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Increases in ornithine decarboxylase activity in the positive inotropism induced by androgens in isolated left atrium of the rat

Carmen Bordallo [a,b], José Manuel Rubín [c], Ana Belén Varona [c], Begoña Cantabrana [b,c], Agustín Hidalgo [b,c], Manuel Sánchez [b,c,*]

[a] *Departamento de Bioquímica y Biología Molecular, Universidad de Oviedo, Oviedo, Spain*
[b] *Instituto Universitario de Oncología del Principado de Asturias, Asturias, Spain*
[c] *Laboratorio de Farmacología (Departamento de Medicina), Universidad de Oviedo, Oviedo, Spain*

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Abstract

It is well established that the intracellular receptors of androgens act as transcription factors upon their activation by androgen binding. However, a growing number of studies have associated androgens with rapid biological responses independent of their classical action mechanism. In this sense, 5α- and 5β-dihydrotestosterone elicited a rapid positive inotropism in the isolated left atrium of the rat via cAMP-dependent mechanisms that may involve genomic effects. In addition, polyamines are mediators of several biological actions including those acute and long-term effects induced by androgens in the heart. The present study analyzed the role of polyamine synthesis in the cardiotonic effect of androgens in the left atrium of male Wistar rats, electrically stimulated (0.5 Hz, 5 ms and supramaximal voltage) and placed in an organ bath in 10 ml of Tyrode's solution. Incubation in the organ bath with an inhibitor of ornithine decarboxylase activity, α-difluoromethylornithine 10 mM, significantly decreased the positive inotropism induced by 5α- and 5β-dihydrotestosterone (0.1–100 μM). This suggests that ornithine decarboxylase seems to be involved in androgen-induced positive inotropism. Furthermore, 6-min exposure to 5α- or 5β-dihydrotestosterone significantly increased the activity of ornithine decarboxylase from 61.81 ± 7.53 (control) to 93.28 ± 9.45 and 80.28 ± 12 pmol/h/mg of protein, respectively. Northern blot analysis showed that 5α- and 5β-dihydrotestosterone did not modify the level of expression of the ornithine decarboxylase gene. Therefore, our results suggest that polyamine synthesis might be involved in the positive inotropism elicited by androgens through the stimulation of ornithine decarboxylase activity without changes in the expression of the ornithine decarboxylase gene.

Keywords: Atrial function; Enzyme; Hormone; Inotropic agent; Signal transduction

1. Introduction

Molecular alternative binding sites of steroids, including androgens, are assumed to be located in the plasma membrane of several cell types (Wehling, 1997; Razandi et al., 1999). Through these, steroids may exert rapid effects on cellular activity that cannot be explained exclusively by genomic mechanisms (Schumacher, 1990; Wong and Moss, 1992). Interestingly, androgens may produce beneficial hemodynamic effects on the clinical parameters of myocardial ischemia by modulating coronary tone and by relaxation of the aorta (Yue et al., 1995), which reduces afterload. Besides this indirect effect on cardiac performance, the androgens, 5α- and 5β-dihydrotestosterone, induce a direct positive inotropism in the isolated left atrium of the rat (García Valencia et al., 1992). This effect may be synergistic to the vascular effect improving cardiac output. Based on the characteristics of the response, the effect was initially interpreted as non-genomic (García Valencia et al., 1992). However, the fact that the incubation with an inhibitor of transcription, actinomycin D, and an inhibitor of protein synthesis, cycloheximide, antagonized the positive inotropism suggests that genomic mechanisms may be involved in the acute response to androgens (Rubín et al., 1999).

Polyamines are mediators of several biological actions (Marton and Pegg, 1995) including those induced by androgens in the heart. Thus, they are involved in different types of experimental cardiac hypertrophies such as that induced by isoproterenol (Bartolome et al., 1982; Nakano et al., 1995), clenbuterol (Cubria et al., 1998), thyroxine

* Corresponding author. Farmacología, Facultad de Medicina, Julian Claveria, Oviedo 33006, Spain. Tel.: +34-985103550; fax: +34-985232255.

E-mail address: sanchezf@correo.uniovi.es (M. Sánchez).

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(Tipnis and Skiera, 1989), angiotensin II (Ibrahim et al., 1995) and androgen treatment (Malhotra et al., 1990; Marsh et al., 1998) or by compensation for arterial hypertension (Ruskoaho and Raunio, 1987) or aortic coarctation (Lipke et al., 1997) since a specific irreversible inhibitor of ornithine decarboxylase, α-difluoromethylornithine (Metcalf et al., 1978), prevents hypertrophy (Bartolome et al., 1982; Lipke et al., 1997; Cubria et al., 1998). In addition to these long-term effects, polyamines are mediators of acute effects caused by androgens, such as Ca^{2+} fluxes and Ca^{2+}-dependent membrane transport (hexose transport and aminoacids) in the rat heart (Koenig et al., 1989). Furthermore, they are also involved in functional responses, as α-difluoromethylornithine inhibited the positive inotropism elicited by ouabain, noradrenaline and Ca^{2+} (Bazzani et al., 1988).

The effect of androgens on polyamine synthesis may be produced at different levels, such as activation of the expression of the ornithine decarboxylase gene (Kontula et al., 1984; Melanitou et al., 1987; Manteuffel-Cymborowska et al., 1997), increases in the half life of ornithine decarboxylase (Laitinen et al., 1984; Murakami et al., 1988) and the direct stimulation of enzymatic activity (Bullock, 1983; Berger et al., 1984; Sertich and Pegg, 1987; Murakami et al., 1988). In view of the putative role of polyamines in the transduction of the effect of androgens in the heart, we have studied whether they participate in the positive inotropism induced by androgens in the left atrium of the rat by means of a pharmacological approach. The level of expression of the ornithine decarboxylase gene was also measured and ornithine decarboxylase activity was assayed.

2. Materials and methods

2.1. Preparation of tissue and incubation media

Three-month-old male Wistar rats, 250–300 g in weight (University of Oviedo, Spain, number 3304-13A), were used. They were killed at the same time of day to avoid circadian changes in ornithine decarboxylase activity (Soliman et al., 1982) by decapitation under anaesthesia with ethyl ether in an inhalation chamber. Afterwards, the left atrium was removed and placed in an organ bath in 10 ml of Tyrode's solution (having the following mM composition: NaCl, 137; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 1.05; NaH_2PO_4, 0.42; $NaHCO_3$, 11.9 and glucose, 5.5) at 37°C and bubbled continuously with a 95% O_2 and 5% CO_2 mixture.

2.2. Experimental procedure

The tissues were allowed to stabilize for 1 h under a basal tension of 1 g before experimentation. After the equilibration period, electrical stimulation of 0.5 Hz with a duration pulse of 5 ms and supramaximal voltage (30–50% above the threshold voltage) was started, using a Grass S11 stimulator and allowing a period of 30 min before the addition of any drug to the organ bath. Contractions elicited by electrical stimulation were recorded on a Letica Unigraph 50 polygraph through isometric transducers TRI 110.

2.3. Pharmacological studies

To study the effect of the androgens, 5α- and 5β-dihydrotestosterone, cumulative concentration–response curves (0.1–100 μM) were made, as previously described (Rubín et al., 1999), by adding the drugs to the organ bath until the maximum effect for each concentration was reached. To avoid the irreversible effects of androgens, only one concentration–response curve was made with each preparation. Thus, to study the effects of α-difluoromethylornithine the drug was added to the organ bath 30 min before performing the concentration–response curve for 5α- or 5β-dihydrotestosterone.

2.4. Biochemical studies

To carry out the biochemical assay, a single concentration (100 μM) of 5α- or 5β-dihydrotestosterone was added into the organ bath, and 6 min after the induction of positive inotropism (when the increase in the contraction reaches the steady state and is maintained) the atria were immediately removed from this bath and placed in liquid nitrogen. These preparations were preserved at −80°C until they were used to perform an ornithine decarboxylase activity assay and Northern blotting analysis.

2.5. Ornithine decarboxylase assay

Ornithine decarboxylase activity was determined essentially as previously described (Lau and Slotkin, 1979). Left atria were homogenized in a Polytron, three times for 10 s, in 1 ml of ice-cold buffer containing 10 mM Tris–HCl, 50 μM pyridoxal 5 phosphate, 2 mM dithiothreitol, pH 7.2. The homogenate was centrifuged for 15 min at 26,000 g, at 4°C. Then, 300 μl of the supernatant and 0.250 μCi of L-[1-^{14}C]ornithine (final concentration 20 μM) were incubated for 60 min at 37°C in a closed tube equipped with a filter paper wetted in 50 μl of 10% KOH to trap released $^{14}CO_2$.

The incubation was terminated by injecting 150 μl of 10% trichloroacetic acid. Then, the tubes were incubated for a further 45 min at 37°C to release $^{14}CO_2$ from the incubation buffer. To estimate the non-specific $^{14}CO_2$ released during the incubation, blank tubes were set up in which α-difluoromethylornithine was added before the supernatant, but otherwise the tubes were treated similarly. The filter papers were removed and the trapped $^{14}CO_2$ was measured by liquid scintillation. Assays were duplicated in

each experiment. The protein content was determined according to the Bradford procedure, and specific ornithine decarboxylase activity was expressed as pmol of $^{14}CO_2$ evolved per h per mg of protein.

2.6. Northern blotting analysis

Total RNA was isolated from the tissues by the guanidine isothiocyanate method as previously described (Chomczynsky and Sacchi, 1987). Twenty micrograms of total RNA were fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde, blotted onto nylon membranes, Hybond N (Amersham), and fixed with ultraviolet light in an ultraviolet cross-linker.

The blot was then prehybridized for 6 h at 42°C in a solution containing 50% formamide, 5× saline sodium phosphate EDTA (SSPE) (1× SSPE: 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA pH 7.4), 10× Denhardt's solution (1× Denhardt's: 0.02% of bovine serum albumin, polyvinylpyrrolidone and Ficoll), 1.5% sodium dodecyl sulphate (SDS) and 100 μg/ml denatured salmon sperm DNA. Membranes were hybridized in the same solution with a cDNA encoding rat ornithine decarboxylase (Blackshear et al., 1989) provided by Dr. Blackshear (Howard Hughes Medical Institute Laboratories), labelled with α^{32}PdCTP for 48 h at 42°C. The blots were washed twice at room temperature in 2× sodium saline citrate (SSC) (1× SSC: 150 mM NaCl, 15 mM trisodium citrate, pH 7), 0.1% SDS for 5 min each, followed by washing at 50°C in 0.1× SSC, 0.1% SDS for 20 min each. Blots were exposed to Kodak X-Omat films at −70°C with intensifying screens. Following exposure to X-ray film, Northern blots were washed in stripping buffer (0.5% SDS, 5 mM EDTA, pH 8) for 3 min at 95°C and hybridized again with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA as an internal control for evaluating RNA lane loading.

2.7. Drugs

The following drugs were used: 5α-dihydrotestosterone (5α-androstan-17-β-ol-3-one) and 5β-dihydrotestosterone (etiocholan-17β-ol-3-one) from Sigma; α-difluoromethylornithine kindly donated by Dr. Böhme from Marion Merrell Dow, and L-[1-^{14}C]ornithine came from Amersham. α-Difluoromethylornithine was dissolved in the incubation media. The androgens, 5α- and 5β-dihydrotestosterone, were dissolved in ethanol (the final concentration of the solvent was lower than 0.1%). At the concentrations used, ethanol did not modify the contractility of the left atria. However, effects of ethanol on ornithine decarboxylase activity have been described (Poso and Poso, 1981; Thompson and Adams, 1994; Davidson et al., 1998). To check this possibility, the effect of ethanol, at the concentrations used as a solvent, was assayed by adding it to the organ bath, allowing 6-min exposure, and to the enzymatic assay in vitro. Ethanol 0.1% decreased the basal activity of ornithine decarboxylase (from 55 ± 4.7 to 34.5 ± 5.5 pmol of $^{14}CO_2$/h/mg of protein) when the atria were exposed to the organ bath and also lowered the enzymatic activity in vitro (48.9 ± 6.4 pmol of $^{14}CO_2$/h/mg of protein). To evaluate the effect of 5α-dihydrotestosterone with a different solvent, it was dissolved in dimethyl sulfoxide (DMSO). This solvent did not modify the basal activity of ornithine decarboxylase when added to the organ bath (52.8 ± 6.3 pmol of $^{14}CO_2$/h/mg of protein). The increases in ornithine decarboxylase elicited by 5α-dihydrotestosterone, using ethanol or DMSO, were

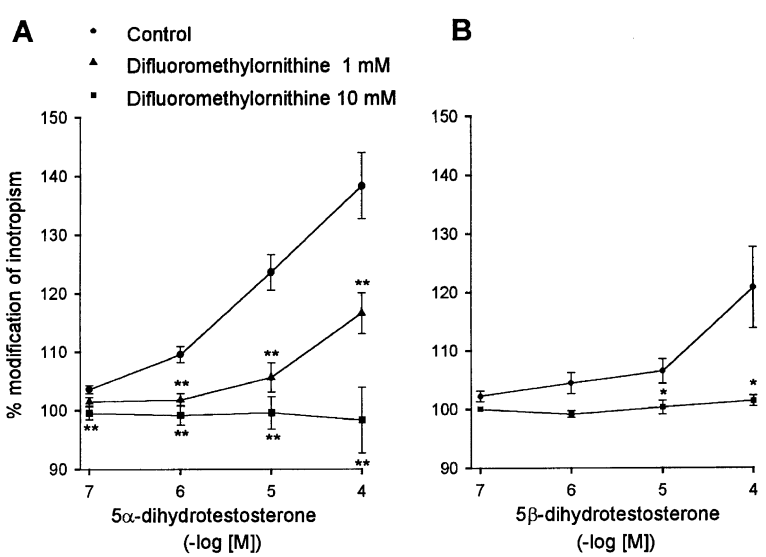

Fig. 1. Effect of α-difluormethylornithine (DFMO), 1 and 10 mM, on 5α-dihydrotestosterone (0.1–100 μM) and 10 mM of α-difluormethylornithine on 5β-dihydrotestosterone (DHT) induced positive inotropism in the electrically stimulated (0.5 HZ, 5 ms, voltage 30–50% above threshold voltage) left atrium of rats. $^{*}P < 0.05$ and $^{**}P < 0.01$, Student's *t*-test.

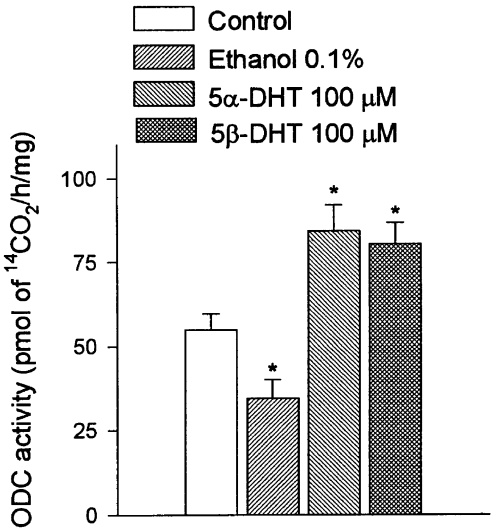

Fig. 2. Effect of 6-min incubation with 5α- and 5β-dihydrotestosterone (DHT, 100 μM), and the effect of ethanol 0.1% on ornithine decarboxylase activity in the left atrium of rats, electrically stimulated (0.5 Hz, 5 ms, 30–50% above threshold voltage). Enzymatic activity is expressed in pmol of $^{14}CO_2$/h/mg of protein, each point represents the mean ± S.E.M. of at least seven items. * $P < 0.05$, Student's *t*-test, with respect to basal activity.

similar (84.2 ± 9 vs. 73.72 ± 6.5 pmol of $^{14}CO_2$/h/mg of protein, respectively). In the experiments shown in Results, ethanol was the solvent for 5α-dihydrotestosterone.

2.8. Calculation and statistical analysis

The data for the contractions were expressed as the means ± standard error of mean (S.E.M.) of the percentage modification of the basal inotropism (100% contraction), the number of points (n) was at least 7 in each case. The enzymatic assays are expressed as the means ± S.E.M. of the values for activity in pmol of $^{14}CO_2$/h/mg of protein. Statistical significance was calculated by means of Student's *t*-test for unpaired values. Test values of $P < 0.05$ were considered significant.

3. Results

3.1. Effect of α-difluoromethylornithine on 5α- and 5β-dihydrotestosterone-induced positive inotropism in the left atrium of rats

α-Difluoromethylornithine, a specific irreversible inhibitor of ornithine decarboxylase (Metcalf et al., 1978; Tabor and Tabor, 1984), was used to pharmacologically evaluate the role of polyamines in the positive inotropism elicited by androgens in the left atrium of rats. The 30-min preincubation with α-difluoromethylornithine, 1 and 10 mM, significantly inhibited the concentration-dependent positive inotropism induced by 5α-dihydrotestosterone. Ten mM of α-difluoromethylornithine also inhibited the increase of the contraction induced by 5β-dihydrotestosterone (0.1–100 μM) (Fig. 1). However, α-difluoromethylornithine did not modify basal inotropism.

3.2. Effect of acute exposure to 5α- and 5β-dihydrotestosterone on the enzymatic activity of ornithine decarboxylase in the left atrium of rats

The exposure to androgens (6 min in the organ bath) significantly increased the activity of ornithine decarboxylase from 55 ± 4.7 (control, $n = 24$) to 84.2 ± 7.8 ($n = 11$) and 80.2 ± 8 ($n = 8$) pmol of $^{14}CO_2$/h/mg of protein, respectively for 5α- and 5β-dihydrotestosterone (100 μM) ($P < 0.05$, Student's *t*-test). Similar to what happened with the contraction, 5α-dihydrotestosterone was also more effective to induce an increase in the activity of ornithine

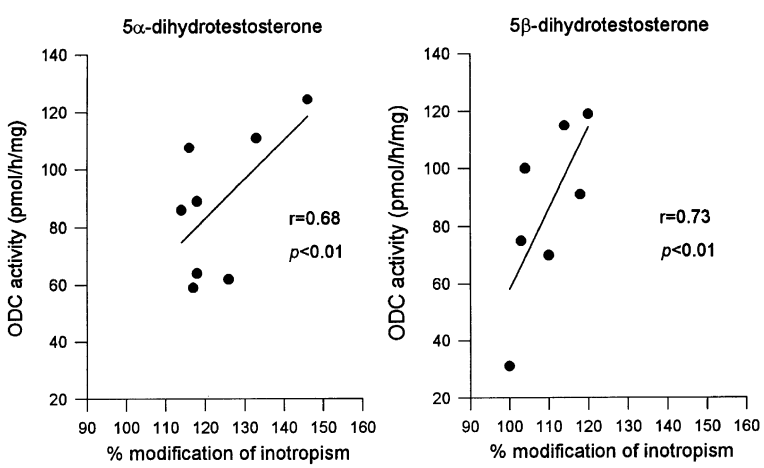

Fig. 3. Regression line between the modification of positive inotropism and ornithine decarboxylase (ODC) activity by acute exposure (6 min) to 5α- and 5β-dihydrotestosterone (100 μM) in electrically stimulated (0.5 Hz, 5 ms, voltage 30–50% above threshold voltage) isolated left atrium of rats.

decarboxylase than was 5β-dihydrotestosterone, although the differences were not statistically significant (Fig. 2).

Since we have recorded the positive inotropism induced by the androgens in each preparation, it was possible to correlate the two variables, ornithine decarboxylase activity and the modification of inotropism. We plotted these values and analyzed the linear regression between the two variables. There exists a positive relation between the increase of inotropism and of enzymatic activity ($P < 0.01$) (Fig. 3).

3.3. Effect of α-difluoromethylornithine on basal values for ornithine decarboxylase and 5α-dihydrotestosterone-induced increase of enzymatic activity in the left atrium of rats

The incubation of the preparation with α-difluoromethylornithine (10 mM), which did not modify basal inotropism, decreased the values for basal activity of ornithine decarboxylase, and, in addition, antagonized the increase of inotropism elicited by 5α-dihydrotestosterone (100 μM) (Fig. 4).

3.4. Effect of acute exposure to 5α- and 5β-dihydrotestosterone on the expression of the ornithine decarboxylase gene in the left atrium of rats

Northern blotting analysis shows that acute exposure (6 min in the organ bath) to 5α- and 5β-dihydrotestosterone did not modify the level of expression of the ornithine decarboxylase gene (Fig. 5).

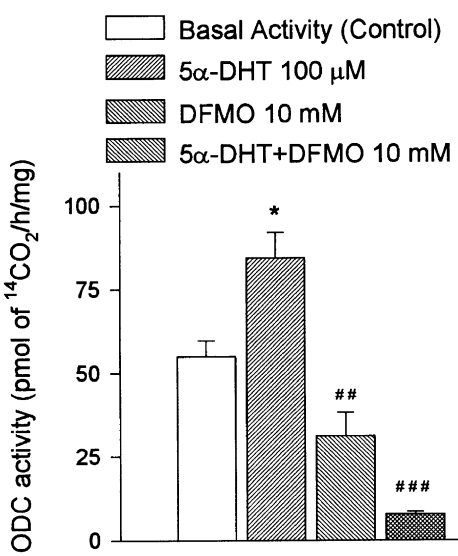

Fig. 4. Effect of α-difluormethylornithine (DFMO) (10 mM) on basal activity and on 5α-dihydrotestosterone (100 μM) induced increases in ornithine decarboxylase activity in electrically stimulated (0.5 Hz, 5 ms, 30–50% above threshold voltage) left atrium of rats. Enzymatic activity is expressed in pmol of $^{14}CO_2$/h/mg of protein; each point represents the mean ± S.E.M. of at least seven items. $^{*}P < 0.05$, Student's t-test, with respect to basal activity.

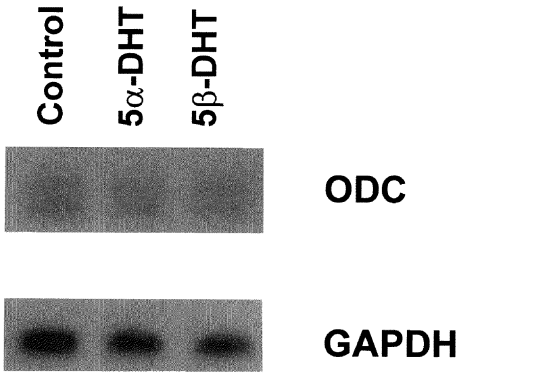

Fig. 5. Exposure for 6 min to 5α- and 5β-dihydrotestosterone (DHT, 100 μM) did not modify the expression of the ornithine decarboxylase gene (ODC). Northern blotting analysis was performed using total RNA from the isolated left atrium of rats. Blots were probed with an ornithine decarboxylase cDNA then stripped and reprobed with a GAPDH cDNA as a control for gel loading.

4. Discussion

According to our results, polyamines may participate in the acute positive inotropism elicited by androgens in the rat left atrium, since the effect was antagonized by an inhibitor of ornithine decarboxylase activity, α-difluoromethylornithine (Metcalf et al., 1978). However, α-difluoromethylornithine did not modify basal inotropism. This suggests that polyamine synthesis did not participate in the basal inotropism of the atrium during electrical stimulation. However, the functional integrity of ornithine decarboxylase and presumably the synthesis of polyamines may participate in the increase of contraction elicited by androgens. As in our results, α-difluoromethylornithine also antagonizes the positive inotropism elicited by ouabain, noradrenaline and Ca^{2+} in ventricle strips of rats (Bazzani et al., 1988). These findings support the hypothesis that polyamines, besides being involved in structural (Matsushita et al., 1972; Caldarera et al., 1974) and metabolic effects (Koenig et al., 1983, 1989; Fan and Koenig, 1988), may also participate in acute functional effects elicited by cardiotonic agents in the heart (Bazzani et al., 1988; Koenig et al., 1989).

The pharmacological findings, and the fact that the intracellular levels of polyamines are highly regulated through the activity of ornithine decarboxylase, led us to study the enzymatic activity in our preparations. The assay of enzymatic activity showed a significant increase in ornithine decarboxylase activity in the rat left atrium after 6 min of 5α- and 5β-dihydrotestosterone exposure. As observed with positive inotropism, 5α-dihydrotestosterone was more effective than 5β-dihydrotestosterone to elicit increases in enzymatic activity. The enzymatic assay seems

to be specific for ornithine decarboxylase, since non-specific decarboxylation was not observed when α-difluoromethylornithine was added to the reaction mixture. Preincubation of the preparations with α-difluoromethylornithine for 30 min, decreased the value for basal activity and antagonized the increase in enzymatic activity elicited by 5α-dihydrotestosterone. Our data are consistent with a previous report by Koenig et al. (1989) of increased ornithine decarboxylase activity in ventricular cubes of rat at 30 s, remaining elevated during a 10-min incubation in the continued presence of testosterone. In addition, polyamines mediate other biological actions induced by androgens (Bullock, 1983; Laitinen et al., 1984; Berger et al., 1984) including those in the heart. In the rodent heart, the treatment with an inhibitor of ornithine decarboxylase, α-difluoromethylornithine, suppresses testosterone-induced increases in ornithine decarboxylase activity and polyamine levels and blocks the hypertrophy (Koenig et al., 1989). Furthermore, polyamines can act as intracellular signals enhancing Ca^{2+} influx through the plasma membrane (Koenig et al., 1983; Fan and Koenig, 1988), and the decrease in polyamine synthesis also reduces Ca^{2+} influx (Fan and Koenig, 1988; Koenig et al., 1989). Therefore, it is possible that the newly synthesized polyamines serve as intracellular messengers in testosterone-mediated Ca^{2+} fluxes and Ca^{2+}-dependent membrane transport, having a modulatory role in the regulation of intracellular Ca^{2+} (Nilsson and Hellstrand, 1993). It has been reported that Ca^{2+} influx seems to participate in the positive inotropism elicited by androgens in the rat left atrium (García Valencia et al., 1992). Besides their role in androgen effects, polyamines also mediate the inotropism induced by other agents such as ouabain and noradrenaline (Bazzani et al., 1988).

Androgens produced an increase in the inotropism and in the ornithine decarboxylase activity in the left atrium of the rat, but pharmacological dissociation was not observed between these two variables. The incubation with α-difluoromethylornithine antagonized both effects, inotropism and enzymatic activity. In addition, a positive relation exists between these two variables according to the linear regression analysis, which shows a significant relation. Thus, polyamines may exert, via several membrane functions (Johnson, 1996; Williams, 1997), significant effects on various physiological parameters of the heart, (Koenig et al., 1989) including positive inotropism.

The functional evidence that polyamines are involved in the effect of androgens in our preparation, and the fact that they regulate the ornithine decarboxylase gene (Berger et al., 1984; Melanitou et al., 1987; Sertich and Pegg, 1987; Manteuffel-Cymborowska et al., 1997) led us to study the possible changes in the level of expression of this gene after acute exposure to androgens. However, the Northern Blotting analysis showed no evidence of changes in the level of expression of this gene after 6 min of exposure to 5α- and 5β-dihydrotestosterone. Therefore, as described, the signalling transduction would affect polyamine pathways rapidly in response to various stimuli prior to an effect on gene expression (Koenig et al., 1983, 1989; Fan and Koenig, 1988). The effects are produced at concentrations above the physiological range. However, we cannot eliminate the possibility that, in vivo, the concentration in the tissues might be higher than the circulating plasma levels. The significance of this finding has to be established, although it may be important in the physiological/pharmacological mechanisms of androgen action on cardiac contractility.

In summary, our results suggest that polyamine synthesis might be involved in the acute positive inotropism elicited by androgens in the left atrium of rat through the stimulation of ornithine decarboxylase activity without changes in the expression of the ornithine decarboxylase gene.

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