide (2-methyl-N-(4-nitro-3-[trifluoromethyl]phenyl)propanamide), IBMX (3-isobutyl-1-methylxanthine), ICI-118,551 hydrochloride ((±)-1-[(2,3-dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol hydrochloride), propranolol (1-[isopropylamino]-3-[1-naphthyloxy]-2-propanolol), salbutamol (α-[(t-butylamino) methyl]-4-hydroxy-m-xylene-α,α′-diol) and testosterone (17β-hydroxy-4-androsten-3-one) from Sigma. α-Difluoromethylornithine (DL-α-difluoromethylornithine) was donated by Dr. Wooster (Wayne University, USA). [$^3$H]dihydroalprenolol was from Amersham.

Testosterone, 5α-, 5β-dihydrotestosterone, flutamide and IBMX were dissolved in dimethyl sulfoxide. The effect of the solvent was studied in several preparations, at concentrations in the organ bath ≤0.1%, it was ineffective, and consequently this was the maximum concentration used. Salbutamol, propranolol, ICI-118,551 and α-difluoromethylornithine were dissolved in purified water with a resistance of 10–15 MΩ cm.

### 2.6. Calculation and statistical analysis

The tracheal contractions to carbachol (3 nM to 30 µM) were expressed as a percentage of the maximum contraction to this agonist (*Emax*), considered as 100%. The effects of testosterone, 5α- and 5β-dihydrotestosterone were expressed as a percentage of relaxation due to carbachol or KCl-elicited contractions, considered as 100% when the baseline was reached. The half effective concentration ($EC_{50}$) of carbachol-elicited contractions and the half inhibitory concentration ($IC_{50}$) of androgen-elicited relaxation in carbachol (0.1 µM)-elicited raised tone, were calculated by fitting the concentration–response curves with the Hill equation (Igor Pro V5.0, WaveMetrics Inc., USA).

To analyse the time constant ($\tau$) of relaxation to androgens, the recording was scanned, digitalised (GetData Graph Digitizer 2.22) and fitted to a single exponential equation (Igor Pro V5.0, WaveMetrics Inc., USA).

The effects of epithelium removal on carbachol (3 µM)-elicited contractions and 5α-dihydrotestosterone (100 µM)-elicited relaxations in carbachol (0.1, 0.3 or 3 µM)-raised tone were expressed as g of contraction/g of tissue weight.

The data obtained were expressed as the mean±S.E.M. for a number ($n$) of at least 5 different animals in each case. Statistical significance was calculated by means of the Student's $t$ test for unpaired values, considering $P<0.05$ as significant.

## 3. Results

### 3.1. Effect of carbachol (3 nM to 30 µM) in preparations of guinea-pig and bovine trachea

Carbachol (3 nM to 30 µM) elicited a concentration-dependent contraction of airway smooth muscle preparations of guinea-pig and bovine trachea. The $ED_{50}$s were 0.58±0.043 µM and 0.28±0.018 µM, respectively. In our experimental conditions, in guinea-pig trachea 0.3 µM carbachol elicited approximately 40% of the maximum contraction, and in bovine trachea 0.1, 0.3 and 3 µM of carbachol elicited approximately 30%, 50% and 88% of the maximum effect, respectively (Fig. 1).

In both preparations, the response to carbachol (3 µM) was higher in the absence of epithelium than in its presence, being, for guinea-pig 170.34±28.6 vs. 126.62±18.91 g contraction/g tissue weight and for bovine trachea 38.76±5.58 vs. 28.05±5.74 g contraction/g tissue weight, respectively.

### 3.2. Effect of 5α-dihydrotestosterone (1–300 µM) on the carbachol (0.3 µM)-raised tone of guinea-pig trachea

Acute exposure to 5α-dihydrotestosterone, 1 to 300 µM, elicited a concentration-dependent relaxation of guinea pig tracheal rings whose tone was raised by carbachol (0.3 µM). Higher concentrations were not used and, consequently, we cannot accurately estimate the maximum effect of 5α-dihydrotestosterone-elicited relaxation, although, based on the concentrations assayed, the estimated $ED_{50}$ was 119.59±15.10 µM (Fig. 2).

This response was reproducible at a 1 h interval between successive exposures to 5α-dihydrotestosterone (100 and 300 µM), and was not affected by epithelium removal.

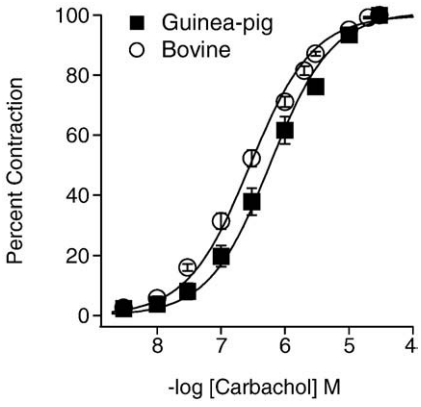


**Fig. 1.** Concentration–response curves of carbachol (3 nM to 30 µM) on guinea-pig tracheal rings and bovine tracheal strips. Data are expressed as the percentage of the maximal contractile response (100%). The symbols are experimental results, vertical bars represent S.E.M., and the solid lines are the data fit to the Hill equation.

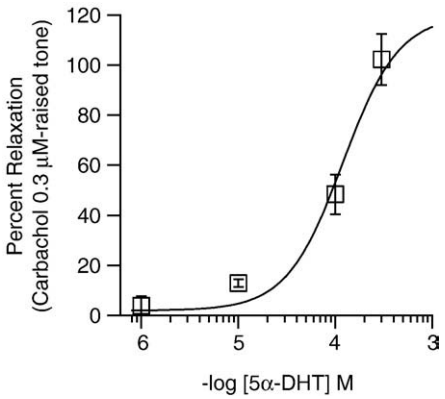


**Fig. 2.** Concentration-dependent relaxant effect of 5α-dihydrotestosterone (DHT, 1 to 300 μM) on guinea-pig tracheal rings precontracted with carbachol (0.3 μM). The values are expressed as a percentage of the maximum relaxation, 100% when baseline is reached. Each point represents the mean ± S.E.M. and the solid lines are the data fit to the Hill equation.

### 3.3. Effect of 5α-dihydrotestosterone (100 μM) on precontracted bovine tracheal strips by carbachol (0.1, 0.3 or 3 μM)

5α-Dihydrotestosterone (100 μM) elicited relaxation of bovine tracheal strips precontracted with 0.1, 0.3 or 3 μM of carbachol. The relaxation was partially reversed (~30%) and maintained as long as the 5α-dihydrotestosterone was present in the organ bath (Fig. 3A). The effect was reproducible by adding the drug at 1 h intervals.

The amplitude of relaxation was inversely proportional to the magnitude of the contraction reached by the preparations, being significantly smaller when they were contracted with 3 μM carbachol. This was demonstrated by plotting the effect in g contraction/g tissue weight (Fig. 3B).

### 3.4. Concentration-dependent relaxation due to androgens (1 to 100 μM) on carbachol (0.1 μM)-raised tone in bovine tracheal strips

The concentration-dependent effect of several androgens (1 to 100 μM) was studied in carbachol (0.1 μM)-raised tone. At 100 μM the time to reach the maximum relaxation was similar for the androgens studied, about 11–13 min and a $\tau$ of 3–4 min. Testosterone (100 μM) was more effective ($P<0.001$) than 5α- and 5β-dihydrotestosterone at eliciting relaxation. The estimated $ED_{50}$s were 61.54 ± 4.32 μM, for testosterone, 150.58 ± 3.98 μM for 5α-dihydrotestosterone and 149.81 ± 15.6 μM for 5β-dihydrotestosterone (Fig. 3C).

The removal of epithelium did not significantly modify the response to androgens. The percentages of relaxation were, in the presence or the absence of epithelium respectively, 57.95 ± 4.97 and 60.06 ± 6.19% for testosterone, 32.07 ± 5.85 and 33.25 ± 4.14 for 5α-dihydrotestosterone and 36.76 ± 3.21 and 33.34 ± 4.7 for 5β-dihydrotestosterone.

### 3.5. Effect of flutamide (10 μM) on 5α-dihydrotestosterone- and testosterone (100 μM)-elicited relaxations in bovine tracheal strips precontracted with carbachol (0.3 μM)

Incubation with an antiandrogen, flutamide (10 μM), for 30 min, neither modified carbachol (0.3 μM)-raised tone nor 5α-dihydrotestosterone or testosterone (100 μM)-elicited relaxation in bovine tracheal strips. The percentage of relaxation for 5α-dihydrotestosterone (100 μM) was 24.48 ± 5.16% and 25 ± 6.53%, respectively, in the

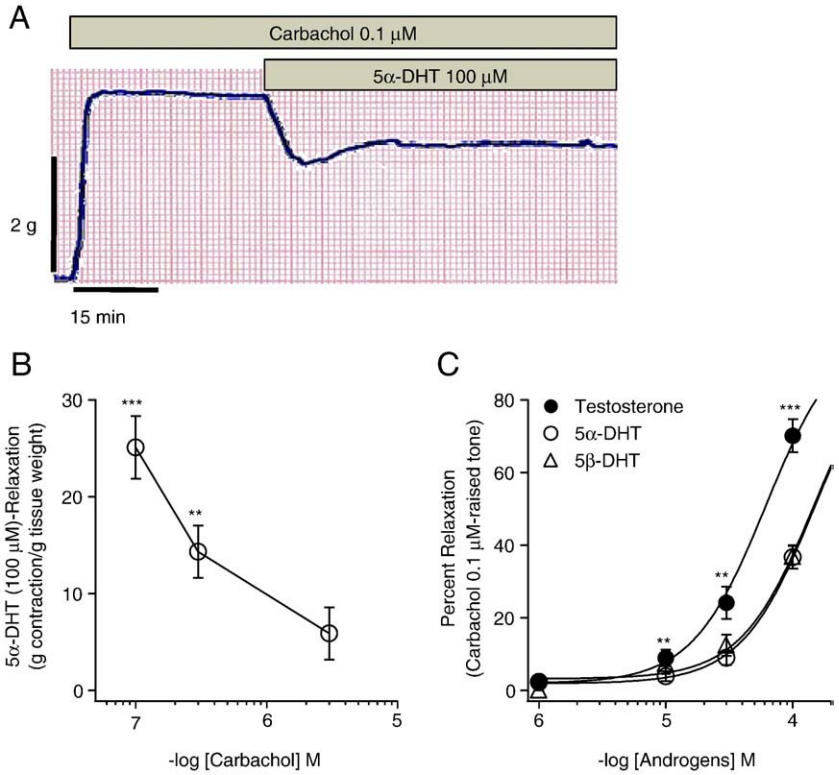


**Fig. 3.** (A) Recording of 5α-dihydrotestosterone (DHT, 100 μM)-elicited relaxation of bovine tracheal strips precontracted with carbachol 0.1 μM. (B) Plot of 5α-dihydrotestosterone (DHT) (100 μM)-elicited relaxation precontracted with carbachol (0.1, 0.3 or 3 μM), expressed as g relaxation/g tissue weight. $^{**}P<0.01$ and $^{***}P<0.001$ vs. carbachol 3 μM. (C) Concentration-dependent curves of 5α-dihydrotestosterone (DHT), testosterone and 5β-dihydrotestosterone (5β-DHT) (1 to 100 μM) in bovine tracheal strips precontracted with carbachol (0.1 μM). Each point represents the mean ± S.E.M. $^{**}P<0.01$ and $^{***}P<0.001$ by means of Student's *t*-test by comparing the effect of the androgens in carbachol (0.1 μM)-elicited contraction.

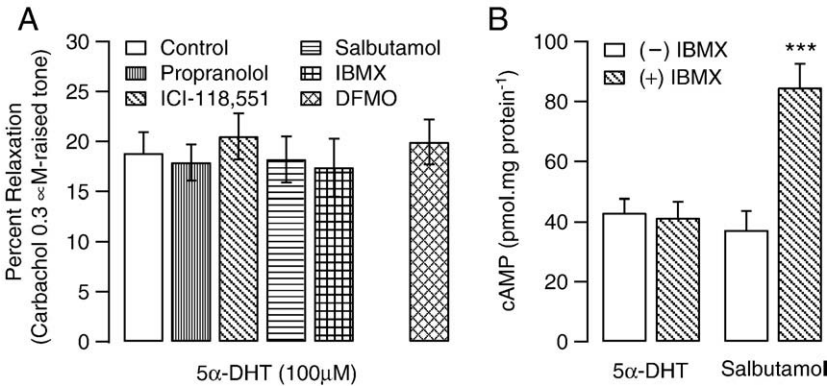


**Fig. 4.** (A) Effect of propranolol (1 μM), ICI-118,551 (0.5 μM), $\beta_2$-adrenoceptors desensitisation by 2 h exposure to salbutamol (30 μM), IBMX (10 μM) and α-difluoromethylornithine (DFMO, 10 mM) on 5α-dihydrotestosterone (DHT, 100 μM)-elicited relaxation of bovine tracheal strips precontracted by carbachol (0.3 μM). (B) Cyclic AMP levels after exposure to 5α-dihydrotestosterone (100 μM)- and salbutamol (3 μM) in carbachol (0.3 μM)-raised tone, in the absence or presence of IBMX (10 μM). Each point represents the mean±S.E.M. ***$P<0.001$ by means of Student's *t*-test by comparing the levels of cAMP in the absence and the presence of IBMX (10 μM).

absence and the presence of flutamide (10 μM), and for testosterone 35.11±3.85% and 34.94±5.07%.

### 3.6. Effect of β-blockers, $\beta_2$-adrenoceptors desensitisation, IBMX and α-difluoromethylornithine on 5α-dihydrotestosterone (100 μM)-elicited relaxation in bovine tracheal strips precontracted with carbachol (0.3 μM)

5α-Dihydrotestosterone (100 μM)-elicited relaxation was not modified by the incubation with the nonselective β-adrenoceptor antagonist, propranolol (1 μM), or by the selective $\beta_2$-adrenoceptor antagonist, ICI-118,551 (0.5 μM). Neither was it affected by the desensitisation of $\beta_2$-adrenoceptors, via long-term (2 h) exposure to the agonist, salbutamol (30 μM), or by incubation with IBMX (10 μM), an inhibitor of phosphodiesterases (Fig. 4). Furthermore, the β-adrenoceptor antagonists did not reverse 5α-dihydrotestosterone (100 μM)-elicited relaxation, as they did in salbutamol (10 μM) relaxed strips.

In addition, the incubation with α-difluoromethylornithine (10 mM), 30 min before 5α-dihydrotestosterone (100 μM), did not antagonise the relaxation due to the androgen (Fig. 4A).

### 3.7. Effect of 5α-dihydrotestosterone (100 μM) on cAMP levels in bovine tracheal strips

Concentrations of intracellular cAMP were determined in bovine tracheal strips, precontracted by carbachol (0.3 μM) and after 5α-dihydrotestosterone (100 μM)-elicited relaxation, in the absence or the presence of IBMX (10 μM). The assay was validated measuring the levels of cAMP elicited by the known agonist of $\beta_2$-adrenoceptors, salbutamol (3 μM).

The incubation for 30 min with IBMX (10 μM) did not modify intracellular levels of cAMP in bovine tracheal strips precontracted with carbachol (0.3 μM), 43.02±4.59 vs. 41.31±5.4 pmol mg protein$^{-1}$. The addition of salbutamol (3 μM) without IBMX did not significantly modify intracellular cAMP levels. However, levels of cAMP were increased in the presence of IBMX plus salbutamol (Fig. 4B).

5α-Dihydrotestosterone (100 μM) did not significantly modify cAMP levels in these preparations (Fig 4B).

### 3.8. Effect of 5α-dihydrotestosterone (100 μM) on salbutamol (3 nM to 10 μM)-elicited relaxation in bovine tracheal strips precontracted with carbachol 0.3 and 3 μM

To study a putative functional interaction between 5α-dihydrotestosterone and $\beta_2$-adrenoceptor activation, the androgen (100 μM) was added to the organ bath 30 min before salbutamol-elicited relaxation curves, in preparations whose tone was raised by carbachol 0.3 or 3 μM, were measured.

The concentration–response curve of relaxation to salbutamol (1 nM to 30 μM) showed $ED_{50}$s values of 64.06±2.3 and 194.15±17.6 nM ($P<0.01$) for carbachol 0.3 μM- and 3 μM-raised tone, respectively. No significant differences were observed in the maximum relaxation to salbutamol, using either of the experimental conditions.

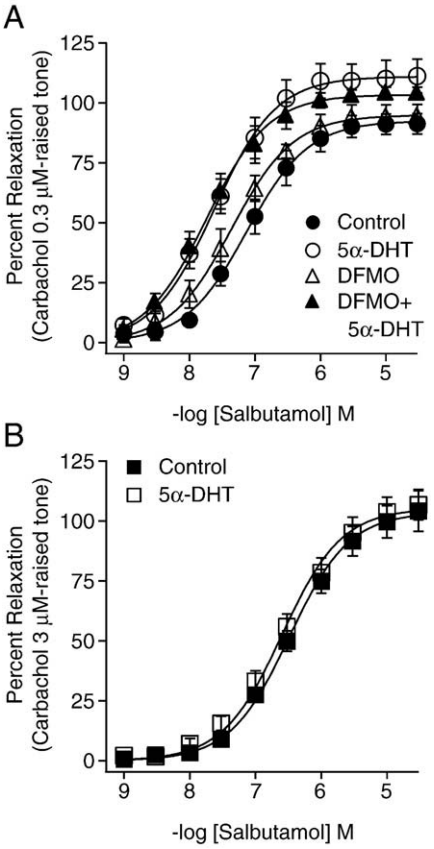


**Fig. 5.** (A) Effect of 5α-dihydrotestosterone (DHT, 100 μM), α-difluoromethylornithine (DFMO, 10 mM) and 5α-dihydrotestosterone plus α-difluoromethylornithine (5α-DHT+DFMO) on salbutamol (1 nM to 30 μM)-elicited relaxation in bovine tracheal strips precontracted with 0.3 μM carbachol. (B) Effect of 5α-dihydrotestosterone (DHT, 100 μM) on salbutamol (1 nM to 30 μM)-elicited relaxation in bovine tracheal strips precontracted with 3 μM carbachol. Each point represents the mean±S.E.M.

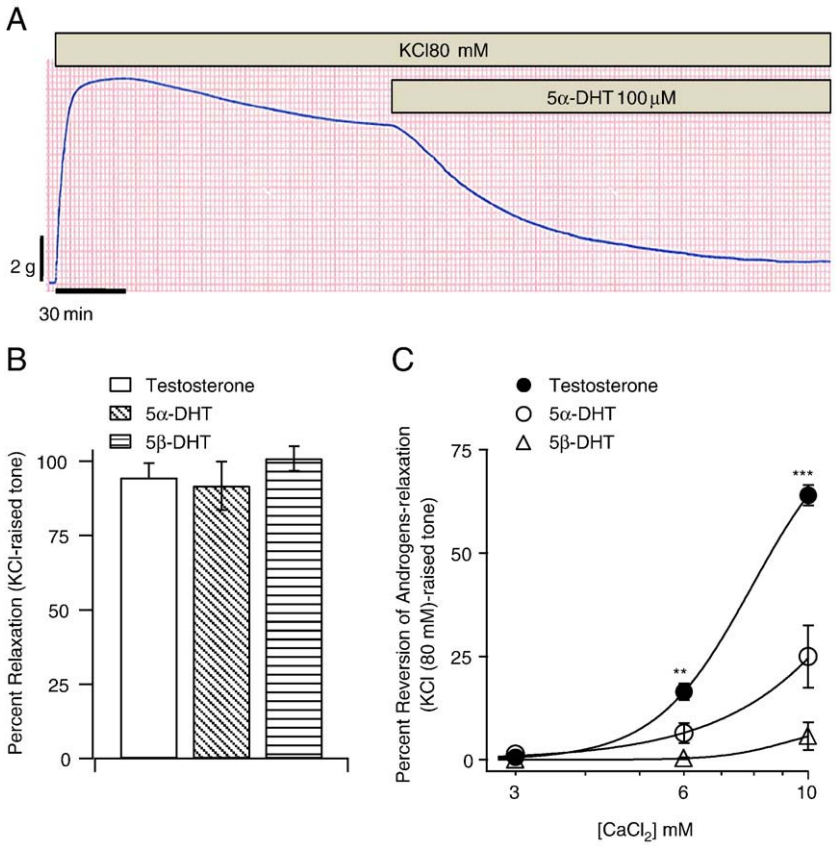


**Fig. 6.** (A) Recording of 5α-dihydrotestosterone (DHT, 100 μM)-elicited relaxation of bovine tracheal strips precontracted with KCl (80 mM). (B) Testosterone-, 5α-, and 5β-dihydrotestosterone (100 μM)-elicited relaxation on bovine trachea precontracted with KCl (80 mM) and (C) its percentage of reversion by extracellular $Ca^{2+}$ increase (3 to 10 mM). Each point represents the mean±S.E.M. $^{**}P<0.01$ and $^{***}P<0.001$ by means of Student's *t*-test, by comparing the effect of testosterone vs. 5α- or 5β-DHT.

The acute exposure to 5α-dihydrotestosterone (100 μM) shifted to the left the concentration–response curve of salbutamol (1 nM to 30 μM) reducing the $ED_{50}$ from 73.42±3.33 nM to 23.54±1.32 nM ($P<0.01$) and increased the maximum relaxation from 91.26±4.25% to 111.11±7.1%, when the bovine trachea was contracted with carbachol (0.3 μM) (Fig. 5A). Using the same experimental conditions, α-difluoromethylornithine (10 mM) had no significant effect on the concentration–response curve of salbutamol (1 nM to 30 μM), performed in the presence or absence of 5α-dihydrotestosterone (100 μM) (Fig. 5A).

In contrast, 5α-dihydrotestosterone (100 μM) did not exert a significant effect when the preparations were contracted with 3 μM carbachol (Fig. 5B).

### *3.9. Effect of 5α-dihydrotestosterone on [$^3$H]dihydroalprenolol binding to bovine tracheal membranes*

To evaluate the possibility of androgen binding to $\beta_2$-adrenoceptors, displacement experiments were performed using increasing concentrations of 5α-dihydrotestosterone (0.1 to 100 μM) in the presence of the specific ligand, [$^3$H]dihydroalprenolol (1 nM). Binding of the radiolabelled ligand to bovine tracheal membranes was saturating (0.1 to 10 nM) and the Scatchard plot showed a receptor density of 0.3 pmol/mg of protein and a $K_D$ of 0.75 nM. The competition assay showed no significant displacement of the ligand by 5α-dihydrotestosterone, binding of which was 98.01±3.2% of the specific binding at 100 μM.

### *3.10. Effect of testosterone, 5α- and 5β-dihydrotestosterone (100 μM) on bovine tracheal strips precontracted with KCl (80 mM)*

KCl (80 mM) elicited a stable contraction of bovine tracheal strips, which was 86.54±5.67% of that produced by carbachol (3 μM) in the same preparation. For this experimental condition, the androgens elicited a relaxing effect greater than 90% with time constants much slower than those produced in carbachol-elicited raised tone (Fig. 6A and B). The response fits a single exponential, with a time constant ($\tau$) for testosterone (20.59±2.80 min)<5β-dihydrotestosterone (31.43±3.91 min)<5α-dihydrotestosterone (36.72±4.12 min).

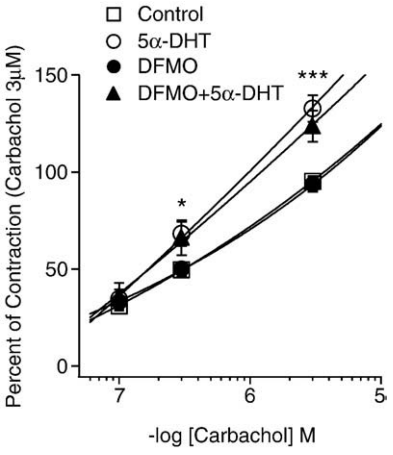


**Fig. 7.** Effect of 5α-dihydrotestosterone (DHT, 100 μM), α-difluoromethylornithine (DFMO, 10 mM) and 5α-dihydrotestosterone plus α-difluoromethylornithine (5α-DHT +DFMO) on carbachol (0.1 to 3 μM)-elicited contractions in bovine tracheal strips, the contraction produced by 3 μM of carbachol in the control curve was considered as 100%. Each point represents the mean±S.E.M. $^{*}P<0.05$ and $^{***}P<0.001$ vs. control by means of Student's *t*-test.

The addition of increasing extracellular $CaCl_2$ (3 to 10 mM) reversed significantly the relaxing effect of testosterone and weakly that of 5α-dihydrotestosterone. But almost had no effect when the tissue was relaxed with 5β-dihydrotestosterone (Fig. 6C).

### *3.11. Effect of 5α-dihydrotestosterone (100 μM) on carbachol (0.1, 0.3 and 3 μM)-elicited contractions in bovine tracheal strips*

Preincubation with 5α-dihydrotestosterone (100 μM), 30 min before performing the second cumulative contraction with carbachol (0.1, 0.3 and 3 μM), significantly facilitated their contractile response. This effect was unmodified in the presence of α-difluoromethylornithine (10 mM) (Fig. 7).

## 4. Discussion

It has been reported that testosterone elicits relaxation of rabbit airway smooth muscle by nongenomic mechanisms (Kouloumenta et al., 2006). Similarly, our results show that the androgen 5α-dihydrotestosterone produces an acute and maintained relaxation of tracheal guinea-pig rings and bovine strips. This androgen, a 5α-reductase active derivate of testosterone, is not a substrate for aromatase (Veldhuis, 1991). Consequently, the potential estrogenic effects of testosterone may be disregarded using this compound. This is an important issue, since it has been reported that estrogens modulate airway smooth muscle tone (Foster et al., 1983; Pang et al., 2002; Degano et al., 2003).

5α-Dihydrotestosterone elicited an epithelium independent relaxation of guinea-pig and bovine trachea with carbachol 0.3 μM-raised tone, a parasympathetic agonist. In our experimental conditions, 5α-dihydrotestosterone seemed to be more effective in guinea-pig than bovine trachea. As discussed below, the differences might be related to the previous tone of the tissues, since carbachol-elicited contraction was slightly more potent in bovine trachea than in guinea-pig, which may determine the magnitude of the response to 5α-dihydrotestosterone. In any case, the response was similar in both preparations, a fact that led us to choose the bovine trachea to further characterise the mechanisms of action of androgens. In addition, due to the gross dimensions of the smooth muscle, the performance of biochemical assays is relatively easier.

The study was carried out at pharmacological concentrations of androgens, which have also been required to elicit acute effects in most isolated tissues of animals (Yue et al., 1995; Costarella et al., 1996; Chou et al., 1996; Rosano et al., 1999; Tep-areenan et al., 2002; Jones et al., 2003) and humans (Malkin et al., 2006; Perusquia et al., 2005, 2007).

The fact that testosterone elicited nongenomic relaxation of bovine trachea and rabbit (Kouloumenta et al., 2006) suggests a general effect of androgens on airway smooth muscles. The order of potency of the androgens studied in carbachol-raised tone was testosterone > 5α-dihydrotestosterone = 5β-dihydrotestosterone. The equiactive effect of the inactive isomer, 5β-dihydrotestosterone, pointed out a different structure–activity relationship than that of the intracellular androgen receptor (Fang et al., 2003). These findings, in addition to the absence of latency in the initiation of the response and the lack of effect of the antiandrogen flutamide, on testosterone and 5α-dihydrotestosterone-elicited relaxation, excluded the involvement of genomic mechanisms in this effect, as previously reported (Kouloumenta et al., 2006).

Nevertheless, our results disagree with the proposed role of epithelium on testosterone-elicited relaxation in rabbit airways (Kouloumenta et al., 2006), as epithelium removal did not modify androgen relaxation in bovine trachea. A possible explanation for these discrepancies could be the absence of a functional epithelium in bovine tracheal preparations. However, this seems not to be the case, since with the removal of the epithelium the response to carbachol was increased, similarly to what has been described in other studies (Folkerts and Nijkamp, 1998).

It is interesting that the magnitude of 5α-dihydrotestosterone and testosterone relaxation depended on the previous tone of bovine tracheal smooth muscle, being more effective when the tone was raised close to the established resting tension, and the effect tended to be counteracted when the contraction of the preparation approached the maximum response to carbachol.

Pharmacological and biochemical evidence suggested that cAMP-dependent mechanisms were associated with the 5α-dihydrotestosterone-elicited acute cardiotonic effect in isolated left atrium of the rat. This was a postsynaptic effect, pertussis toxin-sensitive and required a functional β-adrenoceptor system that might be modulated by intracellular second messengers, such as polyamines (Rubin et al., 1999; Bordallo et al., 2001; Velasco et al., 2002). This was the reason to study the existence of a similar coupling in tracheal smooth muscle which could be compatible with an androgen-elicited spasmolytic effect, since it is well known that cAMP-dependent mechanisms relax airway smooth muscles (Russell, 1986). However, the pharmacological characterisation of the mechanisms involved showed that 5α-dihydrotestosterone-elicited relaxation was not modified by a non-selective β-adrenoceptor antagonist, propranolol, or the selective $\beta_2$-adrenoceptor antagonist, ICI-118,551. The effect was neither reversed by the administration of propranolol, as on salbutamol-elicited relaxation, nor $\beta_2$-adrenoceptor desensitisation or the incubation with IBMX. It was not associated with changes in intracellular cAMP levels. These results excluded the possibility of the 5α-dihydrotestosterone activation of $\beta_2$-adrenoceptors and the involvement of cAMP-dependent mechanisms on bovine trachea relaxation.

However, the incubation with 5α-dihydrotestosterone shifted to the left salbutamol-relaxation, this effect also depended on the previous tone of the trachea in a similar way to that mentioned above for the direct effect of 5α-dihydrotestosterone on carbachol-raised tone. This means that in the presence of 5α-dihydrotestosterone, lower concentrations of salbutamol may be required to produce similar degrees of airway relaxation in increased tone close to the resting tension. The facilitation of salbutamol-elicited relaxation seemed not to be related to an interaction with $\beta_2$-adrenoceptors, since 5α-dihydrotestosterone did not displace the radiolabel [$^3$H] dihydroalprenolol from these receptors.

Intracellular polyamines have been implicated in the androgen-elicited cardiotonic effect in rat (Rubin et al., 1999; Bordallo et al., 2001) and trout heart (Farrar and Rodnick, 2004) and in the 5α-dihydrotestosterone modulation of $\beta_1$-adrenoceptor-mediated effect in the rat heart (Bordallo et al., 2001). However, these polyamines neither mediate 5α-dihydrotestosterone-elicited relaxation nor facilitate salbutamol-elicited relaxation in bovine trachea, as indicated by the lack of effect by an inhibitor of ornithine decarboxylase, α-difluoromethylornithine (Metcalf et al., 1978).

The final mechanisms explored were the roles of $K^+$ and $Ca^{2+}$ permeability in androgen-elicited relaxation in bovine trachea. Ionic permeabilities to $K^+$ and $Ca^{2+}$ are involved in androgen relaxation in a variety of species and blood vessels (Jones et al., 2003), including human arteries (Yildiz et al., 2005; Perusquia et al., 2005; Malkin et al., 2006; Montaño et al., 2008; Cairrao et al., 2008), and in nonvascular smooth muscles (Sanchez Aparicio et al., 1993; Lafayette et al., 2008). The androgens assayed, testosterone, 5α- and 5β-dihydrotestosterone, elicited relaxation of bovine trachea with KCl (80 mM)-raised tone, suggesting that an increase in $K^+$ permeability is not the main mechanism of relaxation. As under this experimental condition, when the cell is depolarised by an increased extracellular $K^+$, the membrane potential approaches the equilibrium potential for $K^+$ and the opening of $K^+$ channels will then have little influence on membrane potential (Quast, 1993), and, consequently, on tracheal smooth muscle tone.

Our results do not agree with previous studies on testosterone-elicited relaxation of rabbit airway smooth muscle, where testosterone

had no effect on KCl-raised tone (Kouloumenta et al., 2006). This discrepancy might be due to the different species studied or for the methods used, in particular the length of the recording of the response. This was longer in our study, conditioned by the fact that in high KCl the relaxation due to the androgens was much slower than that observed in carbachol-raised tone. Therefore, this time should not be taken as reference. Despite the slow time course of the relaxation, it is highly unlikely to be due to genomic mechanisms, as suggested by the fact that it fitted to a single exponential, which implies the existence of one component in the mechanism of action.

The magnitude of the effect was similar for the three compounds, but with differences in the time constants, those being testosterone < 5β-dihydrotestosterone ≤ 5α-dihydrotestosterone. Since the KCl-elicited contraction was significantly more sensitive to androgen-elicited relaxation than that of carbachol, and the contraction of KCl was mainly due to extracellular $Ca^{2+}$ influx, the results suggested a preferential mechanism of blockade of voltage-operated $Ca^{2+}$ channels. Furthermore, the relaxations were partially reversed by increasing external $Ca^{2+}$, especially to testosterone, but not to 5β-dihydrotestosterone. This fact might be related to a slower off rate with 5β-dihydrotestosterone with regards to testosterone and 5α-dihydrotestosterone. Androgen inhibition of L-type $Ca^{2+}$ channel current was reported in functional studies (Perusquia et al., 1996, 2005, 2007; Perusquia and Villalon, 1999; Crews and Khalil, 1999; Jones et al., 2002; Montaño et al., 2008) and also confirmed by electrophysiological recordings of ion channel currents (Scragg et al., 2004, 2007; Hall et al., 2006; Montaño et al., 2008) and/or intracellular $Ca^{2+}$ measurements (Perusquia et al., 2005; Montaño et al., 2008). However, unlike with vascular smooth muscle (Perusquia et al., 2005; Perusquia et al., 2007; Montaño et al., 2008) 5β-dihydrotestosterone was not more potent than testosterone eliciting relaxation of bovine trachea, and was similar to 5α-dihydrotestosterone. However, as hypothesised in vascular smooth muscle (Perusquia et al., 2005; Montaño et al., 2008), 5β-dihydrotestosterone, which lacks hormonal action (Fang et al., 2003), may be biologically active on airway smooth muscle.

On the other hand, androgens facilitated carbachol-elicited contractions of bovine trachea strips. This is an interesting finding since 5α-dihydrotestosterone caused relaxation of this tissue. In this sense, it has also been described that androgens increase the amplitude of carbachol-elicited contractions in mouse ileum (Gonzalez-Montelongo et al., 2006) and that male mice are more sensitive to cholinergic airway responsiveness (Card et al., 2006). The latter has not been related to differences in innate responsiveness of airway smooth muscle but via vagally mediated reflex mechanism (Card et al., 2007).

In summary, this study indicates the importance of testosterone and its metabolites as potential physiological modulators of airway smooth muscle tone, facilitating the effect of agonists of the contraction and the relaxation when tone is raised. This may also be of importance regarding gender differences in the physiology and the pathogenesis of respiratory diseases (Cistulli et al., 1994; De Marco et al., 2000; Caracta, 2003; Carey et al., 2007).

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