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Putrescine modulation of acute activation of the β-adrenergic system in the left atrium of rat

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ABSTRACT

Endogenous polyamines mediate acute metabolic effects and cardiac hypertrophy associated to β-adrenoceptor stimulation. The aim of this study is to characterize the role of polyamines on β-adrenoceptor system mediated responses. To this end, the functional interaction of polyamine modifying drugs on isoproterenol-elicited cardiotonic effect, in isolated left atria of male Wistar rats, and their effects on [^{3}H]dihydroalprenolol (DHA) binding on β-adrenoceptors and on adenylyl cyclase activity of membrane heart were studied. Polyamines interact with β-adrenoceptors in rat heart, as shown by the displacement of [^{3}H]DHA binding. Furthermore, putrescine (but not spermidine or spermine) increased adenylyl cyclase activity, elicited a positive inotropism and increased intracellular cAMP. The putrescine effect on adenylyl cyclase was not antagonized by the β-adrenoceptors blockers, alprenolol and ICI-118,551, and facilitated the isoproterenol effect. Neither alprenolol, atenolol nor ICI-118,551 antagonized putrescine-elicited positive inotropism. However, the effect was abolished in preparations with desensitized β-adrenoceptors. α-Difluoromethylornithine, an inhibitor of ornithine decarboxylase, antagonized the effect of isoproterenol on inotropism and cAMP increase. In addition, putrescine might elicit effects by mechanisms independent of β-adrenoceptor system, since in left atria with functional desensitized receptors an interaction with ouabain-elicited cardiotonic effect was observed. These results suggest that putrescine may act as a low affinity agonist on β-adrenoceptors and modulate acute responses mediated by β-adrenoceptors. These findings may be of importance in the physiology and in diseases involving cardiac β-adrenoceptors.

1. Introduction

Polyamines are involved in several physiological (Seiler, 2005) and pathological conditions (Malhotra et al., 1990; Marton and Pegg, 1995; Moinard et al., 2005), including cardiac hypertrophy and myocardial damage in response to numerous hormonal and trophic stimuli (Flamigni et al., 1986; Tipnis et al., 2000; Shantz et al., 2001).

Their role has been proved by the fact that the decrease in polyamine synthesis confers protection against β-adrenergic mediated cardiac hypertrophy (Tipnis et al., 2000). This was studied by means of treatment of experimental animals with α-difluoromethylornithine, an inhibitor of ornithine decarboxylase (Metcalf et al., 1978), the initial rate-limiting enzyme in the biosynthesis of polyamines.

Polyamines are also mediators of acute effects caused by isoproterenol and androgens in the heart, such as Ca^{2+} fluxes and Ca^{2+}-dependent membrane transport of hexose and amino acids (Fan and Koenig, 1988; Koenig et al., 1989). Cell contractility may also be affected by intracellular polyamines, since ornithine decarboxylase activity is positively correlated with the cardiotonic effect of androgens, whose effect was antagonized by α-difluoromethylornithine (Bordallo et al., 2001). Polyamines potentiated contractile responses by increasing myofilament Ca^{2+} sensitivity in permeabilized cardiomyocytes (Nilsson et al., 1995; Sward et al., 1998). Therefore, cytoplasmic polyamines have a role in contributing to the contractile properties of cardiac muscle (Harris et al., 2000). In addition, polyamines are known to bind with different affinities to a variety of cytoplasmic ligands and many membrane proteins (Ventura et al., 1994), including several types of ion channels (Williams, 1997).

Exogenous polyamines have been reported to produce negative and positive inotropism. Spermidine and spermine have been shown to elicit negative inotropism, presumably by blocking Ca^{2+} channels and reducing intracellular Ca^{2+} (Ventura et al., 1994), whereas

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putrescine-elicited an inotropic response in cardiomyocytes that was associated with an increase in intracellular cAMP (Velasco et al., 2008).

It has not been reported whether polyamines interact with β-adrenoceptors, as has been proposed with other G-protein coupled receptors (Bueb et al., 1992). This is the aim of the present study via a functional and biochemical approach.

2. Materials and methods

2.1. Animals and functional experiments in isolated left atrium of rats

Three-month-old male Wistar rats, 300–350 g in weight (from the University of Oviedo, Spain, number 3304-13A) were sacrificed by decapitation after placing them in an inhalation chamber filled with CO_2 (Directive 2003/65/CE and Spain RD 1201/2005), utilizing a protocol that was approved by the local ethical committee of the University of Oviedo. The left atrium was subsequently removed, placed in an organ bath in 10 ml of Tyrode's solution (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. The contractions were elicited by electrical stimulation with a Grass S11 stimulator (0.5 Hz, 5 ms and a voltage 30–50% above the threshold voltage), and recorded on a Letica Uni-graph 50 polygraph through isometric transducers TRI 110 (Rubin et al., 1999).

The tissues were allowed to stabilize for 1 h under a basal tension of 1 g before experimentation. Subsequently, cumulative concentration–response curves to isoproterenol (0.3 nM to 30 μM) were performed. By the 1 h interval, concentration–response curves to isoproterenol were found to be reproducible. To study the interaction of α-difluoromethylornithine or putrescine on isoproterenol-elicited acute response, these drugs were added to the organ bath prior to performing the second concentration–response curve to isoproterenol: either 6 min for putrescine (10 mM), to allow for a stable increase of inotropism, or 30 min for α-difluoromethylornithine (10 mM).

Additional experiments were carried out in order to pharmacologically characterize the role of β-adrenoceptors on putrescine (10 mM)-elicited positive inotropism. To this end, the effect of 10 min preincubation with the β-adrenoceptor antagonists, alprenolol, atenolol and ICI-118,551 (concentrations up to 1 μM) and the receptor desensitization, were studied on putrescine (10 mM)-elicited positive inotropism in isolated rat left atrium. β-Adrenoceptors were desensitized by long-term exposure (90 min) to high concentrations of isoproterenol (30 μM), as evidenced by the lack of response to subsequent administration of isoproterenol. Furthermore, we studied the tissue responsiveness to ouabain (100 μM), a cardiotonic agent acting via a different mechanism of action than that of isoproterenol, and explored ouabain functional interaction with putrescine.

2.2. Polyamines determination via high performance liquid chromatography (HPLC) in the left atrium of rats

After the incubation period in the organ bath with or without acute exposure (~30 s) to isoproterenol (3 μM) in the presence or absence of α-difluoromethylornithine (10 mM, for 30 min), the left atria were immediately removed, placed in liquid nitrogen and preserved at −80 °C for future use. Putrescine, spermine and spermidine levels were determined using a precolumn derivatization method as previously described (Escribano and Legaz, 1988). Tissues were homogenized in 0.5 ml of distilled water and then treated with sufficient perchloric acid to reach a final concentration of 12% (5 min at 4 °C). The extracts were then centrifuged at 10,000 ×g for 30 min, and 0.2 ml of collected supernatants were then neutralized with 0.3 ml of a saturated solution of $NaHCO_3$. The samples were dansylated overnight with 0.5 ml of a solution containing 5 mg ml^{-1} dansyl chloride in acetone. After one extraction with toluene the toluenic phase was dried under a nitrogen atmosphere, resuspended in 0.4 ml acetonitrile and then chromatographed in a HPLC using a C_{18} reverse-phase column according to the method described above. Quantification of polyamines was performed using 2-hydroxy-diaminopropane as an internal standard. The polyamines were expressed as nmol mg·$protein^{-1}$.

2.3. Determination of intracellular cAMP levels in isolated left atrium of rats

For this, the same protocol was followed with the atria in the organ bath. To determine cAMP levels, atria were homogenized with a Polytron in a buffer containing 4 mM EDTA (to prevent enzymatic degradation of cAMP), followed by heating for several minutes in a boiling water bath to facilitate protein coagulation. The extracts were centrifuged at 18,000 ×g for 15 min. Cyclic AMP in the supernatant was assayed by means of a [^{3}H]AMP radioassay kit following the indications of the manufacturer (Amersham). Cyclic AMP levels were expressed as pmol·mg $protein^{-1}$.

2.4. Preparation of rat heart membranes

Rat heart membranes were prepared as previously described (Hartmann et al., 1995). The hearts were cleaned of fat and connective tissue, cut into small pieces and gently homogenized in an ice cold buffer (mM: sucrose 0.25, Tris 5, $MgCl_2$ 1; pH 7.4) using a Polytron. The homogenates were diluted with an equal volume of KCl (1 M), stirred for 10 min and then filtered through gauze. They were then centrifuged at 700 ×g for 15 min, following which the supernatants were collected and spun first at 10,000 ×g for 15 min and then at 40,000 ×g for 30 min. To perform the binding assay, the membrane pellets were resuspended in a buffer (mM: Tris 50, $MgCl_2$ 10; pH 7.4) at a final protein concentration of 2 mg/ml. To determine adenylyl cyclase activity, pellets were resuspended in a buffer (mM: Hepes/Na 30, Mg Cl_2 5, DTT 2; pH 7.4) at a protein concentration of 1 mg ml^{-1}. Aliquots were frozen in liquid nitrogen and stored at −80 °C.

Some hearts were homogenized by strong pulses in a Polytron in a buffer without sucrose. They were then centrifuged and resuspended as mentioned above and frozen until used to determine adenylyl cyclase activity. These membranes did not respond to isoproterenol and were considered to have an uncoupled β-adrenoceptor system.

Protein concentrations were determined by the Bio-Rad protein assay (Richmond, CA).

2.5. Binding assay in rat heart membranes

Binding assays were carried out in a volume of 500 μl buffer (mM: Tris 50, $MgCl_2$ 10; pH 7.4), containing 200 μg of membrane protein, and incubated at 30 °C for 20 min. The ligand binding was separated by rapid vacuum filtration through Whatman GF/C filters. Radioactivity was determined by liquid scintillation. Cardiac β-adrenoceptor densities (B_{max}) were estimated in saturation experiments using [^{3}H]dihydroalprenolol and specific binding was defined as the portion displaceable by propranolol (1 μM). Polyamine-binding displacement experiments were performed using increasing concentrations of putrescine (30 μM to 60 mM), spermidine (0.3 to 100 mM) and spermine (0.3 to 100 mM) in the presence of [^{3}H]dihydroalprenolol (1 nM). All experiments were conducted in triplicate and repeated at least four independent times.

2.6. Adenylyl cyclase assay in rat heart membranes

This enzymatic assay was performed with minor modifications of previous procedures (Post et al., 2000). Twenty-five to 200 μg samples

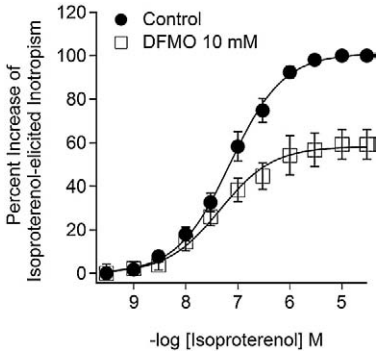

Fig. 1. Concentration-dependent curve for isoproterenol (0.3 nM to 30 μM)-elicited positive inotropism in electrically stimulated left atrium of rats (0.5 Hz, 5 ms, voltage 30–50% above the threshold voltage) and its modification with α-difluoromethylornithine (DFMO). The increase of inotropism was plotted as the percentage of the maximal increase to isoproterenol (30 μM), from the concentration–response curve of the control (100%). Values represent the mean ± S.E.M. of 7 experiments. The ANOVA with repeated measures showed a significant difference between curves of $P<0.01$.

of membranes or total heart protein were incubated in a final volume of 100 μl buffer (containing: 5 mM Mg Cl_2, 1 mM cAMP, 500 μM 3-isobutyl-1-methylxanthine, 100 μM ATP, 5 mM phosphocreatine, 500 μM EGTA, 30 U/ml of creatine phosphokinase, 0.05% BSA, 10 μM GTP, 30 mM Hepes/Na and pH 7.4), 1 μCi [α^{32}P]ATP and β-adrenoceptor agonists and/or antagonists. The reaction was initiated by the addition of membranes, and was terminated after 15 min incubation at 30 °C by adding 100 μl of a 2% sodium dodecyl sulphate solution containing 40 mM ATP and 1.4 mM cAMP. After adding approximately 5.000 cpm of [^{3}H]cAMP in 1 ml of water (for determination of cyclic nucleotide recovery), cAMP was separated from ATP by sequential chromatography on Dowes and neutral alumina (Salomon, 1979). Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay time period, as well as membrane protein concentration. Enzymatic activation was normalized taking into account the basal activity as 100%.

2.7. Drugs

The following drugs were used: isoproterenol (1-[3′,4′-dihydroxyphenyl]-2-isopropyl-aminoethanol hydrochloride), salbutamol (albuterol, α-([*t*-butylamino]methyl)-4-hydroxy-*m*-xylene-α,α′-diol), alprenolol (1-[*o*-allylphenoxy]-3-[isopropylamino]-2-propanolol), atenolol (4-2[2′-hidroxy-3(isopropyl-amino) propoxi]phenyl-acetamide), 2-hidroxydiaminopropane, propranolol (1-[isopropylamino]-3-[1-naphthyloxy]-2-propanolol), ICI-118,551 ((±)-1-[2,3-(ihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride), 2′:3′dideoxyadenosine, forskolin (7ß-acetoxy-1α,6ß,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one), α-difluoromethylornithine (DL-α-difluoromethylornithine), putrescine (tetramethylenediamine), spermidine (*N*-[3-aminopropyl]-1,4-butanediamine), spermine (*N,N*′-bis[3-aminopropyl]-1,4-butanediamine) and ouabain (G-strophanthin; 1ß,3ß,5ß,11α,14,19-hexahydroxycard-20[22]-enolide 3[6-deoxy-α-L-mannopyranoside]) were from Sigma. Sodium fluoride from Probus. [^{3}H]AMP radioassay kit and [^{3}H]dihydroalprenolol from Amersham. Forskolin was dissolved in dimethyl sulfoxide (the final concentration of the solvent was less than 0.1%). Isoproterenol, salbutamol, alprenolol, atenolol, propranolol, ICI-118,551, α-difluoromethylornithine, ouabain, putrescine, spermidine and spermine were dissolved in purified water.

2.8. Calculations and statistical analysis

To plot the concentration–response curves to isoproterenol, the maximum increase of inotropism in the control curves was considered to be 100%. This value was taken into account to plot the second concentration–response curves in the presence of α-difluoromethylornithine or putrescine, without subtracting the cardiotonic effect elicited by this polyamine.

The data obtained were expressed as the mean ± standard error of mean (S.E.M.) of at least 5 atria in each case, unless otherwise indicated. Statistical significance was calculated by means of Student's *t*-test for paired or unpaired values and Bonferroni's test. The comparison for the concentration–response curves was evaluated by analysis of variance (ANOVA) for repeated measures. A $P \leq 0.05$ was considered as significant. The half maximum response to isoproterenol (EC_{50}), on inotropism and on adenylyl cyclase activation, was calculated by fitting the concentration–response curves with the Hill equation (Igor Pro V5.0, WaveMetrics Inc., USA). A linear regression analysis of Scatchard plot data was made by using the computer radioligand program RADLIG (Biosoft). The K_D and B_{max}, maximal binding capacity related to the number of receptors, were also determined.

3. Results

3.1. Effect of α-difluoromethylornithine (10 mM) on isoproterenol-elicited positive inotropism in isolated left atrium of rats

The incubation with α-difluoromethylornithine (10 mM), an inhibitor of ornithine decarboxylase, 30 min before performing the second concentration–response curve to isoproterenol (0.3 nM to 30 μM), significantly antagonized the effect of isoproterenol. The EC_{50} were 71.46 ± 3.73 nM and 44.20 ± 6.19 nM, in the absence and the presence of α-difluoromethylornithine, respectively (Fig. 1).

3.2. Effect of α-difluoromethylornithine (10 mM) on isoproterenol (3 μM)-elicited modification on intracellular polyamines and cAMP in isolated left atrium of rats

The acute exposure to isoproterenol (3 μM) significantly increased the level of putrescine, but not spermidine and spermine. This effect was antagonized by previous incubation with α-difluoromethylornithine (10 mM) for 30 min, which did not modify basal levels of putrescine (Fig. 2A).

The incubation for 30 min with α-difluoromethylornithine did not modify the basal levels of cAMP, but antagonized isoproterenol-elicited (3 μM) increases in cAMP (Fig. 2B).

3.3. Effect of putrescine, spermidine and spermine on [^{3}H]dihydroalprenolol binding to rat heart membranes

The binding of the radioligand, [^{3}H]dihydroalprenolol, to cardiac membranes was saturable (0.1 to 10 nM), and was displaced by propranolol. The Scatchard plot showed a receptor density of

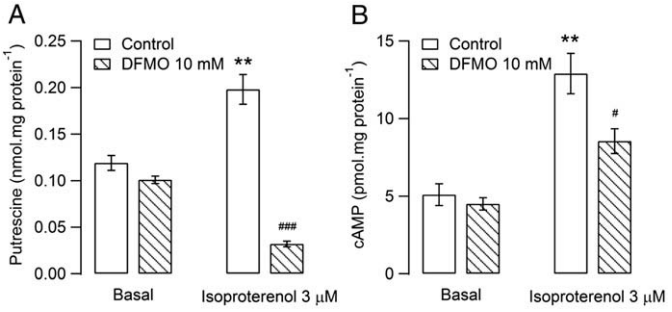

Fig. 2. Effect of α-difluoromethylornithine (DFMO) on basal and isoproterenol-elicited increase of intracellular putrescine (nmol mg protein^{-1}) (A) and of cAMP levels (pmol mg protein^{-1}) (B) in electrically-stimulated left atrium of rats. Each point represents the mean ± S.E.M. for at least 4 data. $^{**}P<0.01$ by comparing the effect of isoproterenol on putrescine or cAMP levels *vs.* basal values; $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ effect of isoproterenol in the absence or the presence of DFMO, by means of Student's *t*-test for unpaired data.

84 fmol·mg protein^{-1} and a K_D of 0.85 nM (Fig. 3A, B). The competition assay with putrescine (30 μM to 60 mM), spermidine (0.1 to 100 mM) and spermine (0.1 to 100 mM) showed displacement of [^{3}H]dihydroalprenolol (1 nM). Putrescine showed a K_D value of 10.41 mM and a maximum displacement of 87.72%. At the highest concentration used, spermidine and spermine displacement was about 50% (Fig. 3C).

3.4. Effect of putrescine (10 mM) on isoproterenol-elicited inotropism and of drugs acting on β-adrenoceptor system on putrescine (10 mM)-elicited inotropism in isolated left atrium of rats

Putrescine (10 mM) facilitated the cardiotonic effect of isoproterenol, without modification of EC_{50}, which was 57.90±4.18 nM in the control curve and 59.90±7.5 nM in the presence of putrescine (Fig. 4A).

Putrescine (10 mM)-elicited cardiotonic effect was not antagonized by the competitive β-adrenoceptor antagonist, atenolol, or by a selective β_2-adrenoceptor blocker, ICI-118,551, at concentrations up to 1 μM. However, dideoxyadenosine (30 μM), an inhibitor of adenylyl cyclase, antagonized putrescine (10 mM)-elicited positive inotropism (Fig. 4B).

The effect of putrescine (10 mM)-elicited inotropism in desensitized β-adrenoceptors was abolished, though not on other cardiotonic agents such as ouabain (100 μM), which increased the response 36.57±5.7%. In these preparations, the preincubation with putrescine (10 mM) facilitated the positive inotropism of ouabain (70.6±11.3%, $P<0.05$ by means of Student's *t*-test for paired data, $n=4$) or elicited arrhythmias and an increase in basal tone (Fig. 5).

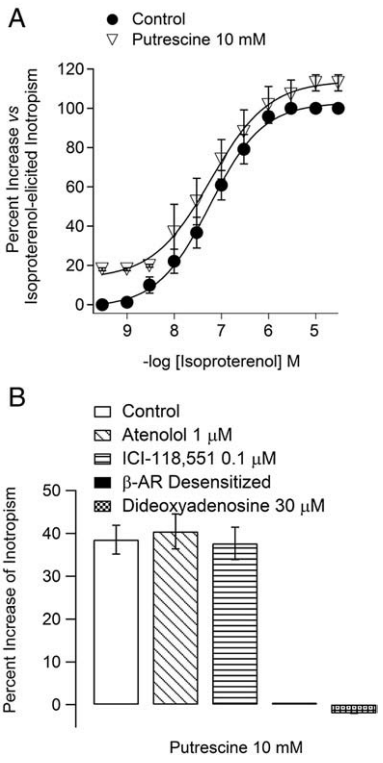

Fig. 4. (A) Effect of putrescine on isoproterenol (0.3 nM to 30 μM)-elicited positive inotropism in electrically-stimulated isolated rat left atrium, considering as 100% the maximum effect to isoproterenol in the control curve. (B) Effect of preincubation with atenolol, ICI-118,551, the desensitization of the β-adrenoceptors (β-AR desensitized) or dideoxyadenosine on putrescine-elicited positive inotropism, whose effects were normalized considering the value of basal inotropism as 100%. Values represent the mean±S.E.M. for at least 5 different experiments. The ANOVA with repeated measures showed a significant difference between curves of $P<0.01$.

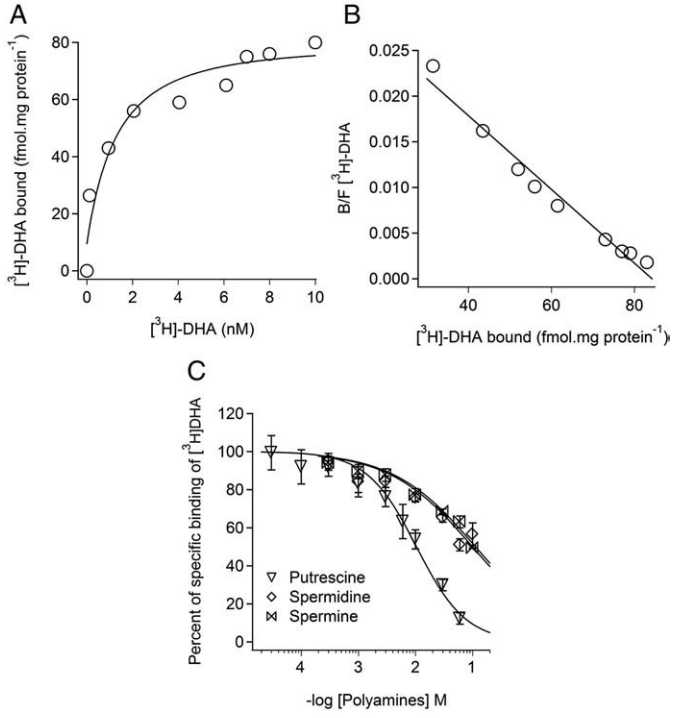

Fig. 3. (A) Specific binding of [^{3}H]dihydroalprenolol (DHA) to rat heart membranes as a function of the concentration of radioligand. The specific binding of radioligand was defined as the portion displaceable by propranolol (1 μM). (B) Scatchard plot obtained from same data. *B*: specific bound of radioligand. *B*/*F*: rate of specific bound and concentration of radioligand free. (C) Competition for specific [^{3}H]DHA (1 nM) binding to heart membranes by the polyamines putrescine (30 μM to 60 mM), spermidine (0.1 to 100 mM) and spermine (0.1 to 100 mM). Values represent the mean±S.E.M. of 4 experiments.

3.5. Effect of polyamines on adenylyl cyclase activity of rat heart membranes

The assay was validated by isoproterenol, which increased in a concentration-dependent way (10 nM to 100 μM) adenylyl cyclase activity, the EC_{50} of activation was 0.19±0.05 μM (Fig. 6A). The effect being antagonized by alprenolol (1 μM), a non-selective β-adrenoceptor antagonist. The selective β_2-adrenoceptor agonist, salbutamol (1 μM), increased enzymatic activity approximately one third of that of isoproterenol, an effect which was absent in the presence of ICI-118,551 (1 μM) (Fig. 6B).

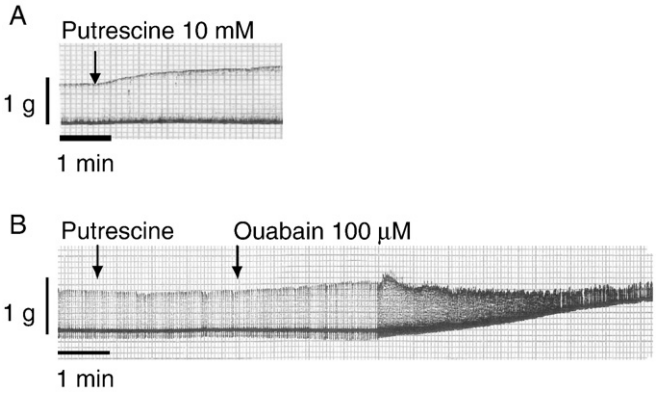

Fig. 5. Examples of putrescine-elicited positive inotropism in isolated left atrium of rats, control, (A) and (B) after β-adrenoceptor desensitization, and posterior addition of ouabain.

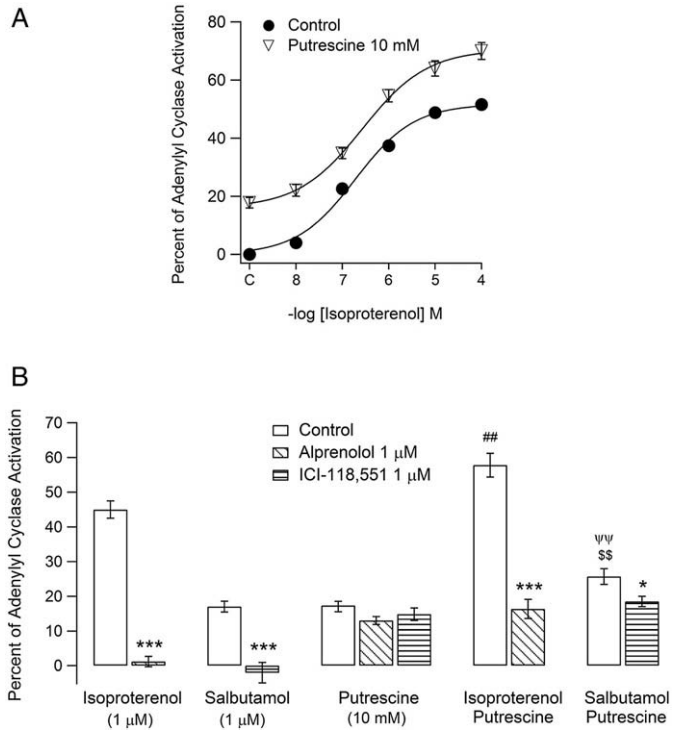

Fig. 6. (A) Effect of putrescine on isoproterenol (10 nM to 100 μM)-elicited activation of adenylyl cyclase. (B) Pharmacological activation of adenylyl cyclase by isoproterenol, salbutamol and putrescine. Effect of alprenolol on isoproterenol- and isoproterenol plus putrescine-elicited activation of adenylyl cyclase and of ICI-118,551 on the effect of salbutamol- and salbutamol plus putrescine. The percentage increase of activation was calculated considering the basal activity as 100%. The values represent the mean ± S.E.M. for at least 5 different assays. $^{*}P<0.05$ and $^{***}P<0.001$ with respect to the corresponding control in each case; $^{\#\#}P<0.01$ isoproterenol in the absence or the presence of putrescine; $^{\Psi\Psi}P<0.01$ comparing the effect of salbutamol in the absence or the presence of putrescine, and $^{\$\$}P<0.01$ comparing the effect of putrescine in the absence or the presence of salbutamol, by means of Bonferroni's *t*-test.

In these membranes, putrescine (1 to 10 mM), significantly increased adenylyl cyclase activity in a concentration-dependent manner (19.25 ± 1.5% at 10 mM), whereas equimolar concentrations of spermidine and spermine had no effect. Neither alprenolol (1 μM) nor ICI-118,551 (1 μM) significantly antagonized putrescine (10 mM) activation of adenylyl cyclase activity (Fig. 6B). Furthermore, putrescine (10 mM) produced an additive effect on isoproterenol-elicited (10 nM to 100 μM) activation, thereby increasing the maximal effect of enzymatic activity along the curve, without a significant modification in the EC_{50}: 0.27 ± 0.05 μM (Fig. 6A). Putrescine (10 mM) effect was also additive to the effect of salbutamol (1 μM) on adenylyl cyclase. Both facilitations of putrescine, on isoproterenol and salbutamol, were partially antagonized by alprenolol (1 μM) and ICI-118,551 (1 μM), respectively (Fig. 6B).

The membranes with an uncoupled β-adrenoceptor system did not respond to isoproterenol nor putrescine (10 mM), but were activated by 30 mM NaF (59.5 ± 12%) and 1 μM of forskolin (105 ± 5.6%).

4. Discussion

Our functional and biochemical studies suggest that polyamines, proposed as mediators in the acute positive inotropism elicited by androgens in the left atrium of the rat (Bordallo et al., 2001; Velasco et al., 2008), might modulate β-adrenoceptor mediated responses.

The three intracellular polyamines assayed, putrescine, spermidine and spermine, displaced [^{3}H]dihydroalprenolol binding from β-adrenoceptors, with putrescine showing more potency and displacement than spermidine and spermine. This interesting finding suggests that polyamines might modulate these adrenoceptors. These effects are demonstrable at millimolar range. Similar concentrations were used in several studies, and it has recently been proposed that 1 mM of spermine might be cardioprotective in ischemia/reperfusion models (Zhao et al., 2007).

To correlate polyamines binding with acute responses mediated by the β-adrenoceptor system in rat heart, we have studied their role on functional responses elicited by isoproterenol, such as the cardiotonic effect and adenylyl cyclase activation. The results show that polyamines may modulate positive inotropism elicited by isoproterenol, since incubation with α-difluoromethylornithine inhibited this effect. Intracellular polyamines have also been proposed as second messengers in the acute effects elicited by isoproterenol in rat heart, similar to the effects of increases in intracellular Ca^{2+} and membrane transport (Fan and Koenig, 1988; Koenig et al., 1989). Besides the heart, polyamines have also been involved in the contraction of rat trachea via G-proteins coupled to 5-HT receptors (Bueb et al., 1992). Additionally, it has been reported that polyamines can modulate G-protein mediated responses in the rat brain, as suggested by the fact that α-difluoromethylornithine treatment decreases β-adrenoceptor activation of adenylyl cyclase (Slotkin et al., 2000). Thus, polyamines may modulate adenylyl cyclase activity in different tissues.

Polyamines are not only involved in acute effects, they also mediate cardiac hypertrophy and myocardial damage induced by different mechanisms, including β-adrenergic stimulation (Tipnis et al., 2000). In this sense, hearts with overexpression of ornithine decarboxylase are more susceptible to induction of hypertrophy in response to isoproterenol than normal hearts (Shantz et al., 2001). On the other hand, inhibition of polyamine synthesis by α-difluoromethylornithine confers protection against isoproterenol-mediated myocardial damage and hypertrophy (Tipnis et al., 2000). Therefore, polyamines are involved in both acute and long-term effects elicited by β-adrenoceptor agonists.

The main β-adrenoceptor in the heart is the β_1 subtype (Rockman et al., 2002). Accordingly, we have pharmacologically characterized that in our preparation the activation of adenylyl cyclase occurred mainly through β_1-adrenoceptors. It is interesting that putrescine, but not spermidine or spermine, increased rat heart membrane adenylyl cyclase activity *in vitro*. This effect is not produced in uncoupled membranes that were unresponsive to isoproterenol, but whose enzymatic activity was increased by NaF or forskolin, activators of G-proteins and adenylyl cyclase, respectively. This result reinforced the hypothesis of selective polyamine modulation of β-adrenoceptor system. However, neither alprenolol nor ICI-118,551 antagonized the effect of putrescine, suggesting the existence of a different binding site in the β-adrenoceptors or a different mechanism of adenylyl cyclase activation. Though this effect may occur via stimulation of the β-adrenoceptor system, since, besides the displacement of the radioligand in the binding assay, putrescine has no effect in those membranes non-activated by isoproterenol.

The effect of putrescine is synergistic with the enzymatic activity of the non-selective β-adrenoceptor agonist, isoproterenol, at all the assayed concentrations, thereby increasing the maximum effect and thus suggesting a different mechanism of action. Similarly, putrescine facilitated salbutamol-elicited activation of adenylyl cyclase. In both cases, when combining either isoproterenol or salbutamol with putrescine as an agonist, the effect of β-adrenoceptor antagonists, alprenolol and ICI-118,551 only account for the activation of isoproterenol or salbutamol, since it remains the percent of increase due to putrescine. Thus, β-adrenoceptor antagonists did not modify the action of putrescine on adenylyl cyclase, in the absence or presence of isoproterenol or salbutamol, suggesting a functional mechanism independent of classical β-adrenoceptor activation.

In addition to its enzymatic effect, the administration of putrescine to the organ bath elicited a positive inotropism and increased intracellular cAMP levels in isolated rat left atrium (Velasco et al., 2008). It has also been reported that there is a lack of effect on putrescine and negative inotropism after extracellular administration of spermidine and mainly with spermine in isolated rat ventricular cardiomyocytes (Ventura et al., 1994). These differences may be due to the different polyamine concentrations utilized in this study or to the specific tissues that were used.

The cardiotonic effect of putrescine may be due to an increase in intracellular polyamines, although we cannot discard the possibility of a combination of extra- and intracellular effects of putrescine. In any case, similar to the effect on adenylyl cyclase, the selective β_1- or β_2-adrenoceptor antagonists, respectively atenolol and ICI-118,551, did not antagonize the positive inotropism to putrescine. On the other hand, the incubation with putrescine facilitated the positive inotropism to isoproterenol. This might indicate an additional mode of action. However, as observed in assessing enzymatic activity, a functional β-adrenergic system is required, since the desensitization of these receptors, by means of long-term exposure to high concentrations of isoproterenol, suppressed the cardiotonic effect of putrescine. However, we cannot discard the possibility that in this experimental condition, the desensitization of β-adrenoceptors may reduce the effects of compounds which also raise cAMP or produce a heterologous desensitization of G-protein coupled receptors (Rockman et al., 2002). In any case, adenylyl cyclase seem to be involved in the functional effect of putrescine, since an inhibitor of it, dideoxyadenosine (Johnson et al., 1989), antagonized putrescine-elicited positive inotropism.

It is very likely that putrescine has an additional mechanism of action independent of the β-adrenoceptor system or cAMP mechanisms, since in those preparations with desensitized β-adrenoceptors, which did not respond to putrescine, it facilitated ouabain-elicited positive inotropism, whose cardiotonic effect is not related to β-adrenoceptors, or induced arrhythmias and increased the basal tone in some preparations by suppressing atria relaxation. Alternative mechanisms for polyamines have been reported, such as modulatory effects observed on calcium sensing receptors (Quinn et al., 1997; Harris et al., 2000; Wang et al., 2003).

The absence of non-permeant analogs of the polyamines studied made it difficult to ensure whether the interaction was at an extracellular level, since intracellular transport of putrescine may yet exist (Aziz et al., 1994). And, as mentioned, intracellular polyamines may modulate isoproterenol-elicited cardiotonic effects and increase in cAMP.

These findings suggest that polyamine may be involved in the complex regulation of the β-adrenergic system in the heart (Feldman et al., 2005), possibly by modulating cAMP-mediated signalling, an important factor in the pathophysiology of cardiomyopathy (Movsesian and Bristow, 2005). It is worthy to mention that cellular polyamine content is at millimolar concentrations (Sarhan and Seiler, 1989) thereby suggesting that in situations of cellular damage the intracellular content might be exposed to the contiguous tissue inducing biological effects. However, the physiological or pathophysiological significance of these results still need to be elucidated.

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