The human carcinoma cell line A431 possesses large numbers of functional β-adrenergic receptors

C. Delavier-Klutchko, J. Hoebeke and A.D. Strosberg

Laboratory of Molecular Immunology, Institut Jacques Monod CNRS, Université Paris VII, 2 place Jussieu, F75251 Paris Cedex 05, France

Received 14 February 1984

The existence of β-adrenergic receptors was demonstrated on whole A431 cells as well as A431 membrane preparations by means of binding assays using the hydrophobic l-[^{3}H]dihydroalprenolol and the hydrophilic antagonist [^{3}H]CGP-12,177 as β-adrenergic ligands. Binding was stereospecific. The receptors, as shown by competition studies, proved to be of the β_2-subtype and appeared functional in the stimulation of adenylate cyclase. The number of receptors per cell and the yield of receptor sites/mg membrane protein render the A431 cell a useful tool for the study of human β-adrenergic receptors.

β-Adrenergic receptor *Human epidermoid carcinoma cell* *l-Dihydroalprenolol* *CGP-12,177*

1. INTRODUCTION

Molecular studies of catecholamine-stimulated β-adrenergic system have mainly been performed on proteins obtained from avian [1] and amphibian [2] erythrocytes. However, the properties of these receptor–cyclase complexes are not necessarily similar to those of mammalian receptors [3]. Several investigators [4–6] have thus turned their attention to tissues and cells from murine and human origin. The paucity of receptor density and the difficulty in growing cells in sufficiently large amounts have often hampered molecular analysis of the receptors. We here describe the existence of large numbers of functionally active β-receptors on the A431 human epidermoid cell line, which is well known as possessing excellent growth properties and having an unusually high number of epidermal growth factor receptors [7]. The binding characteristics of the adrenergic receptor and their interaction with the adenylate cyclase system make these cells an ideal tool for topological and structural studies.

Abbreviations: CGP-12,177, 4-[3-(*t*-butylamino)-2-hydroxypropoxy]benzimidazol-2-one; EGF, epidermal growth factor; Gpp(NH)p, 5′-guanylyl imidodiphosphate

2. MATERIALS AND METHODS

2.1. *Drugs*

[^{3}H]Dihydroalprenolol (49 Ci/mmol) was purchased from New England Nuclear and [^{3}H]CGP-12,177 (34 Ci/mmol) from Amersham. dl-Alprenolol was a gift from Ciba-Geigy and d- and l-propranolol from ICI. l- and d-Isoproterenol, l-adrenaline and l-noradrenaline were acquired from Sigma.

2.2. *Saturation binding curve of dihydroalprenolol and CGP-12,177 on intact A431 cells*

Cells grown in Costar plates (35 mm diameter wells) until confluence (8×10^5 cells/well) were washed 5 times with 2 ml of Hank's balanced salt solution, buffered with 10 mM Hepes at pH 7.4 and supplemented with 0.1% bovine serum albumin and 1 mM ascorbic acid. Increasing concentrations of radioligand in 1 ml of the same buffer were added per well.

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00145793/84/$3.00

Dihydroalprenolol binding was performed by incubation at 25°C for 10 min. Cells were then washed 5 times with cold buffer and lysed in 3 × 0.5 ml of 0.01 M Tris buffer (containing SDS to 0.5% and EDTA to 1 mM). The lysate was transferred to scintillation vials.

Binding of CGP-12,177 was performed as in [8]. Cells were incubated for 60 min at 30°C with the radioligand. The reaction was stopped by addition of 2 ml cold hypotonic buffer [10 mM phosphate buffer (pH 7.4), 1 mM $MgSO_4$] and the cells washed 5 times in the same buffer. Lysis was done with 2% SDS and lysates transferred to scintillation vials.

Non-specific binding for both radioligands was assessed by carrying out incubation in the presence of 100 μM l-isoproterenol.

2.3. *Saturation binding curve of dihydroalprenolol and CGP-12,177 on A431 membranes*

Membranes were prepared as in [9] but omitting Ca^{2+} and supplementing the buffer with EGTA to 0.2 mM. Binding assays were performed in 0.2 ml of 75 mM Tris, 25 mM $MgCl_2$ (pH 7.4) containing varying amounts of radioligands and 50 μg membrane protein. After 25 min incubation at 30°C the reaction was stopped by addition of 1 ml ice-cold buffer and the samples immediately filtered on GF/C glass fibre filters (Whatman). Filters were washed 4 times with 4 ml ice-cold buffer, transferred to scintillation vials and soaked in 1 ml of 1 N HCl before addition of the scintillation cocktail.

Non-specific binding was assessed in the presence of 100 μM l-isoproterenol.

2.4. *Inhibition of the binding of dihydroalprenolol and CGP-12,177 by β-adrenergic agonists and antagonists*

Stereospecificity was assessed by inhibition curves on both intact cells and membrane preparations using the radioligand CGP-12,177. Assay conditions were the same as described for saturation binding. The K_i (inhibition constant) values of the compounds were calculated from the 50% inhibitory concentrations as in [10].

The subtype of the A431 β-receptors was assessed by inhibition of dihydroalprenolol binding on membrane preparations with the agonists l-isoproterenol, l-epinephrine and l-norepinephrine. The K_i values of the agonists were calculated as described above.

2.5. *Adenylate cyclase assay*

Adenylate cyclase activity was assayed as in [11]. The reaction medium contained 100 μM [^{32}P]ATP, 7 mM $MgCl_2$, 1 mM EDTA, 1 mM cAMP, 50 mM Tris–HCl (pH 7.4), an ATP-regenerating system (1.5 mg/ml creatine phosphokinase, 1 mg/ml phosphocreatine) and 40–50 μg membrane protein in a final volume of 80 μl. The reaction was initiated by addition of the membrane preparation and incubation continued for 20 min at 30°C.

3. RESULTS

In a screening of various cell lines, the human epidermoid transformed cell line, A431, was found to bind large amounts of the radiolabelled β-adrenergic antagonist [^{3}H]dihydroalprenolol. The binding was displaceable by excess unlabelled alprenolol (not shown). Since the liposolubility of most β-adrenergic antagonists causes non-specific binding on hydrophobic surfaces, the specificity of the binding to intact A431 cells was verified by displacement with the hydrophilic agonist l-isoproterenol [12]. Due to the high non-specific binding (fig.1), the saturation binding could only be studied up to concentrations of 5 nM. Calculation of the association parameters by Scatchard analysis [13] yielded an association constant (K_a) of 1.1 ± 0.3 × 10^9 M^{-1} for a single type of binding site at a density of 110000 ± 18000/cell.

Since quantitation of dihydroalprenolol binding was hampered by the high non-specific binding, the presence of β-receptors on intact cells was confirmed by using the hydrophilic antagonist CGP-12,177 [8]. This ligand bound to the intact cell with a K_a of 1.4 ± 0.4 × 10^9 M^{-1} for a single type of binding site at a density of 34000 ± 6000/cell (fig.1).

The binding of both radiolabelled β-adrenergic antagonists was confirmed on A431 membrane preparations. Scatchard analysis of the binding curves (fig.2) showed for both ligands 2.2 ± 0.2 pmol bound/mg membrane protein with a K_a for dihydroalprenolol of 1.5 ± 0.3 × 10^8 M^{-1} and for CGP-12,177 of 2.5 ± 0.9 × 10^8 M^{-1}.

The stereospecificity of ligand binding was assessed on intact cells and membranes by the in-

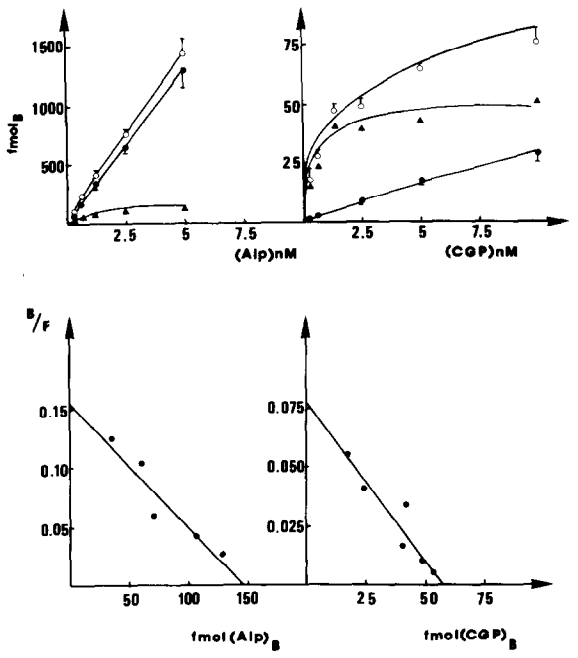

Fig.1. Saturation binding curves of [^{3}H]dihydroalprenolol (left) and CGP-12,177 (right) on intact A431 cells. (Upper plots) Total binding (○—○), non-specific binding (●—●) and specific binding (▲—▲) of the radioligands on A431 cells. Means and SD of 4 independent experiments are given. (Lower plots) Scatchard analysis of the specific binding curves.

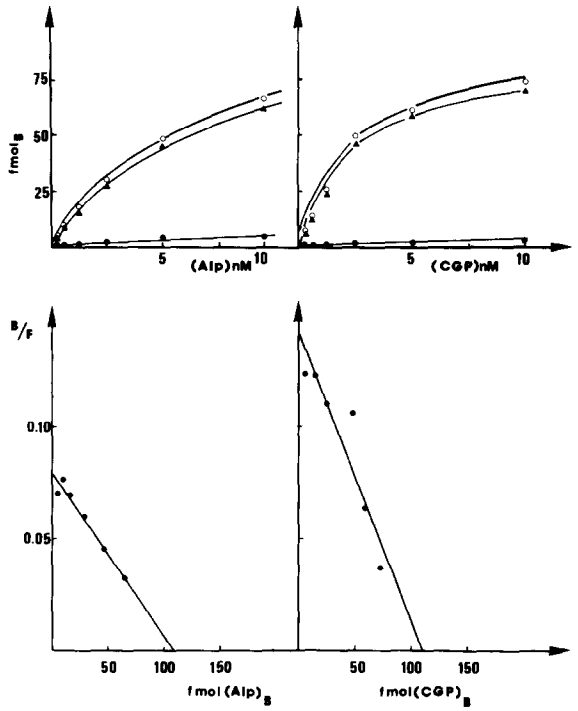

Fig.2. Saturation binding curves of [^{3}H]dihydroalprenolol (left) and CGP-12,177 (right) on A431 membrane preparations. (Upper plots) Total binding (○—○), non-specific binding (●—●) and specific binding (▲—▲) of the radioligands on 50 μg membrane protein samples. The means of 2 independent experiments are given. (Lower plots) Scatchard analysis of the specific binding curves.

hibition of CGP-12,177 binding with the stereoisomers of the antagonist propranolol and the agonist isoproterenol (table 1). On intact cells l-propranolol had a 150-fold higher affinity for the receptor than the d-isomer, while l-isoproterenol had a 30-fold higher affinity. The inhibition constants for l-propranolol and l-isoproterenol were somewhat lower on A431 membrane preparations but the stereospecificity of these two ligands was well conserved (table 1).

Using [^{3}H]dihydroalprenolol as radioligand on A431 membrane preparations, the affinity of 3 agonists for the β-receptor was assessed by means of a competition assay. Isoproterenol showed the highest affinity, followed by epinephrine and norepinephrine (table 2).

The functional coupling of the β-adrenoreceptor to its effector system was verified by studying the stimulation of adenylate cyclase by the agonist l-isoproterenol. As shown in table 3, l-isoproterenol increased cAMP production 2.5-times over basal

Table 1

Stereospecificity of β-adrenergic ligands for the receptor on A431 cells

Ligand	K_i ($\times 10^6$) (M^{-1})	
	Intact cells	Membranes
l-Propranolol	7600	910
d-Propranolol	50	17
l-Isoproterenol	5	3.8
d-Isoproterenol	0.15	0.45

Inhibition constants (K_i) were calculated from displacement curves, using 1.5 nM CGP-12,177 as radiomarker on intact cells and 2.5 nM on membrane preparations. Experimental procedures are described in section 2

Table 2

Subclass typing of the β-receptors of A431 cells

Agonist	K_i ($\times 10^6$) (M^{-1})
l-Isoproterenol	1.6
l-Epinephrine	0.38
l-Norepinephrine	0.053

Inhibition constants (K_i) were calculated from displacement curves using 5 nM [^{3}H]dihydroalprenolol as radioligand on membrane preparations of A431 cells (50 μg protein). Experimental procedures are described in section 2

Table 3

Adenylate cyclase stimulation on A431 membrane preparations

Treatment	pmol cAMP/mg protein per min
–	4.0
+ 100 μM l-isoproterenol	10.0
+ 100 μM Gpp(NH)p	16.8
+ 100 μM Gpp(NH)p and l-isoproterenol	28.0
+ 10 mM NaF	24.9

cAMP levels were determined as described in section 2

cAMP levels, demonstrating the coupling of agonist-occupied receptor sites with the adenylate cyclase system. Additivity of the effects of l-isoproterenol with that of the nucleotide analogue Gpp(NH)p could be shown to reach the levels of activities found by NaF stimulation.

4. DISCUSSION

Most of our knowledge on the properties of mammalian β-adrenergic receptors has been gained from studies on transformed mouse lymphocytes (S49 lymphoma) [4] or rat glial cells (C_6 glioma) [5]. The low number of sites/cell and low yield of receptor sites/mg membrane protein unfortunately limit the use of these cells for purification procedures or visualisation of the β-receptor by fluorescence or immunochemical techniques.

Recently, a human liver cell line carrying 20000 receptors was described, a sufficient number of receptors for use in photobleach recovery studies using a fluorescent β-antagonist [6]. To increase the diversity of homogeneous human cell lines available for the study of β-receptors, a screening of transformed cells was initiated. Among these the A431 epidermoid cell line seemed best suited (see below).

The yield of receptors per mg protein from the A431 cell membrane is equal to or even higher than that found for the commonly used material for β-receptor purification: the turkey erythrocyte [14]. This yield, 2.2 pmol/mg membrane protein, was similar when tested with two different radiolabelled antagonists, [^{3}H]dihydroalprenolol and CGP- 12,177 [8]. The binding sites showed association constants comparable to those found for other β-receptors. The stereospecificity of both agonists and antagonists confirmed the characterisation of the binding sites as β-receptors, and the order of potency of the agonists – isoproterenol > epinephrine > norepinephrine – identifies them as β_2-receptors [15]. Finally, the receptor was shown to modulate the adenylate cyclase stimulation upon binding of isoproterenol. The β_2-receptors of the A431 cell line may thus be exploited for future purification and reconstitution experiments similar to those described for the β_2-receptor of frog erythrocytes or lung tissues [16].

Comparing the yield of EGF-receptor/mg membrane protein (16 pmol) with the number of EGF-receptors/cell (2.6×10^6) for the A431 cell [17], 2.2 pmol β-receptors/mg membrane protein would result from approx. 300000 receptors/cell. The number of receptors obtained by dihydroalprenolol binding on intact cells is one-third of this value, while the number of receptors obtained by CGP-12,177 binding is one-ninth. The discrepancy between the predicted and observed values could be due to the presence on or in the A431 cell of receptors which cannot be reached by hydrophilic agents but become accessible on isolated membranes. When the hydrophilic agonist l-isoproterenol was used for non-specific binding measurements, part of the alprenolol binding sites could not be accounted for. The more hydrophilic antagonist, CGP-12,177, appears even less capable than l-isoproterenol of reaching all the β-receptors of the cell. However, even the lowest density of

receptors detected by CGP-12,177 would permit the use of the A431 cells for visualisation experiments of the type performed on Chang liver cells with the fluorescent label NBD-alprenolol [6].

To summarise, the A431 cell may provide a new tool not only for purification, analysis and reconstitution experiments on a human β_2-receptor, but also for the visualisation of the receptors at the fluorescence microscopy and ultrastructural level.

ACKNOWLEDGEMENTS

We thank Dr G. Todaro for the kind hospitality with which he received one of us (J.H.) in his laboratory at the National Cancer Institute. We also thank Dr M. Waterfield and Dr P. Stroobant (Imperial Cancer Foundation, London) for the A431 membrane preparations used in this work. Dr J.G. Guillet, Ms C. Meo and Mr R. Schwartzmann are thanked for their help in preparing the manuscript. This work was supported by grants from CNRS, DGRST and INSERM. We are grateful to the Association pour le Développement de la Recherche sur le Cancer, the Fonds de la Recherche Médicale Française and the Ligue Française contre le Cancer.

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