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Functional effects of polyamines *via* activation of human β_1- and β_2-adrenoceptors stably expressed in CHO cells

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Abstract:
Polyamines mediate acute metabolic effects and cardiac hypertrophy associated with β-adrenoceptor stimulation. They may also modulate β-adrenoceptors, causing functional responses in rat atria and tracheal smooth muscle. The aim of this study was to determine whether polyamines interact with human β_1- and β_2-adrenoceptors and the functional consequences of such an interaction. Chinese hamster ovary (CHO) cells stably transfected with human β_1- and β_2-adrenoceptors were used to evaluate the effect of polyamines binding to β-adrenoceptors, cAMP production and morphological changes, which were pharmacologically validated by investigating the effects of the β-adrenoceptor agonists, isoproterenol and salbutamol. Polyamines interacted with human β_1- and β_2-adrenoceptors, as shown by the displacement of [^{125}I]iodocyanopindolol in the binding assay. Putrescine showed higher affinity to β_1- than β_2-adrenoceptors. Spermidine and spermine produced partial displacement (approximately 50%), and at the highest concentration, the effect was reversed. Putrescine and spermine acutely increased cAMP and, in a serum-free medium, induced a stellate-like form in cells, which was inhibited by propranolol, a β-blocker. A 10 to 15 h incubation with putrescine produced a spindle-like form and spatial organization *via* β-adrenoceptor activation, evidenced by the antagonizing effect by propranolol and lack of effect in wild-type CHO cells. Additionally, it decreased cell proliferation independently of β-adrenoceptor activation. Spermine caused cell death *via* fetal bovine serum-dependent and -independent mechanisms. The results suggest that putrescine may act as a non-selective and low affinity agonist of human β_1- and β_2-adrenoceptors, eliciting morphological changes. These findings may be of importance in physiology and in diseases involving β-adrenoceptor functionality.

Key words:
polyamines, putrescine, spermine, β-adrenoceptors, cAMP, Chinese hamster ovary (CHO)

Introduction

The polyamines, putrescine, spermidine and spermine, belong to a family of low-molecular-weight organic polycations that are involved in several physiological [34] and pathological conditions [19, 21, 25]. These conditions are due to the interactions of polyamines with DNA, RNA and a variety of cytoplasmic ligands and membrane proteins.

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In the heart, intracellular polyamines are mediators of acute effects caused by isoproterenol, such as the increase of Ca^{2+} flux [9, 15], which may increase contractile responses by increasing myofilament Ca^{2+} sensitivity [27, 41]. The polyamine putrescine elicited cardiotonic effects associated with an increase of cAMP [2]. The inhibition of ornithine decarboxylase activity, the initial rate-limiting enzyme in the biosynthesis of polyamines, by α-difluoromethylornithine [24] antagonized these effects [2], which suggested that cytoplasmic polyamines may have a role in the contractile properties of cardiac muscle [2, 11].

In addition, polyamines are involved in cardiac hypertrophy and myocardial damage induced in experimental models in response to numerous hormonal and trophic stimuli, including β-adrenoceptor stimulation [10, 43], the effect of which was increased in transgenic mice overexpressing ornithine decarboxylase [35]. As reported for the acute effects, prior treatment with α-difluoromethylornithine confers protection against β-adrenergic mediated cardiac hypertrophy [43].

Exogenous polyamines also produced effects on myocardium [2, 44] and tracheal smooth muscle [4]. Spermidine and spermine have been shown to elicit negative inotropism, presumably by blocking Ca^{2+} channels and reducing intracellular Ca^{2+} [44], whereas putrescine elicited an inotropic response in the isolated left atria of rats [2]. This effect may be due to an interaction with β-adrenoceptors in rat heart, as shown by the displacement of a selective ligand, [^{3}H]dihydroalprenolol, and by the fact that a functional β-adrenoceptor system was required to elicit the cardiotonic response [2].

Cyclic AMP-dependent mechanisms are also related to a decrease in intracellular polyamines in cultured cells *via* inhibition of the activities of ornithine decarboxylase and S-adenosylmethionine decarboxylase [13, 14], which may influence cell proliferation, although both regulatory systems are independent [6, 23].

Therefore, there is a lot of evidence suggesting the existence of an interaction between the β-adrenoceptor system and polyamines that may affect tissue function and cell proliferation. This possibility has not previously been studied, nor has the receptor selectivity in human β-adrenoceptors.

The aim of this study was to characterize the effect of polyamines on human β-adrenoceptors, biochemically and using a functional approach, in Chinese hamster ovary (CHO) cells stably expressing human β_1- (CHO-β_1) and β_2- (CHO-β_2) adrenoceptors.

Materials and Methods

Cells and cell culture

Wild-type Chinese hamster ovary cells (CHO-wt) and CHO cells deficient in dihydrofolate reductase (CHO dhfr) stably transfected with human β_1- and β_2-adrenoceptors (donated by Mari Candelore, Merck RL, Rahway, USA) were used [3]. Cells were grown in Iscoves modified Dulbecco's medium with 25 mM HEPES supplemented with 10% fetal bovine serum, hypoxanthine-thymidine (10 mM to 2 mM), 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and 700 μg/l geneticin (G418) from Gibco-Invitrogen at 37°C in a humidified incubator with 5% CO_2.

Subconfluent (70 to 80%) cells were trypsinized and seeded at a density of 8,000 cells/cm^2 and grown under cell culture conditions. The media were replaced every two days.

Cells were counted during passage in a Neubauer chamber.

Drugs

The following drugs purchased from Sigma were used: isoproterenol (1-[3',4'-dihydroxyphenyl]-2--isopropyl-aminoethanol hydrochloride), salbutamol (albuterol, α-([*t*-butylamino]methyl)-4-hydroxy-*m*-xylene-α,α'-diol), propranolol (1-[isopropylamino]-3- [1-naphthyloxy]-2-propanolol), 3-isobutyl-1-methyl- xanthine (IBMX) (3,7-dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2, 6-dione), forskolin (7β-acetoxy-1α, 6β,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one), putrescine (tetramethylenediamine), spermidine (N-[3-aminopropyl]-1,4-butanediamine) and spermine (N,N'-bis[3-aminopropyl]-1,4-butanediamine). α-Difluoromethylornithine (DL-α-difluoromethylornithine, DFMO) was donated by Dr. Wooster (Wayne University, USA). All drugs were dissolved in purified water, except IBMX, which was dissolved in dimethyl sulfoxide (the final solvent concentration in the media was less than 0.1%).

[^{125}I]Iodocyanopindolol and cAMP Biotrack Enzyme Immunoassay were from Amersham. Protease inhibitor cocktail tablets were purchased from Roche. [$^{2,8-3}$H]Cyclic AMP was purchased from Perkin-Elmer.

Microscopic evaluation of morphological changes

To study the morphological changes, 150,000 cells were grown for 14 to 16 h under tissue culture conditions in 35 mm culture dishes with 2 ml media. Next, the medium was replaced with a serum-free medium after washing cells twice with pre-warmed phosphate-buffered saline (PBS) for acute effects. Cells were maintained for 30 min under cell culture conditions and treated by adding the drugs to the culture medium.

Cultured cells were observed before treatment and during the acute exposure to the drugs from 10 min to 6 h and at 24 and 48 h using an inverted optical microscope (Nikon Diaphot 200). Images were captured with a HITACHI KP-111 camera and digitalized with an interface Dazzle DVC 130 and Pinnacle Studio (Version 10.3) software. Changes in cell morphology were judged by blind analysis by two independent investigators.

Binding assay in cell membranes from CHO-wt and CHO-β_1- and β_2-adrenoceptor cells

Cell membranes were prepared at 3 or 4 days after seeding. Membranes were prepared by hypotonic lysis of the cells in 10 ml of buffer (1 mM Tris, pH 7.2 and protease inhibitor cocktail) incubated for 5 min at 4°C in continuous orbital agitation. Then the cells were scraped, and the volume obtained was homogenized for 15 s using a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 200 × g for 5 min (this process was repeated with the pellet), and the supernatant was centrifuged at 48,000 × g for 30 min. All procedures were carried out at 4°C. The final pellets were resuspended in cold buffer (50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4) and stored at –80°C until use. Protein content, when required, was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories GmbH, München).

Receptor binding assays were carried out in a final volume of 150 µl containing 50 µg/ml of membrane protein for 2 h at 37°C with continuous agitation. β-adrenoceptor densities (*Bmax*) and K_D were estimated in saturation experiments using [^{125}I]iodocyanopindolol; specific binding was defined as the portion displaceable by propranolol. For the displacement experiments, the radioligand [^{125}I]iodocyanopindolol was used at a concentration of 40 pM for β_1- or 30 pM for β_2-adrenoceptors, and the drugs of interest were used at various concentrations in competition assays. Binding reactions were terminated by filtration through GF/C filters, washing with 15 ml cold buffer. All experiments were conducted in triplicate and repeated independently at least three times. The radioactivity was measured in a gamma counter (Cobra II auto-gamma Perkin Elmer, PACKARD).

Determination of cAMP levels in CHO cells

For cAMP measurements, the cells were cultured for 15 h before the assay, seeding 50,000 cells/well in a 24-well plate (Orange Scientific) with 2 ml culture medium/well. After seeding, the medium was replaced with fresh culture medium or in some experiments with serum-free media, and the cells were further incubated for 30 min under cell culture conditions before the addition of the assayed drugs. For each experiment, one of the wells with cells was not treated and was used as control to calculate the fold increase of cAMP in the remaining wells. After the 10 min incubation with the assayed drugs at 37°C, cells were immediately lysed. When required, cells were pre-incubated with propranolol for 10 min.

Levels of total cellular cAMP were measured using the cAMP Biotrack Enzyme Immunoassay (EIA) system (Amersham Biosciences) according to the manufacturer's recommended procedure. Cyclic AMP values were expressed in fmol/50,000 cells and fold increase in cAMP ((cAMPdrug – cAMPcontrol)/cAMPdrug).

Polyamine determination *via* high performance liquid chromatography (HPLC) in CHO-β_1-adrenoceptors and CHO-wt cells

Polyamine levels were determined using a pre-column derivatization method as previously described [8]. After a 10 min treatment with DFMO, isoproterenol or putrescine for 48 h, CHO-β_1-adrenoceptors or CHO-wt cells were collected by trypsinization, stopped with culture media, and rinsed with PBS by centrifugation. The cells 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 centrifuged at 10,000 × g for 30 min, and 0.2 ml of collected supernatants were neutralized with 0.3 ml of a saturated solution of $NaHCO_3$. The samples were dansylated overnight

with 0.5 ml of a 5 mg/ml dansyl chloride solution in acetone. After one extraction with toluene, the toluenic phase was dried under a nitrogen atmosphere, resuspended in 0.4 ml of acetonitrile and then chromatographed using a C_{18} reversed-phase column according to the method described above. Quantification of polyamines was performed using 2-hydroxydiaminopropane as an internal standard. The polyamine values were expressed as nmol/10^6 cells.

Long-term effects of the drugs on morphology, proliferation and HPLC determination were studied in cells treated with isoproterenol, propranolol, IBMX, DFMO, putrescine or spermine, depending on the type of experiment, and maintained under tissue culture conditions for 48 h, replacing the medium at 24 h. For pharmacological characterization, propranolol or DFMO was added 1 h before isoproterenol, putrescine or spermine. One dish of untreated cells was used as a control.

Cyclic AMP phosphodiesterase assay in CHO cells

Cell extracts were prepared using a method previously described [18]. Cells were rinsed with ice cold PBS before adding a lysis buffer (25 mM HEPES, 2.5 mM EDTA, 50 mM NaCl, 50 mM NaF, 30 mM Na pyrophosphate, 10% glycerol, 1% Triton X-100, pH 7.5) in the presence of the protease inhibitor cocktail (Roche diagnostics GmbH, Mannheim, Germany). Lysis was performed on ice under gentle agitation for 1 h. The insoluble proteins were removed by centrifugation at 16,000 × g for 10 min at 4°C. The supernatant fraction was used to assay phosphodiesterase activity using a two-step procedure in the reaction buffer (10 mM Tris-HCl, pH 8; 0.5 mM $MgCl_2$, 1 μM cAMP and 0.08 μCi of [$^{2,8\text{-}3}$H]cAMP) for 20 min at 30°C in a total volume of 100 μl as previously described [38]. Reactions were stopped by boiling the samples and were incubated with 50 μg snake venom *Crotalus atrox* (Sigma) at 30°C for 10 min. Then 0.4 ml of anion exchanger slurry (1 part Dowex 1X8-400, 1 part water and 1 part absolute ethanol) was added, and the mixture was incubated on ice for 15 min with frequent agitation. Samples were centrifuged at 16,000 × g for 3 min, and the radioactivity in the supernatant was determined by liquid scintillation. The results were expressed in pmol of cAMP hydrolyzed per min per mg protein. To determine the influence of polyamines on cAMP-phosphodiesterase activity, cell extracts were incubated with putrescine or spermine for 30 min.

Calculations and statistical analysis

Data from receptor binding studies were analyzed by nonlinear regression for receptor binding using Prism 2.0 (GraphPad Software) and by Scatchard plot. The K_D and B_{max} (maximal binding capacity related to the number of receptors) were determined from radioligand saturation curves fitted to a one-site model from at least four separate experiments done in triplicate.

The data obtained are expressed as the means ± SEM for at least four experiments. The significance of differences between multiple means was calculated by analysis of variance, and statistical significance was calculated using the Student's *t* test for unpaired values; $p \leq 0.05$ was considered significant.

Results

Effect of polyamines on [^{125}I]iodocyanopindolol binding to membranes of CHO-wt and CHO-β_1- and β_2-adrenoceptor cells

The binding of a specific ligand, [^{125}I]iodocyanopindolol, to human β_1- and β_2-adrenoceptors was saturable (0.5 to 200 pM) and displaced by propranolol (1 μM). Under our experimental conditions, the results showed a receptor density of 144.9 ± 3.45 fmol/mg of protein and a K_D of 37.25 ± 2.21 pM for β_1-adrenoceptor and 200.3 ± 10.6 fmol/mg of protein and a K_D of 22.8 ± 3.5 pM for β_2-adrenoceptor. The competition assays using putrescine, spermine and spermidine (10 μM to 100 mM) showed displacement of the ligand. These were concentration-dependent for putrescine, IC_{50} = 52.28 ± 4.21 and 7.07 ± 2.06 mM, respectively, for β_1- and β_2-adrenoceptors but not for spermine and spermidine. The maximum displacement obtained with spermine for β_1- (10 mM) and β_2-adrenoceptors (3 mM) was 46 ± 3.4 and 52 ± 2.8%, respectively, for specific binding. Higher concentrations reversed the displacement. Spermidine produced an even smaller effect than spermine, diminishing the effect with higher concentrations (Fig. 1). No binding of the radioligand was observed in membranes from CHO-wt cells.

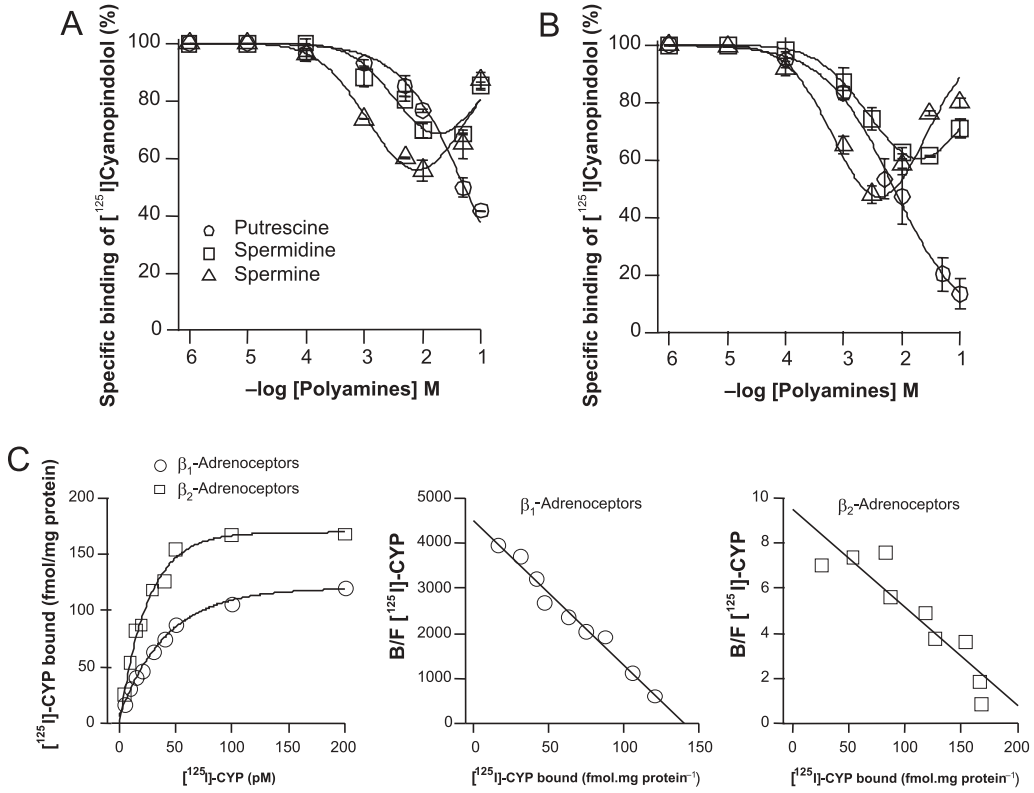

Fig. 1. Competition for specific [^{125}I]iodocyanopindolol (CYP) binding, 40 pM and 30 pM, to membranes from CHO-β_1- (**A**) and CHO-β_2-adrenoceptor cells (**B**) by the polyamines putrescine, spermidine and spermine (10 μM to 100 mM). Panel (**C**) shows specific binding of [^{125}I]iodocyanopindolol to membranes of these cells as a function of the concentration of radioligand. The specific binding of radioligand was defined as the portion displaceable by propranolol (1 μM). Scatchard plot obtained from the same data. *B*: specific bound radioligand. *B*/*F*: rate of specific binding and concentration of free radioligand. Values represent the mean ± SEM for at least four individual experiments performed in triplicate

Effect of β-adrenoceptor agonists, polyamines and IBMX on cAMP levels of CHO-wt and CHO-β_1- and β_2-adrenoceptor cells

CHO-β_1- and CHO-β_2-adrenoceptor cells have basal levels of cAMP higher (781.24 ± 91.82, 649.33 ± 107 fmol/50,000 cells) than the CHO-wt (454 ± 66.68 fmol/50,000 cells). The 15 min incubations with the β-adrenoceptor agonists, isoproterenol (1 μM) and salbutamol (1 μM), elicited 4.27 ± 0.42 and 5.31 ± 0.86 fold increases in cAMP levels in CHO-β_1- and CHO-β_2-adrenoceptors, respectively. Propranolol (1 μM), which did not significantly modify basal levels of cAMP, completely antagonized the β-adrenoceptors agonist-elicited increase of cAMP.

The 10 min incubation with putrescine (3 mM) significantly increased the levels of cAMP in CHO-β_1- and CHO-β_2-adrenoceptor cells (Fig. 2A) but not CHO-wt (Fig. 2B). The effect of putrescine was antagonized by pre-incubation with the β-adrenoceptor antagonist, propranolol (1 μM). The effect was not produced in cells considered β-adrenoceptor desensitized by long term (2 h) exposure to high concentrations (30 μM) of isoproterenol (Fig. 2A).

Spermine (3 mM) (Fig. 2C) also significantly increased the levels of cAMP in CHO-β_1- and CHO-β_2-adrenoceptor cells.

β-Adrenoceptor agonist (1 μM) and polyamine (3 mM)-elicited increases of cAMP were similar in media with serum or in serum-free media.

Isobutyl-methylxanthine (IBMX, 50 μM), an inhibitor of phosphodiesterases, produced 1.84 ± 0.5 and 2.96 ± 0.7 fold increases in cAMP in CHO-β_1- and CHO-β_2-adrenoceptor cells, respectively.

Effect of isoproterenol and polyamines on morphology and proliferation of CHO-wt and CHO-β_1- and β_2-adrenoceptor cells

The treatment with isoproterenol (0.03 to 3 μM), after a change to serum-free media, produced rapid mor-

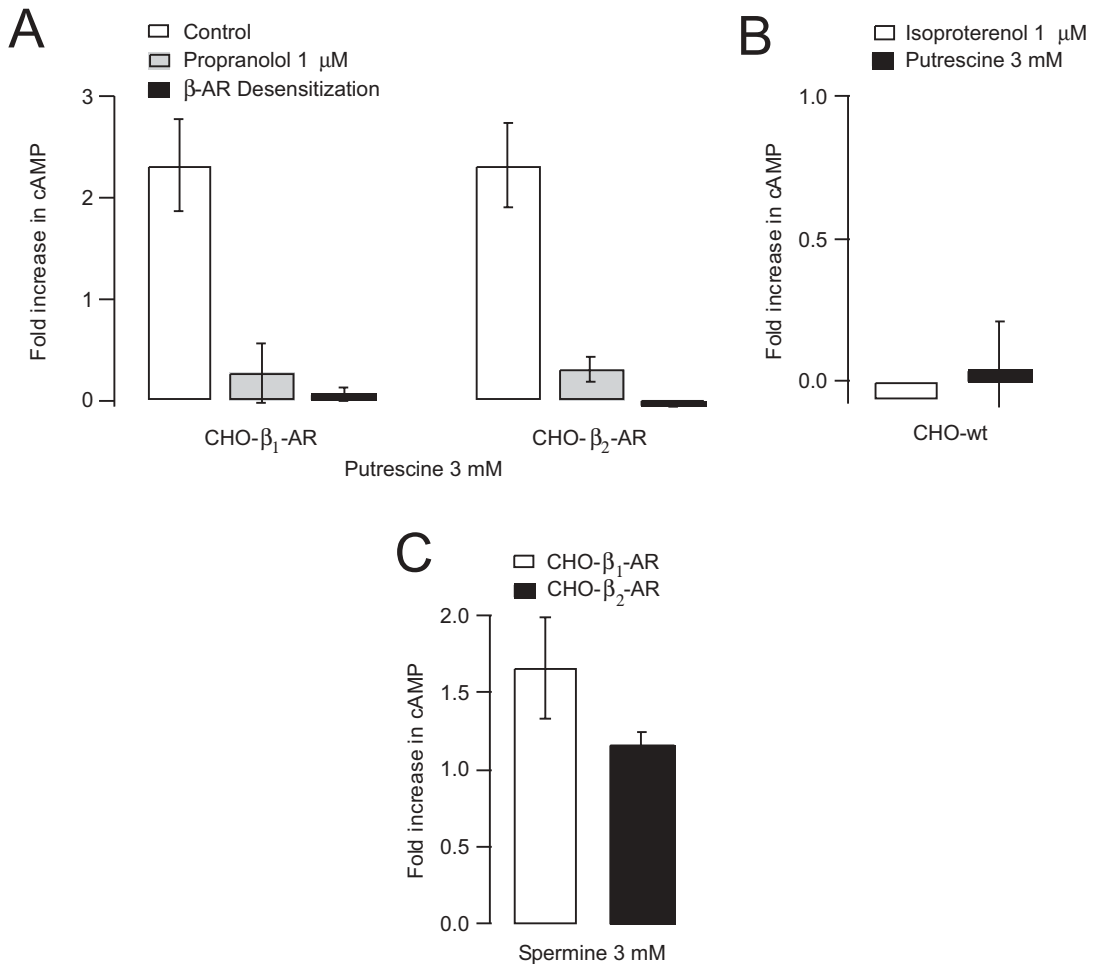

Fig. 2. (**A**) Effect of propranolol (1 μM) and β-adrenoceptor desensitization upon acute exposure to putrescine (3 mM)-elicited increase of intracellular cAMP in CHO- β_1-adrenoceptors (AR) and CHO- β_2-AR cells. (**B**) Effect of isoproterenol (1 μM) and putrescine (3 mM) on wild type CHO-wt cells. (**C**) Spermine (3 mM)-elicited increase of cAMP in CHO-β_1- and CHO-β_2-AR cells. Values represent the mean ± SEM for at least seven individual experiments

phological changes in CHO-β-adrenoceptor cells. As reported with other cAMP raising agents [7], the cells became stellate-like 10 min after the treatment with the drugs. This change was transient, even in the presence of isoproterenol, and cells reverted to a normal phenotype. The duration of this effect in CHO-β_1-adrenoceptor cells was concentration-dependent, lasting for 3 h at 0.1 μM, 4.5 h at 1 μM and longer than 6 h at 3 μM. The maintenance of the β-adrenoceptor agonist in the media with serum, for incubations longer than 8 to 10 h, changed the morphology of these cells to a spindle-like shape as previously described by cAMP increasing drugs [26]. Propranolol (1 μM), a β-adrenoceptor antagonist, prevented both types of morphological changes produced by the β-adrenoceptor agonists.

Similarly, the 10 to 15 min incubation with greater than 100 μM of the polyamines, putrescine and spermine, modified the phenotype of the cells stably ex-

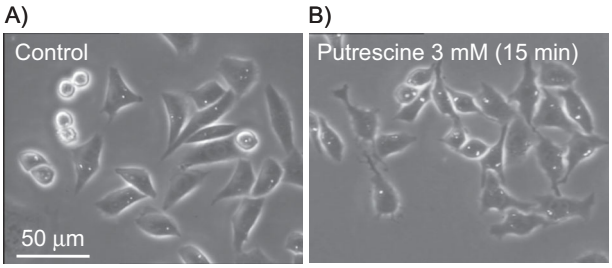

Fig. 3. Effect of putrescine (3 mM) on the phenotype of CHO-β_1-adrenoceptor cells, transforming the characteristic shape (**A**) to stellate-like form after incubation for 15 min (**B**)

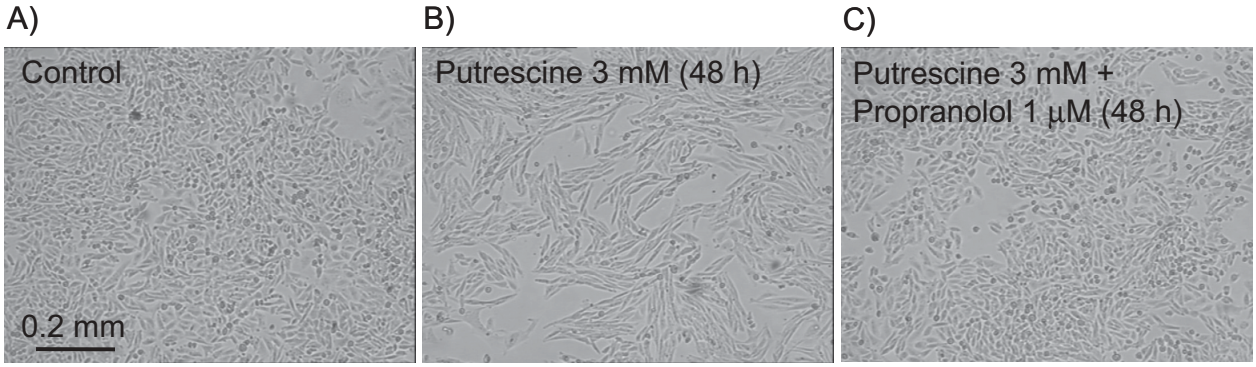

Fig. 4. Morphological effect of putrescine (3 mM) on CHO-β_1-adrenoceptor cells, transforming the characteristic shape (**A**) to spindle-like form after 48 h exposure in the absence (**B**) or the presence of propranolol (1 μM) (**C**)

pressing β_1-and β_2-adrenoceptors to a stellate-like form, identical to that described for isoproterenol (Fig. 3A and 3B). This effect lasted for 2.5 h and more than 6 h at concentrations of 1 and 10 mM of the polyamines, respectively. In these media, incubations with 10 mM spermine for 24 h were lethal.

In cells stably expressing β-adrenoceptors grown in serum containing media, the incubation with putrescine above 300 μM also elicited spindle-like morphological changes. This effect was not reversible after 24 h after the washout of the drug. These changes were also antagonized by prior incubation with propranolol (1 μM). Neither isoproterenol (1 μM) nor putrescine (10 mM) produced those morphological changes in CHO-wt cells.

In media with serum, spermine concentrations above 10 μM produced drastic and non-reversible changes in cell morphology, with a latency of approximately 2 h, and cells exhibiting a shriveled, constricted shape. By 24 h, many round, floating dead cells were observed. In contrast, the control cells were confluent and well spread with a flattened morphology. Spermine at the same concentrations was also lethal in CHO-wt cells, and propranolol (1 μM) did not prevent the lethality of spermine, even at low concentrations of spermine (10 μM) in CHO-β-adrenoceptor cells.

IBMX (50 μM), an inhibitor of phosphodiesterases, produced phenotypic changes (stellate-like and spindle-like) similar to those described for the β-adrenoceptor agonists.

In addition, putrescine (3 mM) and isoproterenol (1 μM) exposure greater than 24 h induced a reproducible, peculiar cellular organization with a tendency to be arranged in circles around an open area clear of cells (Fig. 4A and B). This effect was partially antagonized by previous exposure to propranolol (1 μM) (Fig. 4C).

The counting of the cells showed that a 48 h incubation with isoproterenol (1 μM), putrescine (3 mM)

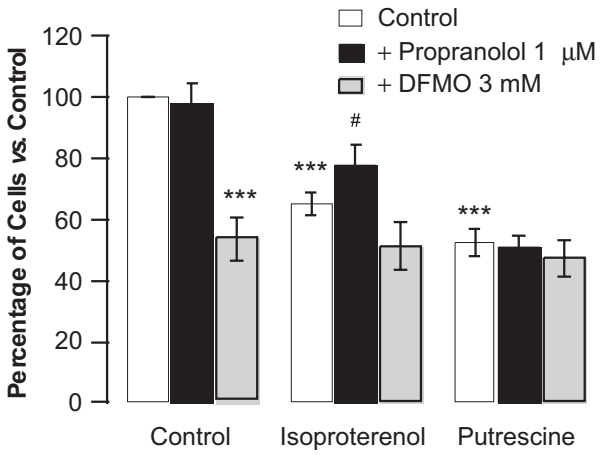

Fig. 5. Modification of the amount of CHO-β_1-adrenoceptor cells after 48 h of exposure to propranolol (1 μM), α-difluoromethylornithine (DFMO, 3 mM), isoproterenol (1 μM) or putrescine (3 mM) and in the absence or the presence of propranolol (1 μM) or α-difluoromethylornithine (DFMO, 3 mM), expressed as percentage of cells with respect to the untreated cells (control). Values represent the mean ± SEM for at least seven individual experiments. *** $p < 0.001$ *vs.* the control, untreated cells, and # $p < 0.05$ compared to the effect of isoproterenol

or DFMO (3 mM) decreased the cell counts compared to untreated cells (Fig. 5A). This effect of isoproterenol (1 μM), but not putrescine (3 mM), was partially antagonized by propranolol (1 μM). α-DFMO (3 mM) neither modified the isoproterenol nor putrescine decrease of proliferation (Fig. 5).

Effect of DFMO (3 mM), isoproterenol (1 μM) and putrescine (3 mM) on intracellular levels of polyamines determined by HPLC in CHO-β_1- and β_2-adrenoceptor cells

The 48 h incubation in the incubation media with DFMO (3 mM) decreased intracellular putrescine and spermidine levels, whereas isoproterenol (1 μM) increased the levels of spermidine and spermine. Putrescine (3 mM) increased intracellular levels of spermidine as determined by HPLC (Fig. 6).

Effect of putrescine and spermine (1 to 10 mM) on cAMP-phosphodiesterase activity assayed in CHO cell extracts

The cAMP-phosphodiesterase activity was 2.46 ± 0.08 pmol of hydrolyzed cAMP/min/mg protein. The incubation with putrescine and spermine (1 to 10 mM) did not significantly modify the basal (100%) enzymatic activity (108.67 ± 4.43 and 106.16 ± 3.99%, respectively, at 10 mM).

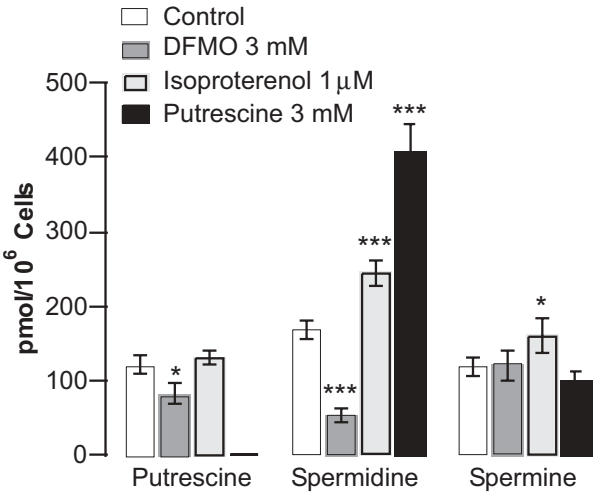

Fig. 6. Effect of α-difluoromethylornithine (DFMO, 3 mM), isoproterenol (1 μM) and putrescine (3 mM) after 48 h exposure on intracellular levels of putrescine, spermidine and spermine in CHO-β_1-adrenoceptors, expressed as pmol/10^6 cells. Values represent the mean ± SEM for at least seven individual experiments. * $p < 0.05$ and *** $p < 0.001$ *vs.* the control, untreated cells

Discussion

It has been reported that polyamines bind and may modulate the β-adrenoceptor system in preparations of rat myocardium *via* functional and biochemical studies where putrescine meets the criteria to be considered an agonist of β-adrenoceptors [2]. However, pharmacological blockade of β-adrenoceptors did not modify this effect. Equally, polyamines bind to β_2-adrenoceptors in bovine trachea membranes (unpublished), suggesting a nonselective interaction with these adrenoceptors. Accordingly, it seems that, at least in isolated preparations, polyamines may be involved in the regulation of β-adrenergic systems of the heart and respiratory smooth muscles. Similarly, because a high degree of homology exists between different species, it should also be expected to have an effect on human β-adrenoceptors [12, 17, 29].

The results obtained in human β-adrenoceptors stably expressed in CHO cells are in agreement with this hypothesis. The three intracellular polyamines assayed, putrescine, spermidine and spermine, displaced [^{125}I]iodocyanopindolol binding from β_1- and β_2-adrenoceptors with quantitative and qualitative differences. Putrescine produces a concentration-dependent displacement more potently on β_2- than β_1- adrenoceptors. However, spermidine and spermine displacement was approximately 50%, with a reversion of binding at the highest concentrations used, which is not compatible with an orthosteric interaction with diverse receptors as described for other compounds [22].

The consequences of the interaction between the polyamines and human β-adrenoceptors were studied by measuring cAMP levels and cAMP-dependent morphological changes, as previously reported [26, 31]. The proof of concept was carried out by assaying the effect of the known β-adrenoceptor agonists, isoproterenol and salbutamol, on these variables in CHO-β_1- and CHO-β_2-adrenoceptor cells. As expected, acute exposure to these agonists increased cAMP levels.

Acute exposure to putrescine and spermine also increased cAMP levels in cell lines stably expressing human β_1- and β_2-adrenoceptors. These effects were antagonized by prior incubation with propranolol and were not produced in CHO-wt cells. Furthermore, pu-

trescine failed to increase intracellular cAMP when β-adrenoceptors were desensitized by long-term exposure to high concentrations of agonists.

Acute increases of cAMP levels in CHO cells cultured in serum-free media have been associated with a transient and characteristic stellate-like form of the cells [7]. This outcome was observed after exposure of CHO-β_1- and CHO-β_2-adrenoceptor cells to the β-adrenoceptor agonist, isoproterenol, and the polyamines, putrescine and spermine.

Exposure for more than 8 to 10 h to β-adrenoceptor agonists or putrescine transformed the cells to a spindle-like shape, which was maintained up to 48 h and was not reversible 24 h after drug removal. Similar cAMP-dependent phenotypic changes have been reported in CHO cells transfected with a G-protein coupled parathyroid hormone/parathyroid hormone-related protein receptor [26].

The β-adrenoceptor agonist- and polyamine-elicited increase in cAMP is produced in the presence of serum or in serum-free media. However, it is important to note that the stellate-like form is only observed in serum-free media. Therefore, despite cAMP production, serum withdrawal is a coadjuvant to elicit this acute morphological change, but independently, lack of serum or the agonists does not elicit this response. Because cAMP-dependent stellation can be prevented by lysophosphatidic acid, it has been proposed that serum phospholipids could play an inhibitory role [7]. In any case, the acute and subacute morphological changes elicited in the CHO-β_1- and CHO-β_2-adrenoceptor cells seem to be characteristic of intracellular cAMP increases and can be used to characterize cAMP mediated responses in these cells.

Both acute and long-term morphological changes after exposure to polyamines were inhibited by prior incubation with propranolol, a β-adrenoceptor antagonist, and were not elicited in CHO-wt cells. All of these data, in association with the binding experiments, suggest that the polyamines, putrescine and spermine, elicit a β-adrenoceptor agonist mediated response, which requires functional β-adrenoceptors.

It has been reported that polyamines may increase intracellular cAMP levels by inhibiting phosphodiesterase activity [5]. In CHO-β-adrenoceptor cells, an inhibitor of phosphodiesterases, IBMX, produced an increase in cAMP levels and acute and long-term morphological changes that resembled those elicited by isoproterenol. However, this is not the mechanism by which putrescine and spermine produced the effects described because no changes in cAMP-phosphodiesterase activity were observed in cell extracts after incubation with these polyamines.

In the presence of serum in the culture media, the polyamine spermine had initiated cell death. This effect was not prevented by propranolol, excluding cAMP-dependent mechanisms in this process. These findings are in agreement with the known fact that polyamines are involved in the initiation of apoptosis in several cell types and the fetal calf serum-dependent toxicity of spermine described in CHO cells [1, 16, 36, 40, 42].

The microscopic observations also showed that CHO-β-adrenoceptor cells treated for more than 24 h with isoproterenol or putrescine, besides the characteristic spindle-like change, tended to organize from randomly oriented cells to forming a leading and trailing edge, suggesting a coordinated cluster, which could reflect cell migration. This phenomenon was not produced spontaneously and was absent in CHO-wt cells treated with isoproterenol or putrescine. This interesting finding suggests that cAMP dependent mechanisms, *via* an activation of β-adrenoceptors, are involved in this effect. This hypothesis is corroborated by the fact that propranolol blocked it.

In addition to the morphological changes, isoproterenol and putrescine decreased proliferation of CHO-β_1-adrenoceptor cells because the number of cells was reduced when compared with untreated cells but not in CHO-wt cells.

An increase in intracellular polyamine levels has been reported after incubation with β-adrenoceptor agonists, through putative ornithine decarboxylase activation [35, 43], and putrescine, *via* transport systems to the cytoplasm [45]. The antiproliferative effect of isoproterenol, but not of putrescine, was partially antagonized by propranolol, pointing out different mechanisms of action between these agents. In addition, the results suggest a dissociation between putrescine-elicited morphological and proliferative changes. These changes may be dependent and independent of β-adrenoceptors activation, intracellular cAMP/polyamines ratio, or a combination of extra/intracellular effects of putrescine that elicit its effects. It is well known that intracellular polyamines regulate cell proliferation [25]. Polyamine depletion, through the inhibition of ornithine decarboxylase, produces an antiproliferative effect [20] and, in some cases, is reversed by incubation with putrescine [23]. However, cAMP-induced growth arrest, which might be the

case in CHO-β-adrenoceptor cells, was not antagonized by putrescine [23].

To our knowledge, this is the first description of an interaction of putrescine with human β-adrenoceptors. This mechanism may help to explain the tonic [2, 9, 11, 15] and trophic [10, 35, 43] effects of polyamines reported in the heart of experimental animals. In addition to the heart, this interaction might be of clinical significance in other tissues. Cellular polyamine content is at millimolar levels [33, 37]. Their extracellular concentrations might be locally high in some areas and circumstances and induce biological effects. For instance, in prostatic fluid, the spermine level is approximately 12 mM [39]. Dietary polyamines and intestinal microflora may increase luminal levels of polyamines, whose concentrations in some parts of the intestine of rat reach 2 to 3 mM for putrescine [30]. Polyamines are growth factors for the enterocytes and intestinal mucosa [28, 32], with putrescine being important in the cecum and colon. Interestingly, the epithelium of rat distal colon and human colorectal tissue expresses β-adrenoceptors, whose activation induces ion transport [46]. Pathological conditions, such as cellular damage or infections, may also locally increase extracellular polyamines [16].

In conclusion, the results demonstrate that putrescine may act as a nonselective agonist of human β_1-and β_2-adrenoceptors, eliciting an intracellular cAMP increase in association with morphological and antiproliferative effects. Further studies should be performed to establish the physiological and/or pathological consequences of these effects in human tissues expressing β-adrenoceptors.

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