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Evaluation of β-adrenergic receptor subtypes in the human prostate cancer cell line-LNCaP

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Abstract

The present study was undertaken to determine the effects of catecholamines, agonists, and antagonists of β-adrenergic receptors (AR) in the LNCaP cell line. Changes in cellular cyclic adenosine-3′,5′-monophosphate (cAMP) levels were quantified by the use of a 6 cAMP response element (CRE)-luciferase reporter gene assay. LNCaP cells were transiently transfected with this gene construct, incubated in 96-well microtiter plates for 24 hr, and then treated with β-AR agonists and/or antagonists for 4 hr. The rank order of potency for catecholamines and known β-AR agonists was terbutaline (3.31 nM) > isoproterenol (8.31 nM) ≥ fenoterol (15 nM) = epinephrine (16.2 nM) > norepinephrine (77.5 nM) > BRL-37344 [(*R**,*R**)-(±)4-[2-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxy acetic acid, sodium salt] (1000 nM) > dobutamine (1770 nM) > CGP 12177 (4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazole-2-one hydrochloride) (inactive). The non-selective β_1-/-β_2-AR antagonists; propranolol and CGP 12177, at 10^{-7} M, inhibited luciferase activity induced by these agonists by 80–96%. Propranolol blocked isoproterenol-induced luciferase responses in a competitive manner (K_B = 1.4 nM). In addition, isoproterenol-activated luciferase expression was blocked more potently by ICI 118,551 [(±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethy) amino]-2-butanol], a β_2-AR antagonist than by ICI 89,406 [(±)-*N*-[2-[3-(2-cyanophenoxy-)]-2-hydroxypropylamino]ethyl-*N*-phenylurea], a β_1-AR antagonist, giving K_B values of 1.07 and 161 nM, respectively. These results suggest that the β_2-AR is the major subtype mediating catecholamine-induced cAMP changes in LNCaP cells.

Keywords: LNCaP; cAMP; Luciferase; Reporter gene assay; Human β-adrenergic receptors

1. Introduction

Prostate tissue has been demonstrated to be a rich source of α_1- and β-AR [1]. Biochemical studies have shown that β-AR agonists are able to stimulate adenylyl cyclase and raise cAMP levels in the rat prostate [2,3]. Accumulation of cAMP and differentiation of neuroendocrine cells have been observed in human prostate cancer cell lines treated with forskolin, dibutyryl cAMP, and catecholamine-like agonists (isoproterenol and epinephrine) [4]. These effects were reversible upon withdrawal of these agents [5]. The pharmacological properties of β-AR agonists with respect to receptor binding and cAMP generation were examined in PC-3 cells, a human prostate androgen-independent cell line [6]. The results of these studies indicated that PC-3 cells contained a large population of β_2-ARs. The role of β-AR subtypes involved in catecholamine-mediated cAMP elevations in a human lymph node carcinoma of the prostate (LNCaP cells), an androgen-sensitive cell line, has not been elucidated.

In this study, we used a reporter gene assay to investigate the effects of catecholamines and known β-AR subtype selective agonists on cAMP levels in LNCaP cells. The

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Abbreviations: AR, adrenergic receptor(s); cAMP, cyclic adenosine-3′,5′-monophosphate; CRE, cAMP response element; LUC, luciferase.

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pharmacological specificity of agonist actions in this cell line was characterized by the use of non-selective and selective β-AR antagonists.

2. Materials and methods

2.1. Cell culture

LNCaP cells were purchased from the American Type Culture Collection. These cells were grown in 150 × 25 mm petri dishes with a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12) supplemented with 10% fetal bovine serum, penicillin G sodium (100 U/mL), or streptomycin (100 μg/mL). When the cultures were 75% confluent, the cells were detached by trypsinization (0.05% trypsin EDTA for 1 min). Cell culture reagents were obtained from Life Technologies. The compounds used in this study were obtained from the following sources: (−)-epinephrine bitartrate, (−)-norepinephrine bitartrate, and BRL 37344 sodium [(*R**,*R**)-(±)-4-[2-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]-phenoxy acetic acid, sodium salt] (Research Biochemicals Inc.); CGP 12177 (4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazole-2-one hydrochloride) (Research Biochemicals International); (−)-isoproterenol bitartrate, (±)-propranolol, and forskolin (Sigma Chemical Co.); (±)-terbutaline and (±)-fenoterol (Receptor Research Chemicals, Inc.); (±)-dobutamine HCl (Eli Lilly Laboratories); and ICI 118,551 [(±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol] and ICI 89,406 [(±)-*N*-[2-[3-(2-cyanophenoxy)]-2-hydroxypropylamino]ethyl-*N*-phenylurea] (Imperial Chemical Industries).

2.2. Transfection and luciferase assay

LNCaP cells were transfected with the firefly luciferase (*LUC*) reporter gene construct containing 6 copies of the cAMP response element (6 CRE-LUC plasmid). The CRE-LUC construct (pADneo2-C6-BGL) was provided by Dr. A. Himmler (Ernst Boehringer Institut). Trypsinized cells were electroporated in 400 μL medium containing the plasmid at 150 V and a single 70 msec pulse using BTX disposable cuvettes (model 640; 4-mm gap) with a BTX model T820 electro square porator (BTX, Inc.). The transfected cells were plated at a density of 60,000/well in 96-well microtiter plates (Culturplate, Packard) and allowed to grow for 24 hr. After 24 hr, the cells were treated with various drug concentrations for 4 hr, which was also found to be an optimum time during the time–course analysis for transcriptional activation in Chinese hamster ovary cells [7]. Antagonists were added 15 min prior to the addition of agonists. Following drug exposures, the cells were lysed, and luciferase activity was measured using a LucLite® assay kit (Packard). Light output was detected by a TopCount® microplate scintillation counter in the single-photon counting mode (Packard Instrument Co.).

2.3. Data analysis

Data are expressed as relative light units, and the changes in luciferase response (cps) are plotted vs. the agonist concentration. Results are also expressed as fold-induction over the corresponding control value. Each agonist or antagonist treatment was done in triplicate or quadruplicate. For each drug, the concentration–response curve and the corresponding molar effective concentration-50 (EC_{50}) value, defined as the drug concentration that produced a half-maximal luciferase response, were analyzed using GraphPad Prism 3 software. Agonist potencies were determined as the negative log molar effective concentration-50 (pEC_{50}) values, and data were expressed as the mean $pEC_{50} \pm$ SEM of N = 3–8 experiments. The pK_B values of antagonists were derived by the concentration-ratio method of Furchgott [8], and calculated by using the formula: $K_B = [I]/(CR-1)$, where pK_B is the negative logarithm of the apparent dissociation constant (K_B) of the antagonist, [*I*] is the molar concentration of the antagonist, and CR is the concentration-ratio of the agonist EC_{50} in the presence of the antagonist to that of the agonist EC_{50} alone. Agonist concentration-ratio shifts were obtained from individual molar EC_{50} values in the presence and absence of a fixed antagonist concentration (e.g. 10^{-7} M). Student's *t*-test was used to determine whether mean values of two treatment groups (pEC_{50} or pK_B values) were significantly different ($P < 0.05$) from each other.

3. Results and discussion

Initial experiments in LNCaP cells showed that forskolin (20 μM) produced on the average a 92-fold induction of luciferase gene expression. This indicates that adenylyl cyclase is involved in the transcriptional activation of the 6 CRE-LUC construct. Subsequent experiments with catecholamines generated concentration–response curves for isoproterenol, epinephrine, and norepinephrine (Fig. 1A) with EC_{50} values of 8.31, 16.2, and 77.5 nM, respectively. The corresponding pEC_{50} values (mean ± SEM) for these drugs were 8.08 ± 0.20 (N = 7), 7.78 ± 0.13 (N = 7), and 7.11 ± 0.27 (N = 7). The maximal effect of norepinephrine was lower than the maximum luciferase activity response to isoproterenol, suggesting that norepinephrine may be a partial agonist in this cell system. These observations are in agreement with those reported for catecholamines on cAMP levels in human lymphocytes, which contain β_2-ARs [9]. Accordingly, the rank order of potency of these catecholamines for transcriptional activation of the luciferase reporter gene in LNCaP cells was isoproterenol = epinephrine > norepinephrine. Therefore, our results with the potencies and rank order of

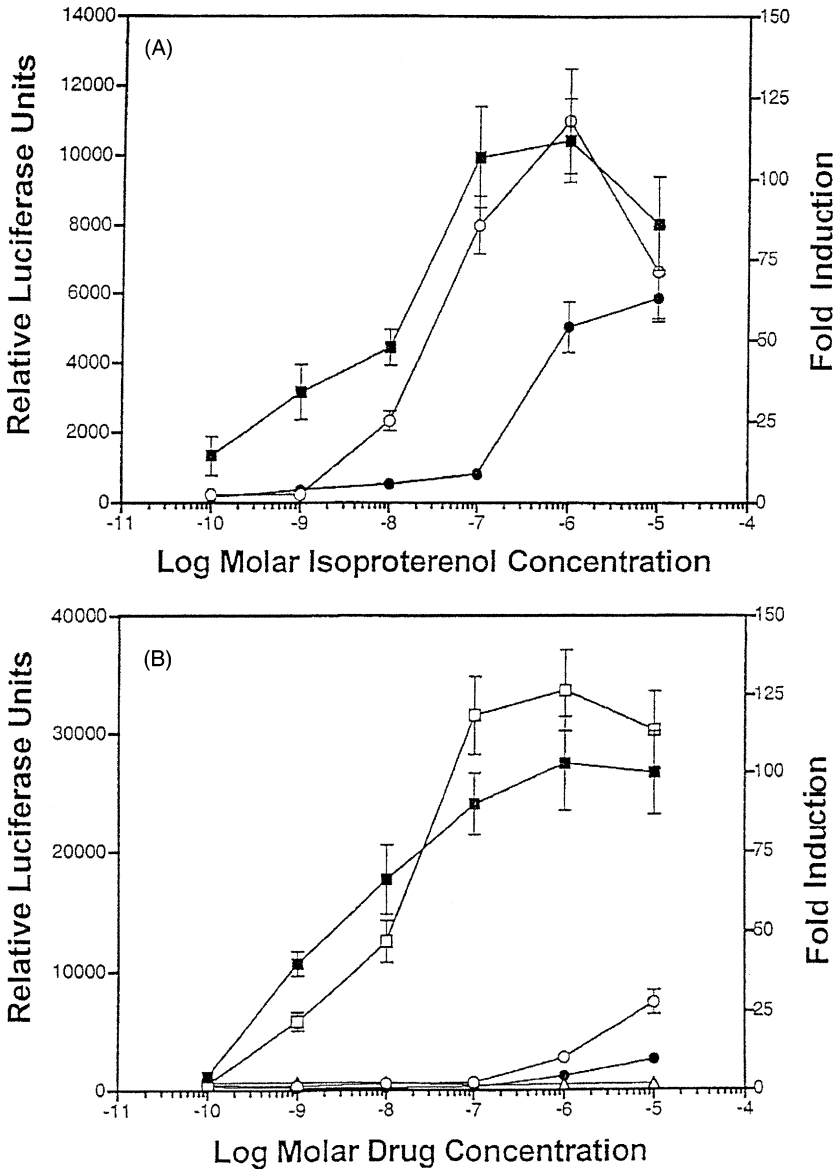

Fig. 1. Concentration dependent luciferase activity responses of selected β-adrenergic receptor agonists in LNCaP cells. Results are given as luciferase activity changes (cps) (left *y*-axis) and as fold-induction over basal activity (right *y*-axis) vs. log molar agonist concentration. Key: (A) (■), isoproterenol; (○), epinephrine; and (●), norepinephrine. (B) (■), terbutaline; (□), fenoterol; (○), BRL 37344; (●), dobutamine; and (Δ), CGP 12177. Data for both panels are expressed as the means ± SEM of N = 3–8, with each concentration determined in quadruplicate.

catecholamines in LNCaP cells are in accord with a β_2-AR subtype classification [10,11].

The comparative concentration–response curves and potencies of the β_1-, β_2- and β_3-AR selective agonists and partial agonists are shown in Fig. 1B. Both terbutaline and fenoterol, as selective β_2-AR agonists, gave maximal luciferase expression (100- to 130-fold inductions) that was comparable to that of isoproterenol (see Fig. 1A). Terbutaline and fenoterol were potent stimulators of luciferase activity giving EC_{50} values of 3.3 and 15 nM, respectively. The corresponding pEC_{50} values (mean ± SEM) of these agonists were 8.48 ± 0.16 (N = 8) and 7.82 ± 0.13 (N = 8). In contrast, BRL-37344, the known β_3-AR selective agonist [12], and dobutamine, a mixed β_1-/β_2-AR agonist [13] showed only 34- and 12-fold inductions of luciferase activity with EC_{50} values of 1000 and 1770 nM. The corresponding pEC_{50} values (mean ± SEM) were 6.00 ± 0.10 (N = 3) and 5.76 ± 0.09 (N = 3), respectively. The pharmacological profile for these drugs is similar to that reported in the β_2-AR containing mouse pineal gland tumor cells [14] and human lymphocytes [9].

It is notable that CGP 12177, a high affinity β_1- and β_2-antagonist and a reported agonist of the β_3- and putative β_4-AR [15,16], did not show any transcriptional activation of luciferase activity at concentrations up to 100 μM (Fig. 1B). Recent evidence has suggested that the β_3-AR independent agonist effects of CGP-12177 on lipolysis are associated with an atypical β_1-AR interaction [17]. Thus,

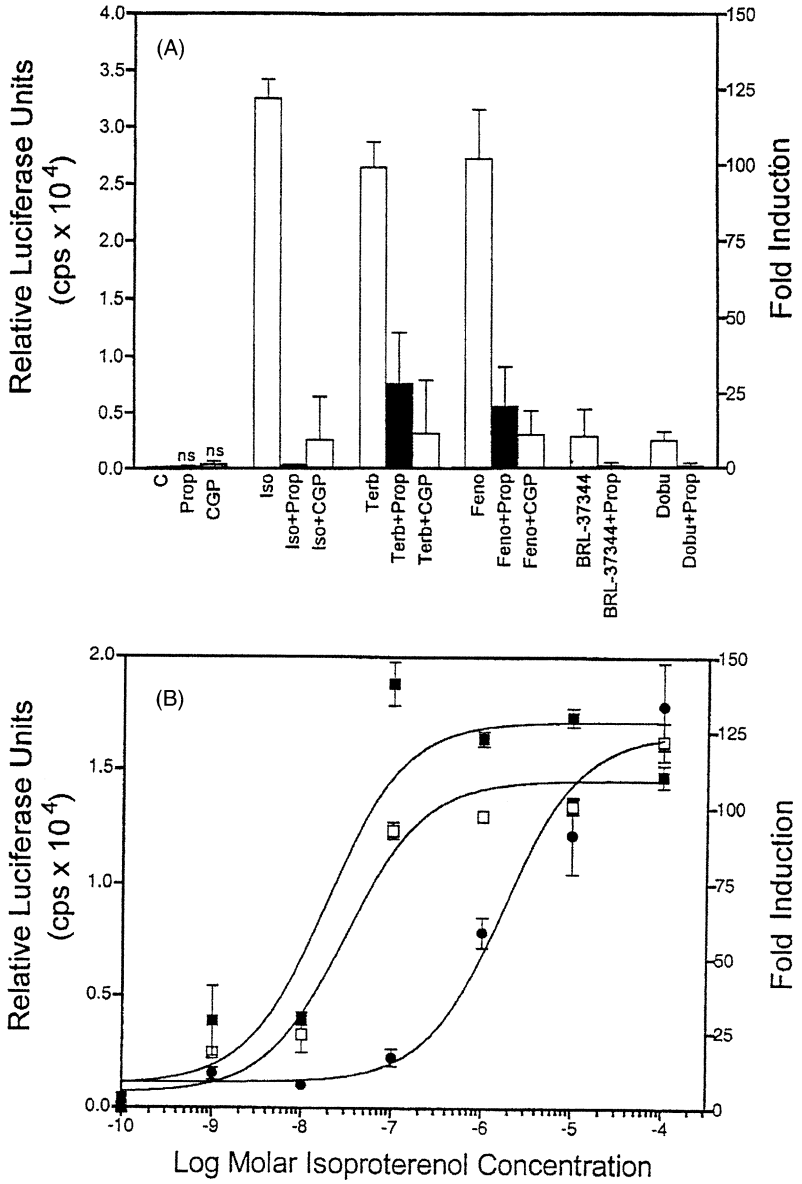

Fig. 2. Inhibitory effects of β-adrenergic receptor antagonists on luciferase responses by selected agonists. Results are given as luciferase activity changes (cps) (left *y*-axis) and as fold-induction over basal activity (right *y*-axis). (A) C = control; Prop = propranolol (10^{-7} M), CGP = CGP 12177 (10^{-7} M), Iso = isoproterenol (10^{-7} M), Terb = terbualine (10^{-7} M), Feno = fenoterol (10^{-7} M), BRL-37344 (10^{-6} M), and Dobu = dobutamine (10^{-5} M). Data are expressed as the means ± SEM of N = 3, each done as four determinations. ns = not significant ($P > 0.05$). The data of the means for agonists in the absence vs. the presence of the antagonists were significantly different ($P < 0.05$) from each other. (B) Effect of ICI 118,551 (10^{-7} M) and ICI 89,406 (10^{-7} M) on the concentration–response curve of isoproterenol. Key: (■), isoproterenol alone; (□), isoproterenol and ICI 89,406; and (●), isoproterenol and ICI 118,551. Data are expressed as the means ± SEM, N = 8.

our findings with CGP 12177 suggest that LNCaP cells do not have responsive populations of the β_3-, putative β_4-, or atypical β_1-ARs.

As shown in Fig. 2A, the non-selective β_1- and β_2-AR antagonists, propranolol and CGP 12177, at 10^{-7} M, nearly completely blocked (inhibitions ranged from 80 to 96%) the luciferase responses of isoproterenol, terbutaline, and fenoterol (at 10^{-7} M), BRL-37344 (10^{-6} M), and dobutamine (10^{-5} M). Propranolol, at 10^{-7} M, caused a competitive rightward shift in the isoproterenol concentration–response curve, resulting in a K_B value of 1.4 nM, giving a pK_B value (mean ± SEM) for propranolol of 8.85 ± 0.28 (N = 3) (data not shown). These results suggest that the effects of these agonists are mediated through the activation of β_1- and/or β_2-AR in these cells.

To further clarify which β-AR is involved, we examined the actions of the selective β_1- and β_2-AR antagonists, ICI 89,406 and ICI 118,551, respectively [18–20], on isoproterenol-induced luciferase activity responses in LNCaP cells. Our results show that ICI 118,551 induced a greater rightward shift in the isoproterenol concentration–response curve than did ICI 89,406 (Fig. 2B). The K_B values for ICI

118,551 and ICI 89,406 against isoproterenol-mediated cAMP changes were 1.07 and 161 nM, respectively, in LNCaP cells. Corresponding $pK_B \pm$ SEM values obtained for ICI 118,551 and ICI 89,406 in our experiments were 8.96 ± 0.25 (N = 6) and 6.79 ± 0.31 (N = 6). Fraundorfer *et al.* [21] showed that the binding affinities (K_i values) of ICI 118,551 and ICI 89,406 on human β_2-AR expressed in CHO cells were 0.63 and 132 nM, respectively, which is nearly equivalent to the results obtained for the blockade of isoproterenol responses by ICI 118,551 and ICI 89,406 in LNCaP cells. Similarly, in membranes derived from human lung mast cells containing β_2-AR [22], ICI 118,551 bound with a high affinity ($K_i = 1.26$ nM). ICI 118,551 has also been shown to exhibit the same potencies in a variety of β_2-AR-containing tissues of the guinea pig ($K_B = 0.55$ nM) [18] and rat uterine ($K_B = 0.5$ nM) [23] smooth muscle. In contrast, the K_B values of ICI 118,551 in β_1-AR containing tissues of rat atrium and right atrial membranes were 68 nM [18] and 280 nM [24]. Taken collectively, the high potency and selectivity of ICI 118,551 vs. ICI 89,406 against the functional responses of isoproterenol indicate that catecholamine-induced effects in LNCaP cells are mediated through the activation of β_2-AR. However, further confirmation of this proposal must await the results of radioligand analyses of the β-AR populations in these cells.

The identification of functional β_2-ARs in the LNCaP cell line represents the first step in determining their role in the control of cell proliferation and differentiation. Cox *et al.* [4,5] have shown that *in vitro* differentiation of two prostate cancer cell lines such as LNCaP and PC-3 to the neuroendocrine cell phenotype can be induced by forskolin and the β-AR agonists, isoproterenol and epinephrine, suggesting that agents that elevate intracellular cAMP regulate neuroendocrine cell differentiation *in vivo*. Our results in LNCaP cells suggest that selective β_2-AR antagonists may regulate neuroendocrine cell differentiation *in vivo*, since the appearance of neuroendocrine cells in the prostate are correlated with tumor grade, loss of androgen sensitivity, autocrine and paracrine activity, and poor prognosis. These studies have documented that the androgen-sensitive LNCaP cell line, like the androgen-insensitive PC-3 cell line [4,5], contains primarily a β_2-AR subtype that mediates the effects of catecholamines by the activation of adenylyl cyclase.

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