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ORIGINAL ARTICLE

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Human breast cell lines exhibit functional α_2-adrenoceptors

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Abstract Adrenergic compounds (epinephrine and norepinephrine) are the most important hormones released during stress. Several different receptors are associated with their action in different tissues. However, α_2-adrenoceptors have not yet been described in either normal or tumour human breast tissue. The aim of this work was to describe and characterize these receptors in several tumour and non-tumour human cell lines. The expression of α_2-adrenoceptors was analyzed at the RNA (RT-PCR) and protein ([^{3}H]-rauwolscine binding and immunocytochemistry) levels in different human breast cell lines, and the biological activity assessed by [^{3}H]-thymidine incorporation. The cancer IBH-6, IBH-7 and MCF-7 and the non-tumour HBL-100 cells line, expressed both α_{2B}- and α_{2C}-adrenoceptor-subtypes. A single subtype was expressed in malignant HS-578T (α_{2A}) and MDA-MB-231 and non-tumour MCF-10A cells (α_{2B}). All cell lines exhibited significant binding for the specific antagonist [^{3}H]-rauwolscine. The α-, α_2-, and the α_1-compounds with known affinity for α_2-adrenoceptors, including epinephrine, norepinephrine, yohimbine, clonidine, rauwolscine and prazosin, competed significantly with binding in MCF-7 cells. In addition, IBH-6, IBH-7 and MCF-7 cells showed significant staining with specific antibodies against α_{2B}- and α_{2C}-adrenoceptor-subtypes, when tested by immunocytochemistry. In all cell lines, the specific agonist clonidine or oxymetazoline stimulated [^{3}H]-thymidine incorporation. EC_{50} values were in the range of 20–50 fM for IBH-6, IBH-7, and HS-578T; 0.14 pM for MCF-7; 2–82 pM for HBL-100 and MCF-10A cells, and a biphasic behaviour with a maximum value at 38.0 pM, was observed for MDA-MB-231 cells. The specific α_2-adrenergic antagonist rauwolscine always reversed this stimulation at 0.1 nM. In conclusion, this study describes for the first time, the presence of α_2-adrenoceptors in human epithelial breast cell lines. Moreover, activation of these receptors was associated with an enhancement of cell proliferation.

Keywords α_2-Adrenoceptors · Breast cancer · Normal breast · Cell proliferation · Clonidine · Rauwolscine

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Introduction

Women who are diagnosed with breast cancer are at high risk of experiencing significant emotional distress, with a subgroup manifesting symptoms of anxiety and depression that meet criteria for psychiatric disorder up to several years after the diagnosis [14, 28]. Typical stress responses include elevated plasma levels of catecholamines (norepinephrine and epinephrine), with the physiological consequences of tachycardia, increased blood pressure, and sweating [28, 64]. New data have reinforced the importance of the immune system on prognosis of early stage breast cancer [9, 51]. Even though some important actions of catecholamines, such as the well documented effects on the immune system [3, 45], may be indirect, their direct effects and the presence of receptors for these compounds on breast cancer cells, could be of importance in the regulation of cell proliferation and thus in the evolution of the disease.

Catecholamines exert their action by binding to different membrane receptors, which have diverse and even opposite actions, as is often the case for α_2- and β-adrenoceptors. Increased cyclic AMP levels, seen with β-adrenergic stimulation inhibit the growth of MCF-7 human breast cancer cells, producing changes in cell morphology [11, 12, 22, 37], and leading to apoptosis [4, 21]. The presence of β-adrenoceptors has been reported in rat mammary carcinomas induced by dimethylbenzanthracene [41] and in human breast cancer cells [60, 65], where receptor stimulation is associated with immediate reduction in DNA synthesis [56].

Although α-adrenoceptors have not been yet described in either normal or tumour human breast cells, they have been found in the milk-purging system of the normal bovine mammary gland, associated mainly with muscular cells [26]. Recently, the expression of the different subtypes of α_2-adrenoceptors was assessed by RT-PCR in lactating bovine mammary gland [31, 32]. The highest mRNA expression of α_2-adrenoceptors was found for the α_{2A}-subtype, followed by α_{2B} and α_{2C}. Our group has demonstrated that α_2-adrenergic agonists significantly increase [^{3}H]-thymidine incorporation into DNA in MCF-7 human breast cancer cells and this effect was associated with a decrease in cyclic AMP levels [63]. However, the α_2-adrenoceptors involved in this response have not yet been described or characterized.

In the present work, we have used the following cell lines: IBH-6, IBH-7, MCF-7, MDA-MB-231, HS-578T, HBL-100 and MCF-10A. IBH-6 and IBH-7, which were developed in our laboratory from primary breast carcinoma cultures, exhibit epithelial characteristics and are oestrogen-sensitive [62]. MCF-7 cells are well differentiated, oestrogen-sensitive and metastatic, derived from pleural effusion [58]. MDA-MB-231 [8] and HS-578T [25], are fast growing cell lines, aggressive and oestrogen receptor negative. HBL-100, a cell line established in vitro from milk of an apparently healthy woman, exhibits several characteristics of transformation [23], and expresses SV40 large T antigen, which is defective in some functions [61]. MCF-10A is an immortal cell line that arose spontaneously from human diploid breast cells of extended life span, obtained from a pre-menopausal woman with fibrocystic disease [57].

The present manuscript describes and characterizes for the first time, the presence of α_2-adrenoceptors in both tumour and non-tumour human epithelial breast cell lines, both at the RNA (by RT-PCR followed by Southern blotting) and protein levels (by binding assays and immunocytochemistry). Moreover, these receptors prove to be functional, as judged by the increase in [^{3}H]-thymidine incorporation, when breast cells are stimulated by α_2-adrenergic agonists.

Materials and methods

Drugs and chemicals

Foetal calf serum was purchased from Gen S.A. (Buenos Aires, Argentina) or Invitrogen Life Technologies (Carlsbad, CA, USA). Glutamine, oxymetazoline HCl, clonidine HCl, L-phenylephrine HCl, yohimbine HCl, rauwolscine HCl, WB-4101 [2-(2,6-dimethoxy phenoxy ethyl)amino methyl-1,4-benzodioxane HCl] were purchased from ICN Biomedicals Inc (Ohio, USA). [6-^{3}H]-thymidine, [^{3}H]-rauwolscine and [^{32}P]-dATP were from Dupont-New England Nuclear (Boston, MA, USA). Liquid scintillation cocktail was Optiphase 'Hisafe' 3, (Wallac, Turku, Finland). Culture media, antibiotics, trypsin, insulin, RNAse inhibitor, DNA Taq polymerase and random primer labelling kit were from Invitrogen Life Technologies (Carlsbad, CA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). MMLV reverse transcriptase was from Promega (Madison, WI, USA), random hexanucleotides from Byodinamics (Bs. As., Argentina) and Zeta Probe nylon membranes were from BioRad (Richmond, CA, USA). Anti-human α_{2B} (C-19) and α_{2C}-RA (C-20) goat polyclonal antibodies, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-goat peroxidase conjugate and the Catalyzed Signal Amplification System were from DakoCytomation (Carpinteria, CA, USA).

Cell cultures

The MCF-7, MDA-MB-231, HS-578T, HBL-100 and MCF-10A human breast cells and the human colon HT-29 cell line, were obtained from the American Type Culture Collection (Manassas, VA, USA). The IBH-6 and IBH-7 cell lines have been developed in our laboratory [62]. All cells (except MDA-MB-231) were routinely cultured in phenol red free [2] Dulbecco's Modified Eagle's Medium:Ham F12 (1:1) supplemented with 10% foetal calf serum, 2 mM glutamine, 2 μg/ml bovine insulin, 100 UI/ml penicillin, 100 μg/ml streptomycin, 15 mM HEPES and 10 nM estradiol. MCF-10A medium was supplemented with 20 ng/ml epidermal growth factor. Cells were sub-cultured once weekly after trypsinization (0.25% trypsin–0.025% EDTA) and seeded at a concentration of 80,000 cells/25 cm^2 flask. Medium was changed twice weekly. Oestrogen-sensitive cells were routinely monitored. MDA-MB-231 cells were incubated in Leibovitz's L-15 medium with 2 mM L-glutamine and 10% foetal calf serum [8].

RT-PCR and Southern blot

Total RNA was isolated according to the method described by Chomczynsky and Sacchi [13] and quantified by absorbance at 260 nm. cDNA was obtained incubating 5 μg of each total RNA with 200 U of MMLV reverse transcriptase, 10 μg RNase inhibitor, 0.5 mM dNTPs and 0.2 μg of random hexanucleotides for 1 h at 37°C. To amplify each subtype of α_2-adrenoceptors, the primers described by Eason and Liggett [20] were used: 5′-AAACCTCTTCCTGGTGTCTC-3′ and 5′-AGACGAGCTCTCCTCCAGGT-3′ for α_{2A}, 5′-CCTGGCCTCCAGCATCGGAT-3′ and 5′-CAGAGCACAAAAACGCCAT-3′ for α_{2B} and 5′-GTGGTGATCGCCGTGCTGAC-3′ and 5′-CGTTTTCGGTAGTCGGGGAC-3′ for α_{2C}. These primers amplify a sequence of 691 bp for α_{2A}, 630 bp for α_{2B} and 574 bp for α_{2C} subtypes. Full-length cDNA clones for these receptors (kindly provided by Dr. J. Lefkowitz, Duke University Medical Center, Durham, NC, USA) were used as positive controls. Amplification of β-actin was

performed in order to assure the quality control of RNA and to detect any possible genomic DNA contamination. For that reason, a pair of oligonucleotides priming in two contiguous exons of the β-actin gene was used, rendering PCR products of 444 or 554 bp, when amplified from cDNA or genomic DNA, respectively. The primers used for this amplification were: 5′-ATCATGAAGTGTGACGTGGAC-3′ and 5′-AACCGACTGCTGTCACCTTCA-3′ [20].

For amplification of cDNA, reverse transcription (RT) products from 0.5 μg of RNA were incubated with 500 nM of each primer, 200 μM of each dNTP, 2 mM $MgCl_2$, 10% dimethylsulphoxide and 2 U of DNA Taq polymerase, for 33 cycles of 1 min at 94°C, 50 s at 55°C and 100 s at 72°C. The products were analyzed by electrophoresis in 1.5% agarose gels, stained with ethydium bromide and vacuum transferred to Zeta Probe nylon membranes. The full-length cDNAs from each receptor subtype [35, 39, 48] were used as probes and labelled with [^{32}P]-dATP with a random primer from a commercial kit. In all cases, the specific activity of the probes was $>10^9$ cpm/μg. Membranes were incubated 18 h in hybridization buffer with heat-denatured probes at 65°C according to the manufacturer's instructions. Filters were washed, exposed to autoradiographic film and developed.

[^{3}H]-rauwolscine binding

Binding assays for α_2-adrenoceptors were carried out as described for histamine receptors [54] and modified by Jansson et al. [34]. Cells were incubated in 24 well plates in Dulbecco's modified Eagle's medium/F12 (or Leibovitz's L-15 for MDA-MB-231 cells) with 10% foetal calf serum until near confluence, followed by 48 h incubation with 2% charcoal-stripped foetal calf serum. Cells were washed with TME buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4) and incubated 30 min for Scatchard analysis, with increasing concentrations of [^{3}H]-rauwolscine at 37°C. Non-specific binding was evaluated by parallel incubation with 50 μM WB-4101 (which binds to both α_1- and α_2-adrenoceptors) or rauwolscine, with similar results. The reaction was stopped by washing three times with TME buffer and 2 ml scintillation cocktail were added directly over the cell monolayer. Radioactivity was measured in a liquid scintillation counter. Cell number was determined in parallel incubations.

Competition studies were performed to evaluate binding specificity. Cells were processed as described for Scatchard analysis, except that they were incubated with 4 nM [^{3}H]-rauwolscine with or without increasing concentrations of the different agonists or antagonists. Results are expressed as percentage of binding obtained in the absence of unlabelled compounds. K_i values were determined according to the equation described by Cheng and Prusoff [10].

Immunocytochemical studies

Cells were cultured in Lab-Tek Chamber Slide System (Nunc) with 10% foetal calf serum in phenol red free Dulbecco's modified Eagle's medium:F12 (1:1). Two days before immunocytochemistry medium was changed to RPMI with 2% charcoal-stripped foetal calf serum. The cells were fixed in 10% buffered formalin containing 0.2% triton X−100 in phosphate buffer saline (PBS) for 30 min at room temperature and washed with PBS, three times, 5 min each. The chambers were incubated 20 min at room temperature with 3% H_2O_2 in distilled water to quench endogenous peroxidase activity, washed extensively with PBS and incubated in 2% albumin-PBS. Cells were incubated for 60 min at 37°C with anti-human α_{2B} (C-19) or α_{2C}-RA (C-20) goat polyclonal antibody (Santa Cruz Biotechnology) diluted 1:100 and 1:60, respectively in 1% albumin-PBS. These antibodies were raised against a peptide corresponding to amino acids on the internal carboxy-terminal regions of α_2-adrenoceptor. Chambers were washed with PBS and subsequently incubated for 30 min at room temperature with anti-goat peroxidase conjugate (DakoCytomation). The Catalyzed Signal Amplification System (DakoCytomation) was used to amplify the intensity of receptor staining. The reactivity was visualized with DAB. Controls were performed omitting primary antibodies.

Proliferation assays

Cells were seeded at 7,000 cells/well in 96 well plates and incubated in RPMI 1640 phenol-red free medium (or Leibovitz's L-15 for MDA-MB-231 cells) with 2% charcoal-stripped foetal calf serum, 2 mM glutamine, 100 UI/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B and 15 mM HEPES. After 48 h, cells were considered synchronized, the medium was changed and adrenergic/antiadrenergic agents were added, together with 0.2 μCi [^{3}H]-thymidine/well. All solutions of adrenergic compounds were prepared in 10 mM ascorbic acid, frozen and diluted immediately before use. Antagonists were added 20 min before the agonists. After approximately 20 h incubation, cells were harvested in a Nunc Cell Harvester 8, and filters were counted in a liquid scintillation counter. MCF-10A cells were trypsinized prior to harvesting.

Statistical analysis

Statistical analysis of the effect of agonists/antagonists and binding competition was performed by ANOVA followed by Tukey–Kramer test [19]. Differences were considered statistically significant when $P<0.05$.

Results

Expression of α_2-adrenoceptor subtypes

The expression of α_2-adrenoceptors was analyzed at the RNA level, by RT-PCR from total RNA from different human epithelial breast cell lines. PCR product specificity was examined by Southern blot hybridization with the different probes for each adrenoceptor subtype. Oestrogen-sensitive cancer cells IBH-6, IBH-7 and MCF-7 and transformed but not tumour at low passages HBL-100 cells, expressed both α_{2B} and α_{2C} subtypes (Fig. 1). On the other hand, a single subtype was evidenced by the aggressive cancer oestrogen-insensitive MDA-MB-231 (α_{2B}) and HS-578T cells (α_{2A}) and the non-transformed MCF-10A cells (α_{2B}). Probes for each receptor subtype were used as positive control, in addition, for the α_{2A} subtype, human colon HT-29 cell line [7] was also included in the study. Genes for α_2-adrenoceptors are continuous, without introns [49]. Accordingly, the risk of contaminating the reversed transcribed mRNA with genomic DNA was examined with primers for β-actin whose product spans an intron and thus provides a product 110 bp larger than would be apparent by RT reaction from RNA [20]. Our experimental data show only one fragment of 444 bp without any contamination with the product of 554 bp, indicating that there was no amplification of genomic DNA, ensuring both the real nature of the adrenoceptor subtypes found and the quality of the RNA extracted (data not shown).

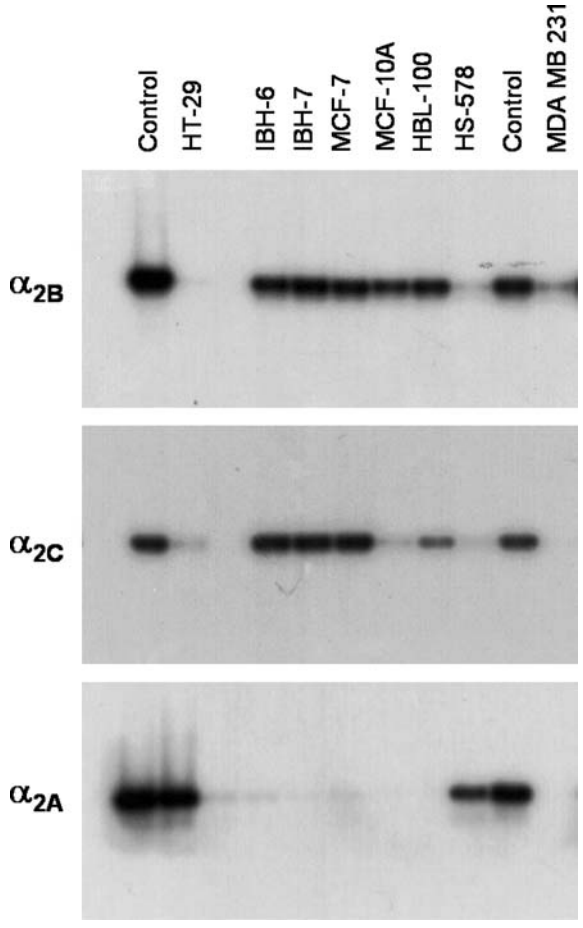

Fig. 1 Expression of α_{2B}-, α_{2C}- and α_{2A}-adrenoceptors in different human breast epithelial cell lines. Total RNA extracted from IBH-6 IBH-7, MCF-7, MCF-10A, HBL-100, HS-578T and MDA-MB-231 cells was reverse transcribed and cDNA products were subjected to PCR amplification using specific primers for the different α_2-adrenoceptor subtypes, as described in Materials and methods. HT-29 cells were used as positive controls, as well as the cloned probes for each subtype of receptors. RNA quality and possible contamination with genomic DNA were assessed by analysis of cDNA with β-actin primers (not shown). Southern blot analysis was performed on PCR amplification products

Scatchard analysis of α_2-adrenoceptors

In order to assess whether RNA expression correlated with gene translation, two approaches were undertaken. First, we monitored the binding of a specific α_2-adrenergic antagonist. Scatchard analysis for all the cell lines is depicted in Fig. 2. A single binding site was found in each cell line, although the RT-PCR analysis showed the presence of two isoforms in most of them. All cell lines showed similar K_d values, with slight differences in the binding capacity (Table 1).

Binding specificity

The specificity of [^{3}H]-rauwolscine binding was assayed as described in Materials and methods on MCF-7 cells (see Fig. 3). All α-, α_2- or α_1-compounds with known affinity for α_2-adrenoceptors, efficiently competed for [^{3}H]-rauwolscine binding. Compounds assayed included: α_2-adrenergic agonists (Fig. 3a): norepinephrine and epinephrine (natural α-agonists) and clonidine (α_2-agonist) and antagonists (Fig. 3b): prazosin (α_1-antagonist with affinity for α_2-adrenoceptors, particularly α_{2B} and α_{2C}), rauwolscine and yohimbine (α_2-antagonists). EC_{50} and K_i values obtained for all compounds are detailed in Table 2. The α_1-agonist, phenylephrine and the β-agonist isoproterenol, caused a significant and striking enhancement of rauwolscine binding (Fig. 3c). These results are in general agreement with classical affinity for α_2-adrenoceptors, particularly α_{2B} and α_{2C} subtypes, except for the increase caused by non-α_2-compounds (a possible explanation of this effect is discussed below).

Immunocytochemical studies

The second approach used to confirm the presence of adrenoceptors at the protein level was immunocytochemistry. When MCF-7 cells were exposed to specific antibodies to α_{2B}- and α_{2C}-adrenoceptors subtypes, nearly all cells showed positive reactivity (Fig. 4). The

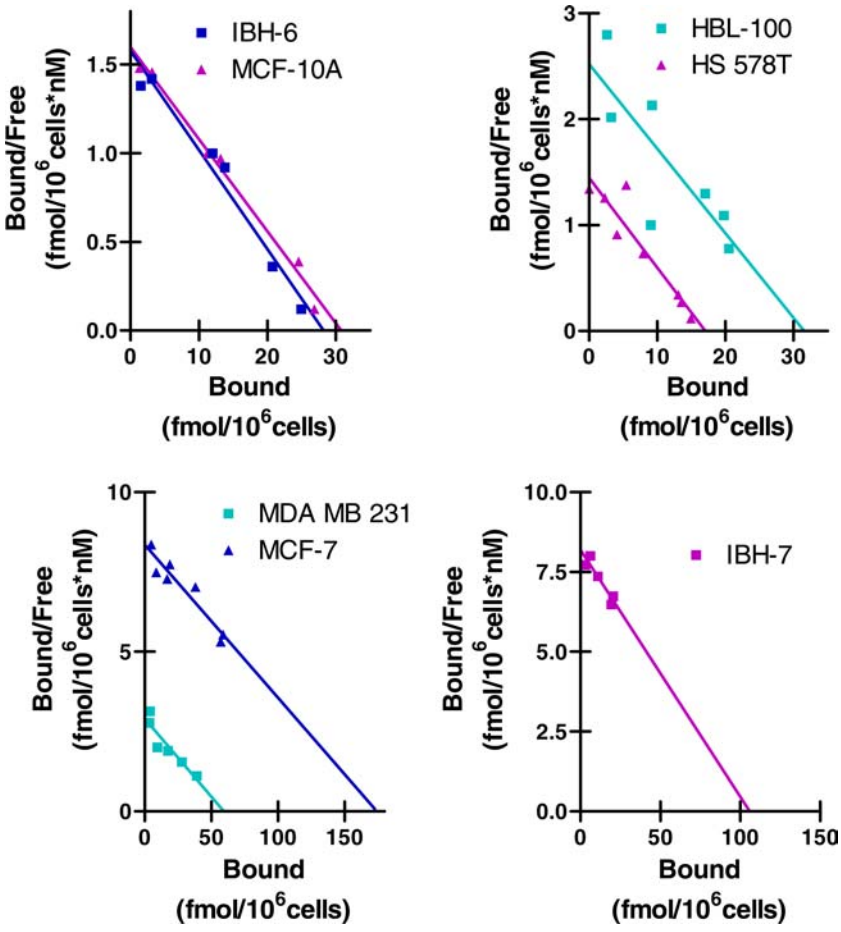

Fig. 2 Scatchard analysis of the binding of [^{3}H]-rauwolscine to different cell lines. Scatchard analysis was performed, as described in Materials and methods, with increasing concentrations of [^{3}H]-rauwolscine and parallel incubations with 50 μM WB-4101 or rauwolscine (with similar results) in whole cell assays. Binding was performed in triplicate during 30 min at 37°C in buffer. The cells were then washed, exposed to scintillation cocktail and counted. The slope was calculated by linear regression. Experiments were repeated at least twice for all cell lines with similar results

Table 1 Binding parameters of [^{3}H]-rauwolscine to human breast cells (MCF-7, IBH-6, IBH-7, MDA-MB-231, HS-578T, HBL-100 and MCF-10A)

Cells	K_d (nM)	Binding capacity (fmol/10^6 cells)	r^2
MCF-7	20.87 ± 3.05	171.20 ± 39.37	0.90
IBH-6	17.86 ± 1.56	28.13 ± 6.75	0.97
IBH-7	12.94 ± 2.74	105.90 ± 25.4	0.88
MDA-MB-231	25.97 ± 6.74	68.0 ± 19.72	0.85
HS-578 T	15.20 ± 8.95	14.96 ± 3.01	0.88
HBL-100	12.52 ± 3.99	31.51 ± 2.05	0.86
MCF-10 A	16.78 ± 1.99	32.06 ± 6.07	0.98

Scatchard analysis of [^{3}H]-rauwolscine binding was performed for each cell line as described in Materials and methods. Values were calculated from the curves shown in Fig. 2

commercial goat polyclonal antibody directed against human α_{2B}-adrenoceptors (Fig. 4a) produced positive staining mainly on the cell surface, although some reactivity was also observed in the cytoplasm. Conversely, when the goat antibody directed against α_{2C}-adrenoceptors was used (Fig. 4b), localization was mainly cytoplasmatic, with some reactivity at the level of the cell surface. Staining in controls was negligible (Fig. 4c).

These results are in agreement with the previous RT-PCR and confirm the presence of both α_{2B}- and α_{2C}-adrenoceptors at the protein level in MCF-7 cells. Moreover, localization of these receptors is similar that described in other tissues.

Proliferation studies

The biological activity of the specific α_2-adrenergic agonist clonidine was assessed analyzing cell proliferation as described in Materials and methods, in HBL-100, IBH-6, IBH-7 and MCF-10A (Fig. 5a) and MCF-7, MDA-MB-231 (Fig. 5b) cell lines, whereas oxymetazoline, a more specific α_{2A} agonist, was used in HS-578T cells (Fig. 5b). These agonists significantly stimulated [^{3}H]-thymidine incorporation with an EC_{50} of 18.8 fM for IBH-6 cells, 19.3 fM for IBH-7, 0.14 pM for MCF-7 and 50.9 fM for HS-578T. In the non-tumourigenic cells the values were: 2.38 pM for HBL-100 and 82.1 pM for MCF-10A. In MDA-MB-231 cells, enhancement of thymidine incorporation by clonidine achieved a

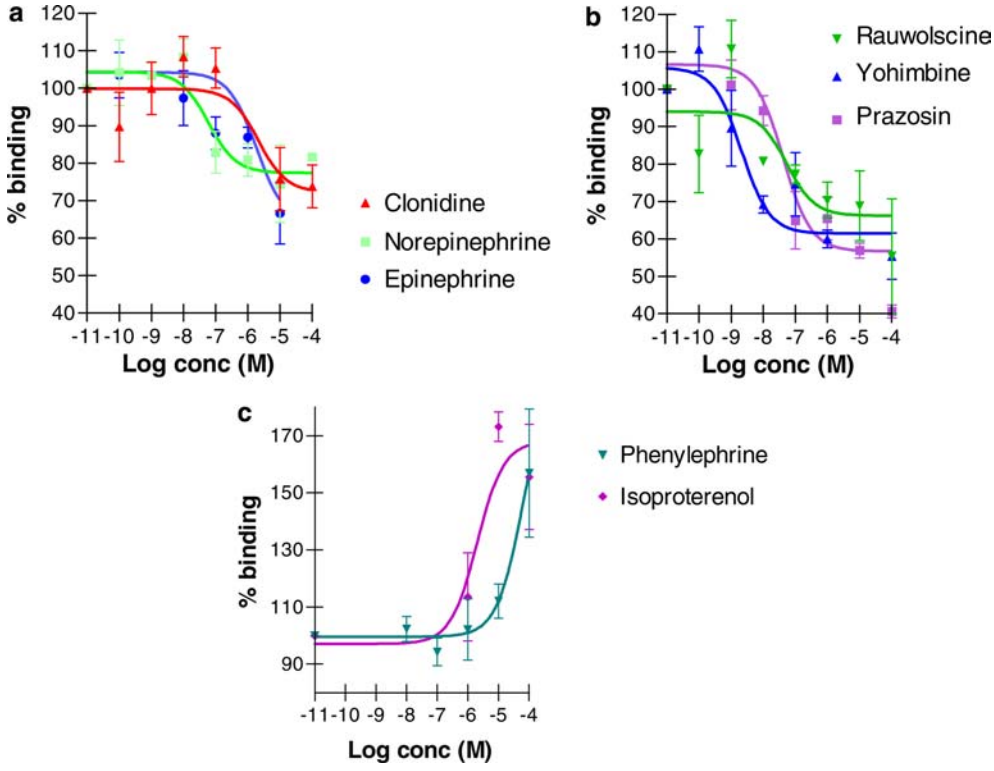

Fig. 3 Competition analysis of the binding of [^{3}H]-rauwolscine to MCF-7 cells. Competition analysis was performed in whole cells, as described in Materials and methods, with a constant concentration (4 nM) of [^{3}H]-rauwolscine in the presence or absence of increasing concentrations of different compounds. α-agonists **a** were clonidine (*filled triangle*), epinephrine (*filled circle*) and norepinephrine (*filled square*). α-antagonists **b** were: yohimbine (*filled triangle*), rauwolscine (*inverted filled triangle*) and prazosin (*filled square*) and non-α_2 compounds **c** were: phenylephrine (*inverted filled triangle*) and isoproterenol (*filled diamond*). Binding was performed during 30 min at 37°C in triplicate and counted as previously described. Experiments were repeated twice with similar results

Table 2 Binding displacement of [^{3}H]-rauwolscine by different adrenergic agonists in human breast cells MCF-7

Compound	Action	EC_{50}	K_i (nM)
Yohimbine	α_2-antagonist	2.1 nM	1.87 nM
Rauwolscine	α_2-antagonist	53.9 nM	48.0 nM
Clonidine	α_2-agonist	2.13 μM	1.90 μM
Epinephrine	α-agonist	1.84 μM	1.69 μM
Norepinephrine	α-agonist	58.6 nM	55.3 nM
Prazosin	α_1- and α_2-antag	41.7 Nm	38,2 nM

Competition analysis of [3H]-rauwolscine binding was performed in the MCF-7 cell lines as described in Materials and methods. Values shown were calculated from the curves shown in Fig. 3

maximum value at 38.0 pM, which decreased at higher agonist concentrations. The α_2-adrenoceptor antagonist rauwolscine per se had no significant effect on [^{3}H]-thymidine incorporation (not shown). However, in the presence of an agonist, rauwolscine concentrations as low as 0.1 nM reversed the stimulatory effect on cell proliferation in all cell lines assayed (Fig. 5c, d). In summary, a specific α_2-adrenergic agonist was able to stimulate [^{3}H]-thymidine incorporation at very low concentrations in every cell line tested, and this effect was reversed by low concentrations of a specific antagonist.

Discussion

Expression of α_2-adrenoceptor subtypes

The expression of α_2-adrenoceptors in different human breast cell lines was clearly demonstrated at the RNA level by RT-PCR. The cancer hormone-responsive, the transformed and one of the non-tumor cells expressed both α_{2B} and α_{2C} subtypes, whereas malignant, hormone-insensitive HS-578T and MDA-MB-231 and other non-tumour cell line showed expression of a single subtype: α_{2A} and α_{2B}, respectively. These results contrast with those reported for normal lactating bovine mammary glands [31], where although α_{2B} and α_{2C} subtypes were (also) present, the maximal expression corresponded to α_{2A}-adrenoceptors. α_2-adrenoceptors were minimally expressed in epithelial cells as well [26].

Scatchard analysis of α_2-adrenoceptors

The presence of α_2-adrenoceptors at the protein level was assessed by different techniques. According to the observations made in cells transfected with the α_{2C} gene [66], a special feature of this receptor is its intracellular localization, thus its presence would be underestimated with the use of the membrane-binding assay. In agreement with the finding, whole cell analysis at 37°C revealed significant binding of the tritiated antagonist rauwolscine in all cell lines tested. Scatchard analysis of

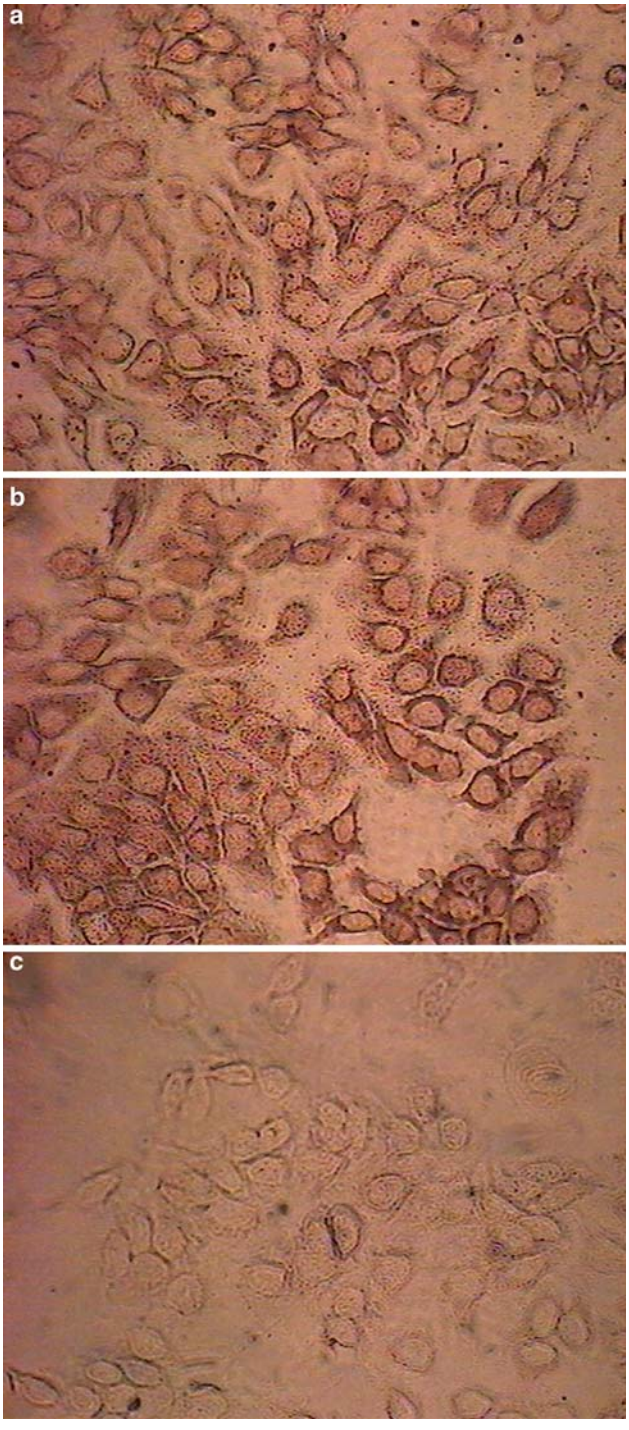

Fig. 4 Immunocytochemistry of MCF-7 cells. Immunocytochemistry was performed as stated in Materials and methods. Incubations were performed in the presence of commercial specific polyclonal antibodies directed against α_{2B}- (**a**) or α_{2C}-adrenoceptors (**b**) or in the absence of antibody (control, **c**). Antibodies were used at a concentration 1:100 for α_{2B}- and 1:60 for α_{2C}- adrenoceptors. Original magnification was 400×. Experiments were repeated twice with similar results

α_2-adrenoceptors in all cell lines, suggested an apparent single binding site; however, two receptor subtypes were found for the majority of them by RT-PCR. K_d values reported for rauwolscine in S115 cells transfected with each receptor that they are 3.4 nM for α_{2B} and 0.9 nM for α_{2C} [33]; since these affinities are relatively similar, the presence of two different binding sites could be easily masked. Accordingly, RNA interference for α_2-adrenoceptors, mediated by short interfering RNAs, will be performed in our laboratory in the near future in order to differentiate and analyze each receptor subtype. It is worth noting that the K_d values found in the present study were higher than those described in transfected cells. Comparison of binding parameters in mouse mammary tumour cell lines stably transfected with the different human α_2-adrenoceptor subtypes [42] with K_d values described herein, indicates a lower affinity in human breast cells. Both the cell system used and differences in experimental conditions, could account for this discrepancy. Moreover, a study performed in intact cells at 37°C, reported a K_d value of 11 nM for α_{2A}, similar to those described in this paper [34]. One reference was found in the literature describing α_2-adrenoceptors in normal bovine mammary gland with a K_d value of 4.42 nM in the teat and 4.38 nM in muscle cells of mammary duct, with no binding in the parenchyma [26].

Binding specificity

Competition studies performed with [^{3}H]-rauwolscine to assess α_2-adrenoceptor binding specificity clearly showed two patterns of events that characterize the tested compounds. The classical α_2-adrenergic agonists and antagonists, and some α_1-compounds with known affinity for α_{2B}- and α_{2C}-adrenoceptors, competed for [^{3}H]-rauwolscine binding. In studies of α_2-adrenoceptors in normal bovine mammary gland, the K_i value described for epinephrine was 3.5 μM, which is very similar to the one described here, for prazosin, 3.3 μM [26]. A higher sensitivity to norepinephrine than epinephrine was found in the present investigation, as revealed by a higher apparent K_i was described for the latter (1,690 nM) in comparison to the former (55.3 nM). This is in agreement with results obtained when CHO cells were transfected with wild type α_{2B}-adrenoceptor [24]: K_i was higher for epinephrine with a value of 1,495 nM, similar to the one reported in this paper, and K_i for norepinephrine was 479 nM, an order of magnitude different from the one described here. The α_2-adrenoceptor antagonist yohimbine proved to be the most efficient compound in competing for [^{3}H]-rauwolscine binding. This compound is known to be fourfold to 15-fold more selective for α_{2C}-adrenoceptors [36] and a 4.8-fold more selective for α_{2B}-adrenoceptors than for α_{2A}-adrenoceptors [69]. The low competition achieved with clonidine could be related to its partial agonist effect when acting through α_{2B}-adrenoceptors [38]. It has been reported that α_{2C}-adrenoceptors is similar to the α_{2B}-adrenoceptors with respect to their relatively high affinity for prazosin [6].

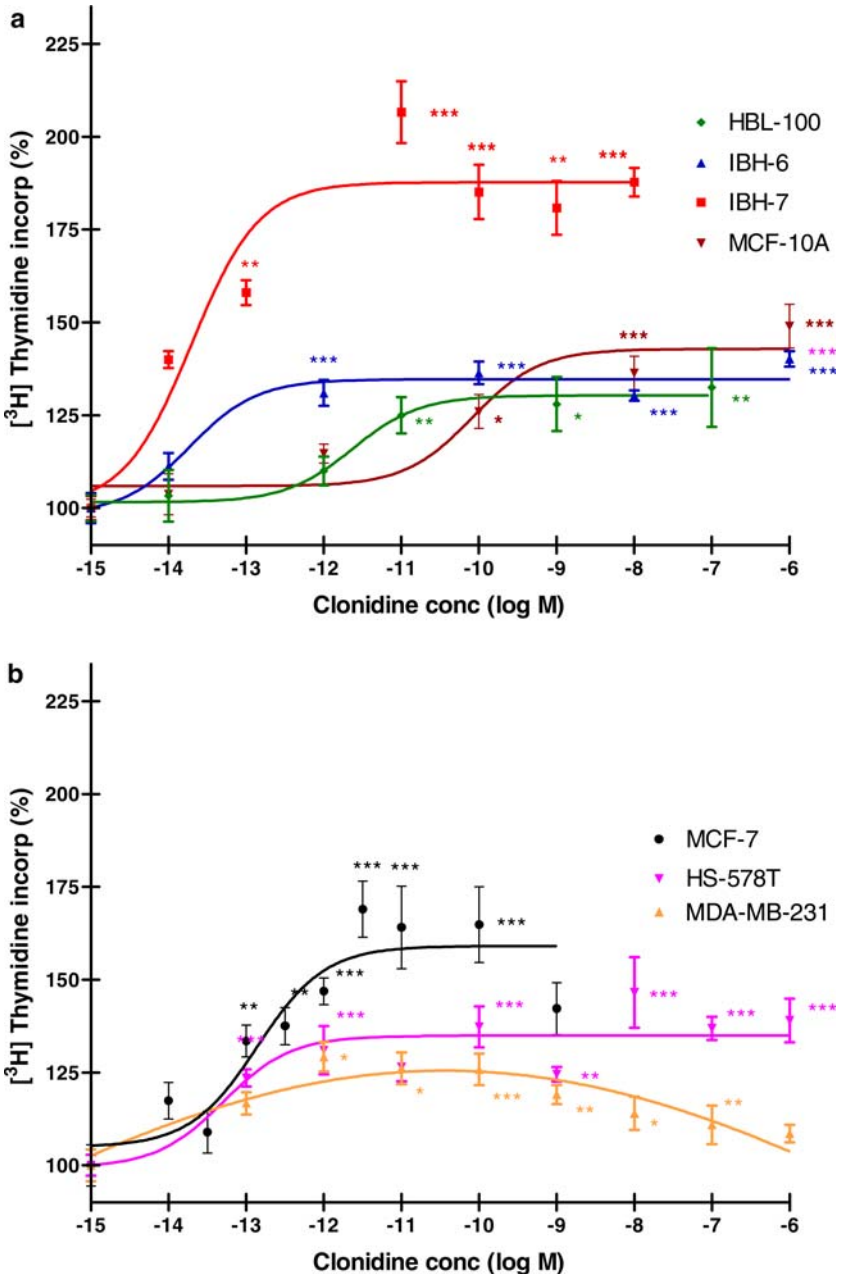

Fig. 5 Effect of the α_2-adrenergic agonist oxymetazoline (in HS-578T cells) or clonidine (in the remaining cell lines) in the presence (**c, d**) or absence (**a**, **b**) of the α_2-adrenergic antagonist rauwolscine on [^{3}H]-thymidine incorporation to synchronized human breast cell lines. Proliferation analysis was performed as described in Materials and methods. In **a**, increasing concentrations of the agonist clonidine were incubated as already stated on IBH-7 (*filled square*), IBH-6 (*filled triangle*), HBL-100 (*filled diamond*) and MCF-10A (*inverted filled triangle*) cell lines. In **b**, increasing concentrations of the agonist (oxymetazoline or clonidine) were incubated as already stated on MCF-7 (*filled circle*), MDA-MB-231 (*filled triangle*) and HS-578T (*inverted filled triangle*) cell lines. In **c**, 1 nM agonist (clonidine or oxymetazoline for HS-578T cells) was incubated in the presence or absence of increasing concentrations of the specific antagonist rauwolscine on HBL-100 (*filled circle*), MDA-MB-231 (*filled diamond*), HS-578T (*filled triangle*) and MCF-10A (*inverted filled triangle*). In **d**, 1 nM clonidine was incubated in the presence or absence of increasing concentrations of the specific antagonist rauwolscine on IBH-7 (*filled square*), IBH-6 (*filled triangle*) and MCF-7 (*filled circle*) cell lines. The value obtained in the absence of antagonist was considered 100% for each cell line, and remaining values were calculated with respect to this one. Values are the mean ± SEM of eight wells for treated and 16 for untreated cultures. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ represent significant differences between groups, as analyzed by ANOVA followed by Tukey–Kramer comparison test. Experiments were repeated twice with similar results

Most of the responses described in the literature for canonical α_2-agonists and antagonists are related to α_{2A} receptors, completely absent in the majority of the breast cell lines here examined. The competition curves described in this paper are compatible mainly with the presence of α_{2B}-adrenoceptors found by RT-PCR and

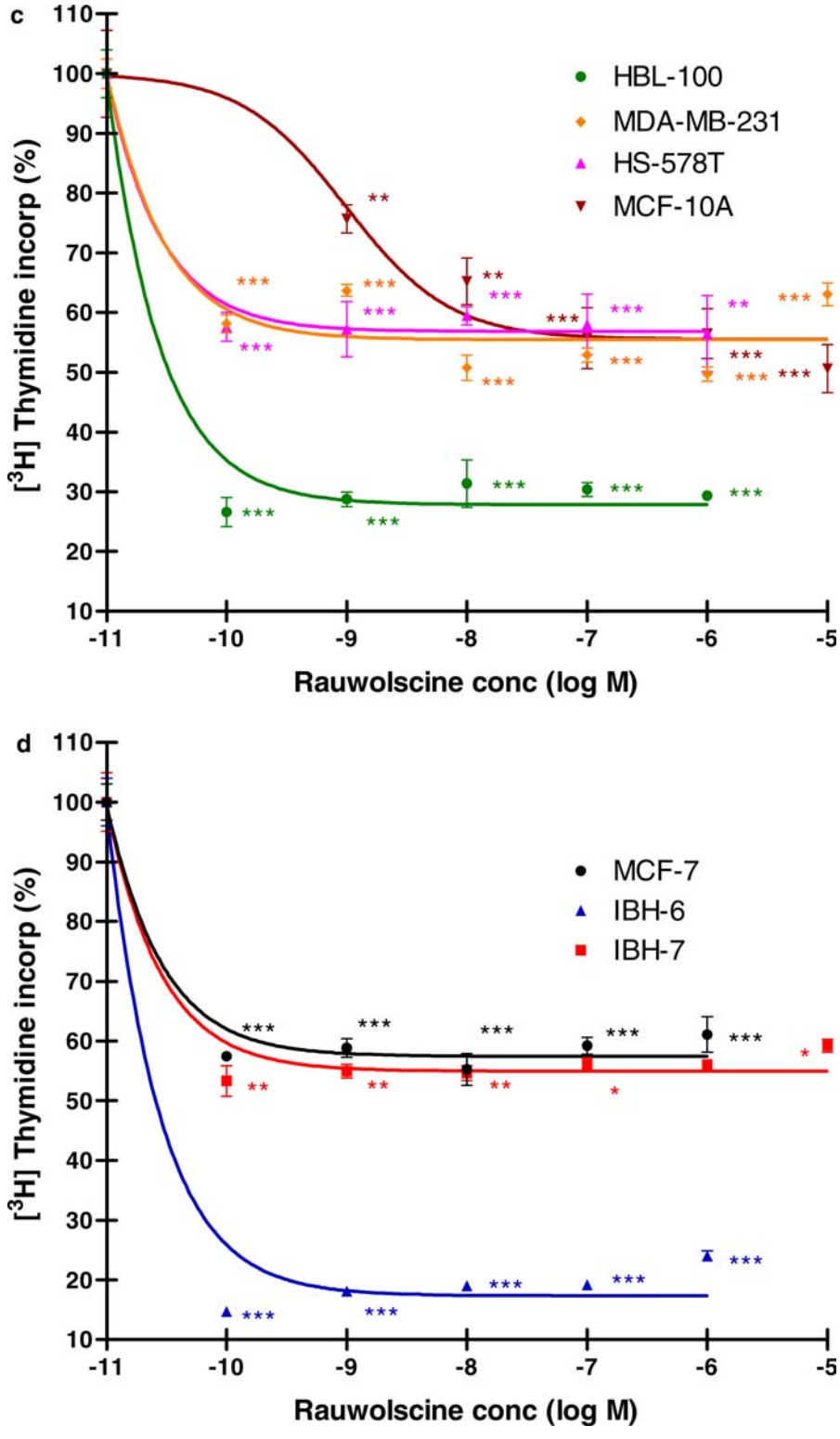

Fig. 5 (Contd.)

immunocytochemistry. A striking stimulation on binding was found with both α_1- and β-adrenergic compounds. One possible explanation for this could be the existence of a ''cross talk'' between α- and β-adrenoceptor as previously proposed [44, 55]. An oligomerization of G-protein-coupled receptors, either as homo or hetero oligomers, has also been described [43, 52, 67].

Immunocytochemical studies

The presence of α_{2B}- and α_{2C}-adrenoceptors was confirmed at the protein level with specific antibodies. Immunocytochemical studies showed both subtypes of receptors in nearly all cell lines studied. The α_{2B}-adrenoceptors was found mainly in the cell membrane with some positive cytoplasmatic reactivity. Although there is major consensus on membrane localization of the α_{2B}-adrenoceptors [46], a similar localization to the one described in this paper was shown in prenatal rat spinal cord [29]. Additionally, α_{2B}-adrenoceptors have been shown to localize in the plasma membrane at steady state, but internalize after agonist treatment [16]. Thus, localization found in our study could be ascribed in part to internalization of the receptor. Alternatively, intracellular localization could be due to synthesis of new receptors. The α_{2C}-adrenoceptors was located intracellularly as has already been described elsewhere [30, 46, 53].

Proliferation studies

The α_2-adrenoceptors mediates a great variety of physiological effects including vasoconstriction, platelet aggregation, gastrointestinal secretion, neurotransmitter release, and inhibition of insulin secretion [1, 5]. α_2-agonists can promote proliferation of intestinal crypt cells in vivo and act as co-mitogens in fibroblasts transfected with the α_2-adrenoceptor-gene [1]. Transactivation of receptor tyrosine kinase(s) by α_{2B}-adrenoceptors expressed in trophoblast giant cells is required to initiate blood vessel formation in the placenta [5]. α_{2B}-adrenoceptors activate MAPK and modulate proliferation of primary cultured proximal tubule cells [15]. In all breast cell lines studied, the α_2-adrenergic agonist (clonidine or oxymetazoline) caused a significant increase of [^{3}H]-thymidine incorporation, which was reversed by the simultaneous addition of the specific α_2-adrenergic antagonist, rauwolscine. As stated before, an increase in cyclic AMP levels, due to either incubation with analogues of this compound with the β-adrenergic agonist isoproterenol or to inhibition of cyclic AMP degradation, is associated with inhibition of growth in the breast cancer cells. In a previous report, our group showed that both intracellular and extracellular concentrations of cyclic AMP paralleled cell proliferation in MCF-7 cells incubated with clonidine and yohimibine [63]. The increase in cell proliferation was moderate but highly significant. The cell lines studied in this paper were extremely sensitive to clonidine as judged from cell proliferation studies, when compared to other cells previously described although not strictly related to this parameter. For example, 60 nM UK 14304 increases $[Ca^{++}]_i$ in HEL cells [18], or 20 nM for half-maximal regulation of GTPase by the same compound in α_{2A} receptors stably expressed in Rat-1 fibroblasts [40]. It should be noted, however, that the EC_{50} found by our group for [^{3}H]-thymidine incorporation in MCF-7 was much higher for the natural neurotransmitter epinephrine (10 pM) than for clonidine [63]. The EC_{50} found for the non-tumour cells HBL-100 and MCF-10A is more in the range of concentrations described for other functions of α_2-adrenoceptor. Scatchard analysis showed binding affinities in the nM order. However, the possibility of higher affinity binding sites (with apparent K_d in the fM order) cannot be ruled out by binding assays for experimental reasons. With this methodology cpm differences between specific and non-specific binding would be in the order of magnitude of experimental error.

The presence of combined activity of α_{2B} and α_{2C} receptor subtypes in most cell lines precludes the assignment of cell proliferation to any particular subtype of adrenoceptors. However, since MDA-MB-231 and MCF-10A cells, possess only the α_{2B} subtype, and since the agonist effect was similar in these and other cell lines studied, it is likely that the α_{2B} is indeed involved in this phenomenon. Nevertheless, we cannot rule out the participation, either direct or indirect, of the α_{2C} subtype because we have not found breast cell lines with the exclusive expression of this subtype. The mitogenic role of α_{2A}-adrenoceptor in HS-578T was also similar to that of α_{2B}-adrenoceptor-containing cells, suggesting redundant action on cell proliferation for α_2-adrenoceptor subtypes. There is evidence in the literature [47] that mice lacking all three α_2-adrenoceptor subtypes do not survive beyond day 11.5 of embryonic development, whereas all knock-out mice lacking a single subtype of α_2-adrenoceptor are viable, suggesting a certain degree of redundancy in function.

Even though only two non-tumour cell lines were examined, an interesting difference was found between tumour cells and non-tumour cells regarding the EC_{50} for the stimulatory effect on cell proliferation. In fact, neoplastic cell lines were much more sensitive to α_2-adrenergic agonist stimulation than non-tumour cells. It may be speculated that this would be an additional advantage for cell growth in these cells.

In conclusion, the present study clearly shows the expression of at least one α_2-adrenoceptor subtype in several breast cell lines, both at the RNA and protein levels. Moreover, these receptors are functional and associated with an increase in cell proliferation. Adrenergic agonists and antagonists are useful compounds for the treatment of certain diseases, with minimal side effects [50]. Considering the already known side effects of α-adrenergic derivatives in other human disorders [17, 68], the results presented in this paper highlight the possibility of clinical trials. The direct control of cell proliferation shown in vitro could eventually open new avenues for adjuvant therapies in the treatment of breast diseases.

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