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Expression of functional β_2-adrenergic receptors in the lung epithelial cell lines 16HBE14o$^-$, Calu-3 and A549

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Abstract

Adrenergic drugs acting through the β_2-adrenoceptor (β_2-AR) adenylate cyclase (AC) signal transduction system elicit a variety of responses within the mammalian airway epithelium; however, its composition of multiple phenotypically differentiated cell types complicates the understanding of the regulation cascades within this tissue. The present study evaluates β_2-AR mRNA level, number, subtype and the cyclic adenosine-3′,5′-monophosphate (cyclic AMP) response to isoproterenol (iso) in the human airway epithelial cell lines 16HBE14o$^-$, Calu-3 and A549, using reverse transcriptase polymerase chain reaction (RT-PCR), radioligand binding studies, [^{3}H]-radioimmunoassay and immunocytochemical staining.

After 4–5 days in culture, all three cell types produced β_2-AR mRNA and protein at a magnitude of gene expression levels Calu-3 $\geq$ 16HBE14o$^-$>A549, whereas control cells Cos-1 and Caco-2 were negative. The β_2-AR adenylate cyclase system was highly expressed and functional in the human airway epithelial cells Calu-3 and 16HBE14o$^-$. The mean β_2-AR density (B_{max}), equilibrium dissociation constant (K_D), and the percentage of β-AR subtypes assessed by radioligand binding were approximately 9908 ± 1127 and 6423 ± 895 binding sites/cell, 32 ± 2.7 pM and 25 ± 1.1 pM, and approximately 100% in Calu-3 and 16HBE14o$^-$cells, respectively. However, in the alveolar cell type A549 the cell surface β_2-AR was virtually undetectable by (−)-[^{125}I]-iodocyanopindolol (ICYP) binding. Stimulation of cultured cells with (−)-isoproterenol enhanced the basal cyclic AMP accumulation only in Calu-3 and 16HBE14o$^-$ cells, which was blocked by the β_2-selective antagonist ICI 118,551, but not by the β_1-selective antagonist CGP 20712A, confirming functional coupling of the β_2-AR to adenylate cyclase in these cells. Immunocytochemical staining localised the receptor on the cell membrane and the cytoplasm in Calu-3 and 16HBE14o$^-$ cells, while it was confined to the cytoplasm only in A549 cells. In conclusion, the β_2-AR expression and its functional coupling to adenylyl cyclase was very high in the human airway epithelial cells Calu-3 and 16HBE14o$^-$, but not in A549, suggesting that the cell lines Calu-3 and 16HBE14o$^-$ present suitable models to study function and regulation of the β-adrenoceptor signalling in the respiratory system.

Keywords: β_2-Adrenoceptor; β_2-AR mRNA; Cyclic AMP; Lung epithelial cells; Cell culture

1. Introduction

β_2-Adrenoceptor agonists are the most widely used bronchodilator drugs in the treatment of obstructive respiratory diseases like asthma [1]. Primarily, they elicit relaxation of airway smooth muscle, but their effects may include a stabilisation of inflammatory cells [2]. A number of studies have shown that bronchial epithelial cells may be an additional target of β_2-AR agonists because they are involved in the pathophysiology of asthma and, at the same time, can be influenced by β_2-AR agonists [3,4].

The lung epithelium is an important part of the nonspecific defence mechanism in the respiratory system also in allergen-induced airway hyperreactivity [5,6] and can modulate the underlying smooth muscle cell metabolism [7]. Lung epithelial cells have the capacity for releasing anti-inflammatory mediators and inactivation of pro-inflammatory mediators. They may thus initiate and perpetuate inflammatory reactions by recruitment of inflammatory

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cells, cell–cell adhesion and modulation of the activity of inflammatory or parenchymal cells, and thus determine the severity of airway disorders.

These activities, including the secretion of mediators provided by lung epithelial cells, are mediated in an intracellular second messenger-dependent manner by the cyclic adenosine monophosphate (cAMP), which is in turn selectively activated by the β_2-AR G-protein adenylate cyclase system [5]. The β_2-AR G-protein adenylate cyclase system, which is stimulated by endogenous catecholamines and β_2-AR agonists, is accepted to facilitate clearance of alveolar fluid, to influence ion fluxes (Na^+- and K^+-channels), and to modulate the release of bronchodilating mediators such as prostaglandin E_2 and nitric oxide [8,9]. The ability to raise intracellular cAMP via the β_2-AR might be involved in anti-inflammatory cascades, since drugs that elevate intracellular cAMP, such as selective β_2-AR agonists and phosphodiesterase inhibitors, decrease pro-inflammatory cytokines and chemokines [10,11].

The β_2-AR, but not the β_1-AR, are known to be expressed at high density within the lung on multiple cell types, including bronchial smooth muscles, bronchial epithelial cells and multiple immune cells [12,13]. Therefore, it remains difficult to assign the observed physiological and pathophysiological responses following beta-adrenergic stimulation to a single cell type and to resolve the complex regulatory pathways. On the other hand, the only permanent lung epithelial cell culture model that has been carefully investigated with regard to β_2-AR expression and function is the BEAS-2B cell line [14]. Other cell lines have been used to investigate the influence of β-agonists and glucocorticoids without precise knowledge about receptor functionality [15–17]. Therefore, the aim of the present study was to systematically evaluate the frequently used permanent human lung epithelial cell lines Calu-3, 16HBE14o$^-$ and A549 with regard to expression of β_2-AR mRNA, protein and, most importantly, the receptor coupling to adenylate cyclase, the enzyme which activates the intracellular cAMP formation.

2. Materials and methods

2.1. Materials

(−)-[^{125}I]-Iodocyanopindolol (ICYP; specific activity, 81.4 TBq mmol^{-1}) was purchased from Perkin-Elmer Life Sciences (Zaventem, Belgium). CGP 20712A [1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-imidazolyl)-phenoxy]-2-propanol], (±)-CGP 12177 [(±)-4-(3-tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-ol], (−)-isoproterenol bitartrate and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich-Chemie (Deisenhofen, Germany). [^{3}H]-cAMP-Radioimmonoassay-Kit was purchased from Amersham-Buchler (Braunschweig; Germany). Other substances were obtained at p.A. quality from commercial sources. The SV40-transformed human bronchial lung cell line 16HBE14o$^-$ was obtained from Dieter C. Gruenert (Department of Medicine, University of Vermont, Burlington, VT) and used at passages 2.88 to 2.98. The number before the dot gives information about the number of passages prior, and the last two digits describe the number of passages after immortalisation. Caco-2, Calu-3, Cos-1, and A549 were from ATCC (Manassas, VA). FluorSave anti-fade medium was purchased from Calbiochem (Schwalbach, Germany).

2.2. Cell lines and culture conditions

16HBE14o$^-$ and Calu-3 cells were grown in Eagle's minimum essential medium (EMEM) and Caco-2 in Dulbecco's modified essential medium (DMEM), both supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids and 2 mM L-glutamine at 37 °C in 5% CO_2. EMEM with 10% FCS was used for A549 and Cos-1 cell culture.

2.3. Preparation of mRNA and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was prepared from 10^6 freshly trypsinized cells using the Qiagen RNeasy mRNA Mini-Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. On-column DNase I digestion was applied to minimize contamination with genomic DNA. The quality of the isolated RNA was controlled by agarose gel electrophoresis and the RNA concentration was determined spectrophotometrically at 260 nm. Omniscript Reverse Transcriptase (Qiagen) was used as suggested by the supplier to transcribe equal amounts of total RNA (1 μg) into cDNA. Aliquots (1 μl) of the different cDNAs were analyzed for the presence of β_2-AR sequences by PCR using sense (TCGTCATGTCTCTCATCGTC) and anti-sense primers (AATGGCATAGGCTTGGTTCG) previously published by Kelsen et al. [14]. The samples were amplified in an Applied Biosystems GeneAmp 9700 thermal cycler in reaction volumes of 25 μl containing 1.25 U Taq DNA polymerase, 0.1 mM dNTPs (both Peqlab, Erlangen, Germany) and 0.5 μM of each primer in 10 mM Tris–HCl (pH 8.8), 50 mM KCl and 1.5 mM $MgCl_2$. After initial denaturation at 94 °C for 3 min, a 25- or 30 cycle-program of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min was followed. The identity of the amplified product was confirmed by TA cloning into pCR4 TOPO, followed by sequencing (IZKF Leipzig, DNA services unit). Sense (ACCACAGTCCATGCCATCAC) and anti-sense (TCCACCACCCTGTTGCTGTA) primers detecting glycerol-3-phosphate dehydrogenase (G3PDH) were used as positive control (30 cycles). DNA size standard III (Peqlab) was used to determine the sizes of the products, which were identical with the expected values.

2.4. Radioligand binding studies

The number of surface β_2-adrenoceptors (B_{max}) and the dissociation constant (K_D) were measured on intact lung epithelial cells by the radioligand binding technique, using the β-AR antagonist (−)-[^{125}I]-iodocyanopindolol (ICYP) with high β_2-subtype selectivity as recently described by Abraham et al. [18]. For this purpose, cells were trypsinized and washed twice in phosphate buffered saline solution (PBS) and pellets were resuspended in incubation buffer (10 mM Tris–HCl, 154 mM NaCl, 0.55 mM ascorbic acid, pH 7.4 at 25 °C). Cell count and viability were determined by trypan blue exclusion. Saturation of radioligand binding was determined by incubating aliquots of 10^5 cells (in 150 μl of incubation buffer) in disposable polypropylene tubes with six different concentrations of ICYP, ranging from 10 to 150 pM in a total volume of 250 μl for 90 min at 37 °C in a shaking water bath. In parallel, nonspecific binding was measured by displacement of ICYP with 1 μM of the hydrophilic nonselective β-AR antagonist (±)-CGP 12177. The reactions were terminated by dilution with 10 ml of ice-cold 10 mM Tris–HCl, 154 mM NaCl buffer, pH 7.4, followed by rapid filtration over pre-soaked Whatman GF/C glass fibre filters (Whatman, Inc., Clifton, NJ) using a Millipore cell harvester. Filters were washed with additional 10 ml of ice-cold Tris–HCl buffer. The radioactivity of the wet filters was counted with a gamma counter (1470 WIZARD Automatic Gamma Counter, Perkin-Elmer Life Sciences) at an efficiency of about 80%. "Specific binding" of ICYP was defined as difference between total binding and nonspecific binding and usually amounted to about 90% of the total binding at a concentration of radioligand equal to the K_D value. At low receptor densities, specific binding decreases steadily and was approximately 10% for β_2-AR negative cells (A549, Cos-1, Caco-2). Intracellular ICYP binding was evaluated using the lipophilic propranolol instead of CGP 12177. All measurements were performed at least twice and in duplicate.

To assess the percentage of β_1- and β_2-adrenoceptor subtypes, displacement experiments were performed with constant concentrations of ICYP (40 pM) and 8 to 22 different concentrations (10^{-10} to 10^{-4} M) of β-adrenergic subtype selective competing antagonists (ICI 118,551 as β_2- and CGP 20712A as β_1-selective antagonist). Nonspecific binding was determined in the presence of 1 μM (±)-CGP 12177. The reaction was stopped by adding ice-cold Tris–HCl buffer and rapid separation of the reaction mixture as described above.

2.5. Intracellular cyclic AMP production

To determine whether the expressed β-adrenoceptors are functional, the isoproterenol-induced cAMP accumulation was further assayed in intact cultured cells. In brief, harvested cells were washed with PBS and adjusted to a density of 200,000 cells/tube. Cell suspensions were incubated for 15 min at 37 °C in PBS containing phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 100 μM) in the presence or absence isoproterenol in five different concentrations ranging from 10^{-9} to 10^{-5} M in a final volume of 330 μl. In addition, isoproterenol-stimulated cyclic AMP was measured in the presence and absence of a selective β_2-adrenoceptor antagonist ICI 118,551 (100 nM) or a selective β_1-adrenoceptor antagonist CGP 20712A (300 nM). Basal levels of cAMP accumulation were determined in PBS for each treatment. Incubation was terminated by placing the incubation tubes in boiling water for 5 min. After cooling, samples were centrifuged at $12{,}000 \times g$ for 10 min. Cyclic AMP was extracted and measured by radioimmunoassay (Amersham-Buchler) as described elsewhere [19].

2.6. Immunocytochemical staining

For immunocytochemical staining, cells were seeded on glass cover slips at a density of 50,000 cells/cm^2 and grown for 5 days. Cell layers were fixed for 10 min with 2% paraformaldehyde, washed with PBS and treated for 10 min in 50 mM NH_4Cl, followed by permeabilization for 8 min with 0.1% Triton X-100. After 60-min incubation with a 1:100 dilution of a rabbit polyclonal β_2-AR antibody, clone H-73 (Santa Cruz Biotechnology, CA), the layers were washed three times with PBS and reacted with a 1:200 dilution of FITC-labelled goat anti-rabbit IgG (Sigma) in PBS containing 1% BSA. Nonspecific staining was evaluated by replacement of the polyclonal β_2-AR antibody with normal rabbit antiserum. After 60-min incubation, the specimens were washed again three times with PBS and embedded in FluorSave anti-fade medium. Images were obtained by confocal laser scanning microscopy on a Zeiss LSM 510.

2.7. Data analysis

Data reported are mean ± S.E. Saturation and competition binding curves were established by least-square curve fitting based on the mass action law using the software GraphPad Prism (GraphPad Software, San Diego, CA).

The maximal number of ICYP binding sites (B_{max}) and the equilibrium dissociation constant (K_D) were calculated from saturation curve fitting for one-site binding model: $Y = B_{max} * X/K_D + X$, where Y as the specific binding, X as ligand concentration, B_{max} as maximum number of binding sites and K_D as the equilibrium binding constant were defined. For convenience, data were also analyzed from Scatchard plots [20].

Concentration inhibition curves with antagonist ligands were obtained from multiple regression analysis with variable hill slope using the iterative nonlinear least-square curve fitting program GraphPad Prism. The inhibition concentrations (IC_{50} values) for inhibition of binding by competing ligands were calculated from concentration–inhibition curves which were fitted with the equation $Y =$

Bottom+(Top − Bottom)/1 + 10^(*X* − LogEC50). The inhibition constants (K_i) were calculated from the equation of Cheng and Prusoff [21]: $K_i = IC_{50}/([L]/K_D) + 1$, where IC_{50} is defined as the concentration of competing agonist and antagonist agents required to inhibit 50% of the specific ICYP binding, [*L*] the concentration of ICYP in the assay. The K_D values for ICYP were independently estimated from saturation studies.

Dose response increases to isoproterenol in intracellular cAMP accumulation were analyzed by fitting the sigmoidal curves to the experimental data by equating *Y*= Bottom+ (Top − Bottom)/1 + 10^((LogEC50 − *X*)*Hill Slope)).

The *F*-ratio test was carried out to determine the significance of multiple regression and for measurements of the goodness of fit for either one- or two-site ICYP binding sites.

3. Results

3.1. RT-PCR

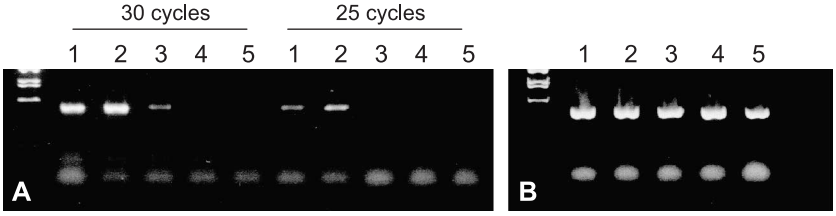
Total RNA was obtained from polarized Calu-3 and 16HBE14o$^-$ as well as 4–5-day-old A549, Cos-1 and Caco-2 cells. Amplification of complementary cDNA by PCR over 30 cycles revealed the presence of β_2-adrenoceptor mRNA in the cell lines Calu-3, 16HBE14o$^-$ and A549 (Fig. 1). No β_2-AR gene expression was observed in Cos-1 fibroblasts and the human adenocarcinoma cells Caco-2, although all samples produced a comparably strong signal for G3PDH used as reference (Fig. 1B). An additional PCR analysis was performed over 25 cycles. In this case, a positive signal was observed only in Calu-3 and, with slightly weaker intensity, in 16HBE14o$^-$ cells, whereas the cDNA generated from A549 cells remained negative. This allows to rank the β_2-AR mRNA levels in the order: Calu-3 ≥ 16HBE14o$^-$>A549.

3.2. Characteristics of β-adrenoceptors in lung epithelial cells

3.2.1. β_2-Adrenoceptor density

To determine the number of specific ICYP binding sites on the cell surface of Calu-3, 16HBE14o$^-$, and A549 and, as negative control, on Cos-1 and Caco-2 cells, saturation binding studies were performed with the labelled β-AR antagonist (−)-[^{125}I]-iodocyanopindolol (ICYP) and, in parallel, nonspecific binding was determined in the presence of a non-labelled hydrophilic β-AR antagonist (±)-CGP 12177 (Fig. 2). The characteristic ICYP binding pattern suggests the presence of only a single class of β-ARs in Calu-3 and 16HBE14o$^-$ cells. For these cell types, the extrapolated number of maximal binding sites (B_{max}) was 9908 ± 1127 and 6423 ± 895, respectively. Numbers of receptors per cell were calculated after conversion of specific ICYP binding (cpm, not corrected for efficiency) in fmol/10^6 cells and multiplication with Avogadro's number. In these cell lines, the specific ICYP binding was saturable, reversible and of high affinity with a dissociation constant (K_D) of 32 ± 2.7 and 25 ± 1.1 pM (Fig. 2, Table 1). Representative Scatchard plots of the specific ICYP binding are given in the insets of Fig. 2A and B, which indicate the presence of one receptor population, i.e. β-AR. Saturation of specific ICYP binding was obtained at concentrations between 80 and 150 pM. In contrast, we were not able to detect specific ICYP binding sites in cell lines A549 and Cos-1 (Table 1), as the specific ICYP binding was frequently lower than 10% of the total binding. Similar data set was found for Caco-2 cells (data not shown).

The lipid-soluble β-AR antagonist propranolol (1 μM) was used instead of the hydrophilic nonselective β-AR antagonist (±)-CGP 12177 to displace specifically membrane-bound ICYP regardless of the intracellular localisation of the binding sites. Interestingly, A549 cells



	Cell line	origin	β_2-AR expression
1	16HBE14o-	human bronchial epithelium	++
2	Calu-3	human bronchial carcinoma	++(+)
3	A549	human alveolar type-II like	+
4	Caco-2	human colon carcinoma	-
5	Cos-1	African Green Monkey fibroblast	-

Fig. 1. Analysis of β_2-adrenoceptor gene expression by RT-PCR. Total RNA was isolated from 16HBE14o$^-$, Calu-3, A549, Cos-1 and Caco-2 cells, reverse transcribed into cDNA and analyzed for (A) β_2-adrenoceptor specific sequences by 30 (left) or 25 (right) cycles of amplification as described. Primers specific for G3PDH (B) were used as positive control (30 cycles).

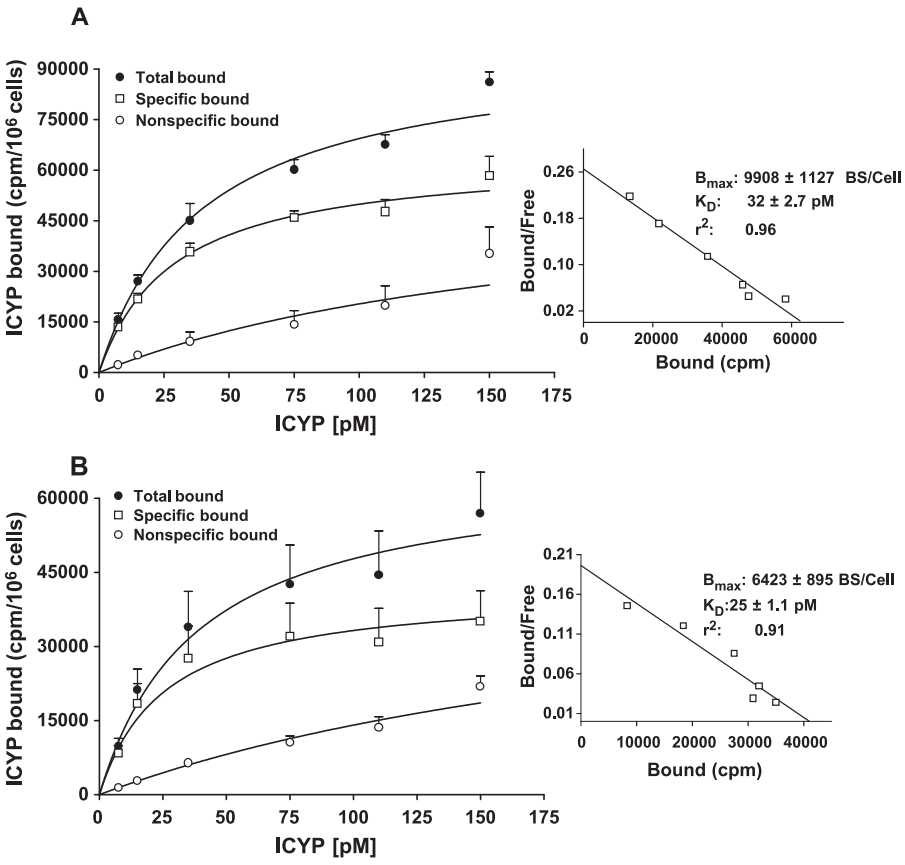


Fig. 2. Equilibrium ICYP binding to beta-adrenergic receptors on cultured lung epithelial cell lines, 16HBE14o$^-$ (A) and Calu-3 (B). 100,000 cells/tube were incubated for 90 min at 37 °C with six different concentrations of ICYP; total binding (●) and nonspecific binding (○) were quantified from counted wet filters. Nonspecific binding was defined as the ICYP binding not displaced by 1 μM (±)-CGP 12177. Specific binding (□) was obtained as difference between total binding and nonspecific binding. B_{max} and K_D were calculated from specific ICYP binding. Data are means of three separate experiments performed in duplicate.

revealed approximately 4000 binding sites per cell using this methodology.

3.2.2. β-Adrenoceptor subtyping

In order to characterize the β-AR subtypes, present in the human lung epithelial cells under investigation, competition studies were performed with several antagonist ligands. In this study, the radiolabelled ligand for the β-AR (ICYP, 40 pM) was displaced by increasing concentration of non-labelled highly selective β_2-AR antagonist, ICI 118,551, and selective β_1-AR antagonist, CGP 20712A. Representative displacement curves are shown in Fig. 3. In Calu-3 and 16HBE14o$^-$ cell lines, both ICI 118,551 and CGP 20712A inhibited ICYP binding with steep and monophasic competition curves fitted significantly better to a one-site model than to a two-site model (F-test: $P<0.01$; Fig. 3A and B). The concentration of ligand causing 50% inhibition of binding (IC_{50}) was calculated by nonlinear regression analysis. The IC_{50} served as an estimate of the displacement potency of the ligand. This value was inversely related to the affinity. Based on this calculation, the β_2-AR antagonist ICI 118,551 [22] inhibited ICYP binding with about >1000 times more potency than the β_1-AR antagonist CGP 20712A (Table 1; [23]). As shown in Fig. 3, the competition binding curves of both antagonists for ICYP binding were similar in Calu-3 and 16HBE14o$^-$ cell lines, and had comparable potencies across both cell lines (Table 1). The inhibition constant (K_i) calculated from the inhibition con-

Table 1
Properties of beta-adrenergic receptors in lung epithelial cells

	n	Calu-3	16HBE	A549	Cos-1
(1) Dissociation constant (K_D) and β-adrenoceptor density (B_{max}) of specific ICYP binding					
K_D (pM)	4	32.12±2.69	25.51±1.08	no BS	no BS
B_{max} (binding sites/cell)	4	9908±1127	6423±895	no BS	no BS
(2) Inhibition constants (K_i: nmol/l for selective β-adrenoceptor antagonists ICI 118.551 and CGP 20712A					
ICI 118.551	3	2.07±0.51	1.34±0.36	n.d	n.d.
CGP 20712A	3	2760.50±314.50	1431.67±185.30	n.d	n.d.

BS: binding sites; n.d.: not determined.

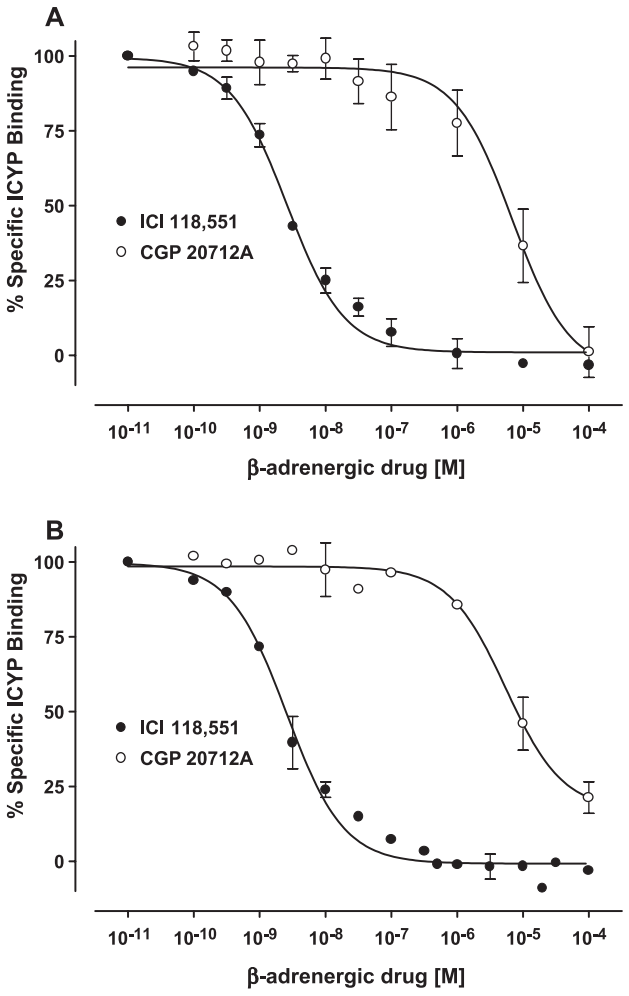


Fig. 3. Competitive ICYP displacement by the β_2- and β_1-selective adrenoceptor antagonists ICI 118,551 and CGP 20712A in 16HBE14o$^-$ (A) and Calu-3 (B) cells. Cells were incubated with 40 pM ICYP for 90 min at 37 °C in the absence (100%) or presence of the selective β_2-antagonist ICI 118,551 (●) and the selective β_1-antagonist CGP 20712A (○) or 1 μM (±)-CGP 12177 to define nonspecific binding. Data are expressed as the percentage of total binding in the absence of competing agents and are means of three experiments, each performed in duplicate.

centration (IC_{50}) was 2.07 ± 0.51 nM for ICI 118,551 and 2760.50 ± 314.50 nM for CGP 20712A in Calu-3, and 1.34 ± 0.36 nM for ICI 118,551 and 1431.67 ± 185.30 nM for CGP 20712A in 16HBE14o$^-$, respectively. In contrast, high affinity displacement binding curves could neither be generated with ICI 118,551 nor with CGP 20712A in A549, Cos-1 and Caco-2 cells, suggesting the absence of surface β-ARs on these cell types.

3.2.3. β-Adrenoceptor function

Further, the function of the expressed β-adrenoceptor protein was studied at the signal transduction level. For this purpose, we determined the concentration dependent stimulation of adenylate cyclase activation by (−)-isoproterenol as measured by intracellular cAMP production in all five cell lines. As shown in Fig. 4, treatment with (−)-isoproterenol in concentrations from 10^{-9} to 10^{-5} M for 15 min resulted in a marked and reproducible cAMP accumulation that increased steeply from basal levels (7.90 ± 0.48 pmol/10^6 cells) up to a maximum cAMP response. Strong cyclic AMP responses to isoproterenol were observed only in Calu-3 with 64.61 ± 0.52 pmol/10^6 cells and in 16HBE14o$^-$ cells with 57.07 ± 2.53 pmol/10^6 cells, respectively. Cos-1 cells showed significant but very low cAMP formation in response to isoproterenol (10 μM) (12.81 ± 0.26 pmol/10^6 cells). A549 and Caco-2 cells did not show detectable cAMP induction. To verify the role of the β_2-AR subtype and its coupling to the adenylate cyclase in lung epithelial cells, (1 μM) (−)-isoproterenol-stimulated cAMP production was discriminated by the β_2-selective antagonist ICI 118,551 (100 nM) and the β_1-selective antagonist CGP 20712A (300 nM). The β-adrenergic agonist isoproterenol (1 μM) acting through β-adrenoceptors led to an increase in intracellular cAMP generation with about 275% in Calu-3 cells and 602% in 16HBE14o$^-$ cells above basal values (Fig. 5). Treatment of cells with 100 nM ICI 118,551 inhibited completely the isoproterenol-mediated cAMP response. This β_2-selective antagonist decreased even the cAMP content in Calu-3 and 16HBE14o$^-$ cells to 86% and 30% below basal values, respectively (Fig. 5), while pretreatment with the β_1-selective antagonist CGP 20712A (300 nM) had no effect. In all other cell lines, neither ICI 118,551 nor CGP 20712A affected the cAMP responses to isoproterenol stimulation (see also Fig. 5).

3.2.4. Immunocytochemical staining

Immunocytochemistry was performed to confirm the presence of β_2-AR protein and to collect evidence for its

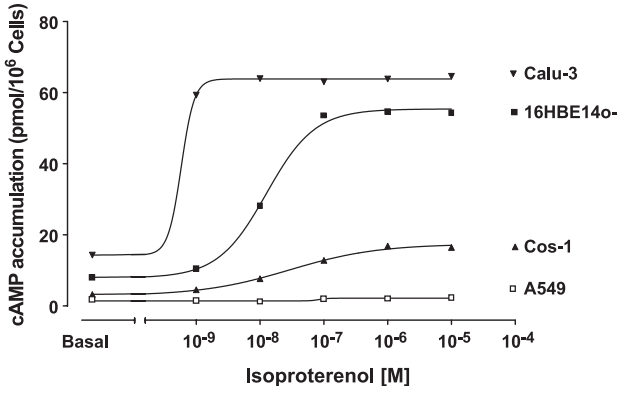


Fig. 4. Characterization of β-adrenoceptor function: concentration dependent stimulation of cAMP by (−)-isoproterenol in 16HBE14o$^-$ and Calu-3, but not in A549 and Cos-1 cells. Production of intracellular cAMP was stimulated by incubation either with isoproterenol at the indicated concentrations or without drugs (basal cAMP level) for 15 min at 37 °C. cAMP was measured in supernatants obtained from lysed cells. Data represent means ± S.E. of two experiments performed in duplicate.

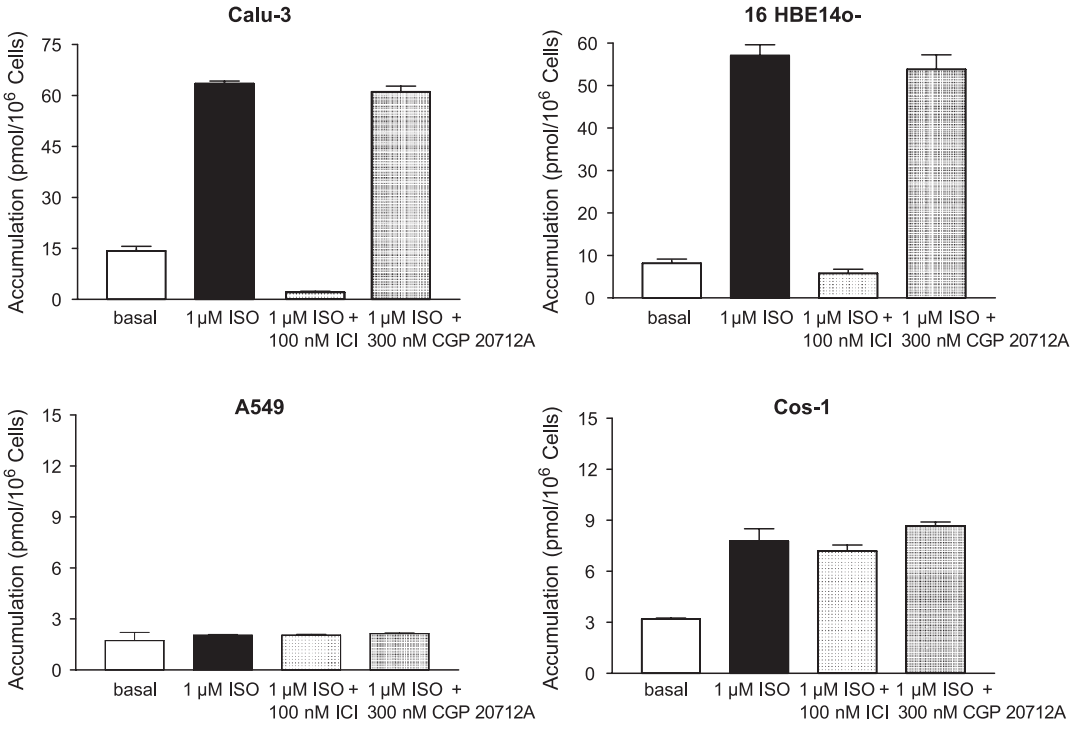


Fig. 5. Characterization of β-adrenoceptors function: Inhibition of (−)-isoproterenol-induced intracellular cAMP accumulation in 16HBE14o$^-$ and Calu-3, but not in A549 and Cos-1 cells. The activity of adenylate cyclase for cAMP production was determined in the absence (basal) or in the presence of only (−)-isoproterenol (1 μM) or in the presence of (−)-isoproterenol and the selective β_2-antagonist ICI 118,551 (100 nM) or in the presence of (−)-isoproterenol and the selective β_1-antagonist CGP 20712A (300 nM).

intracellular distribution in all five cell lines investigated. In accordance with mRNA analysis by RT-PCR and radioligand binding studies, no receptor protein was detectable in Cos-1 and Caco-2 cells. Staining of A549, Calu-3 and 16HBE14o$^-$, however, resulted in significant and specific fluorescence (Fig. 6). Interestingly, the fluorescence was apparently confined to the cytoplasm in A549 cells, while the cell assemblies of Calu-3 and 16HBE14o$^-$ cultures did stain within the cytoplasm and along the cell membrane.

4. Discussion

The main findings of the present study were that, in three permanent human lung epithelial cell lines (Calu-3, 16HBE14o$^-$, A549), (1) β_2-adrenoceptor mRNA and protein were produced; (2) ICYP binding studies confirmed the presence of β-adrenoceptors on the cell surface of Calu-3 and 16HBE14o$^-$, but in A549 cells β-adrenoceptors were localized in the cytoplasm. In addition, (3) competition binding studies showed the presence of a

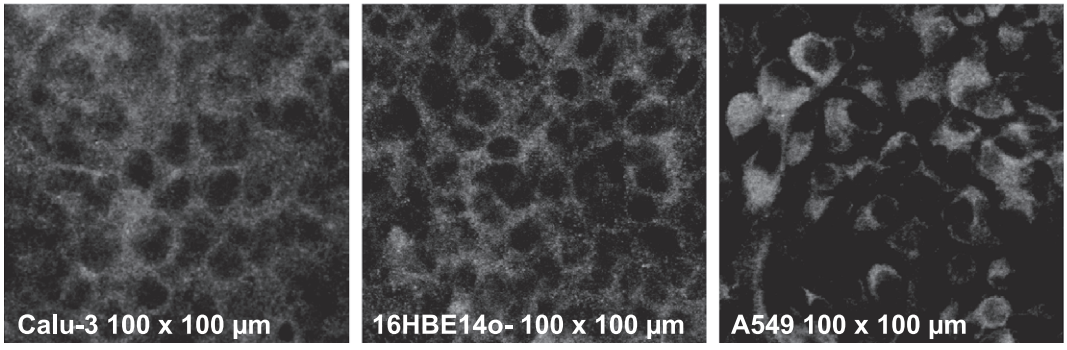


Fig. 6. Immunocytochemical staining of 16HBE14o$^-$, Calu-3 and A549 for β_2-adrenoceptor protein. Cells were grown for 4–5 days on glass cover slips, fixed with paraformaldehyde, permeabilised with Triton X-100, incubated with a 1:100 dilution of receptor specific polyclonal antiserum and stained with a 1:200 dilution of a FITC-labelled goat-anti-rabbit IgG. Images were obtained by confocal laser scanning microscopy on a Zeiss LSM 510.

homogeneous population of the β_2-ARs in Calu-3 and 16HBE14o$^-$, which (4) are coupled to adenylate cyclase to produce typical levels of cAMP in response to β-adrenergic agonist stimulation.

Within the normal lung, β_2-AR is known to be expressed not only in the airway smooth muscle but also in epithelial cell types [24,25]. Previous studies have shown that airway epithelial cells take part in the pathophysiology and therapy of obstructive respiratory diseases as they release pro- or anti-inflammatory mediators [5]. Hence, they may represent an important target of the class of β_2-adrenergic drugs [1,4]. However, no appropriate cell culture models of the airway epithelium are available except one, the BEAS-2B cell line, which expresses functional β_2-ARs and thus, could give insight in the underlying mechanisms of β-adrenergic drug responses. This human bronchial epithelial cell line BEAS-2B was obtained by immortalization with SV40 from normal lung tissue [26]. According to Kelsen et al. [14], this cell line expresses about 4360 ± 528 surface receptors per cell and accumulates up to 226 pmol cAMP/10^6 cells upon stimulation with 10 μM isoproterenol. Although other cell lines, including Calu-3, 16HBE14o$^-$ and A549, have been employed to study the properties of β-AR agonist responsiveness [15,16], the mechanisms by which the β-agonist-mediated phenomena were achieved at cellular receptor level had not been investigated. The aim of this study was, therefore, to characterize and identify functional β-ARs and to follow up the generation of a second messenger in the permanent human epithelial cell lines of the respiratory system, Calu-3, 16HBE14o$^-$, and A549.

The cell line Calu-3 was isolated from an adenocarcinoma of the lung and is frequently used in biopharmaceutical studies. When grown to confluency, this cell line forms a functional barrier and starts to exhibit properties of differentiated sub-mucosal gland cells [27]. Previous studies suggested that functional adrenoceptors exist, as treatment with (nonselective) agonists influenced secretion of defensins [16] and chloride secretion [15]. However, receptor density, functionality and the receptor subtype that mediate the response were not known.

16HBE14o$^-$ was generated by transformation of normal bronchial epithelial cells with a replication defective SV40ori − [28]. Lectin typing suggests a classification as basal cell of the bronchial epithelium [29], and indeed, this line has the ability to form layers with functional tight junctions, when grown under appropriate culture conditions [30]. It has previously been used to study the effect of the nonselective β-AR agonist isoproterenol on chloride ion conductance [31] and IL-8 release [32]. Receptor subtypes and the signaling pathway involved were not investigated.

As primary human alveolar epithelial cells are not easily accessible, the type-II pneumocyte-like A549 tumor cell line remains the most frequently used model of the alveoli. Although they express some biochemical features of differentiated tissue, these cells cannot form polarized epithelial cell assemblies or transform into type-I pneumocytes [33]. They have been used to study the regulation of β_2-AR mRNA and protein levels [34,35]. In addition, cell type-specific effects of cAMP upregulation were postulated based on the observation that cell growth was stimulated in 16HBE14o$^-$ and NCI-292 but not in A549 cells by the β_2-agonist salbutamol [17]. However, conclusions were drawn without detailed knowledge about receptor localization and functional coupling. The results obtained here would suggest that the observed difference was due to the fact that A549 cells produce β_2-ARs but do not display these at the cell surface where they should couple to G-protein and adenylate cyclase and produce cAMP upon agonist stimulation.

To close this gap in background information, we carried out the studies described above. RT-PCR analysis of β_2-AR gene expression confirmed that all three lines do still produce significant levels of β_2-AR mRNA. Interestingly, the amounts detected in the bronchial epithelial cells were considerably higher than those found in the A549 cells. It remains unclear whether this difference is reflecting their origin as airway versus alveolar, or is the result of differentiation.

We further sought to provide confirmation at the protein level by radioligand binding (ICYP). In Calu-3 and 16HBE14o$^-$ cell lines, ICYP binding clearly showed the criteria expected of ligand binding to cell surface β-AR. It was saturable and reversible and of high affinity (K_D value: 28.82 pM), agreeing closely with prior studies of ICYP binding to β-AR in the lung [24,36] and airway epithelium [37]. The obtained data demonstrate a high density of β_2-ARs in Calu-3 and 16HBE14o$^-$ cells with 9908 ± 1127 and 6423 ± 895 binding sites/cell (B_{max}), respectively. This is close to the numbers found in human airway epithelial cells harvested by bronchoscopic procedures in vivo (7000–9000 β_2-AR/cell [38,39] and in primary cultures of bovine bronchial epithelial cells (13000 β_2-AR/cell [40]). On the other hand, the BEAS-2B cell line, which has been used previously to study the regulation of β_2-adrenoceptor–G-protein–adenylate cyclase system function [41], contained lower, but well in the range, numbers of β_2-ARs (2500 to 4000 receptors/cell) [9,14].

Competitive binding assays employing the β_2-selective adrenoceptor antagonist, ICI 118,551, further showed that the radioligand ICYP binding to Calu-3 and 16HBE14o$^-$ cells was inhibited concentration-dependently by ICI 118,551 with steep monophasic concentration–inhibition curves, which fitted significantly better to a one-site model than to a two-site model. These monophasic inhibition curves favour the presence of one β-adrenoceptor subtype, the β_2-subtype. The data obtained from the concentration–inhibition curves with the highly β_1-selective adrenoceptor antagonist, CGP 20712A, reconfirmed this finding, as the affinity of CGP 20712A for the β_2-subtype was >1000-fold lower than that of ICI 118,551. These findings from both selective β-adrenoceptor antagonists thus provided a homogeneous population of β_2-subtype (~ 100%) in Calu-3

and 16HBE14o$^-$ cells. The present results are in good agreement with reports in the literature that the β_2-adrenoceptors represent the mere binding sites as for both antagonists. Identical results were found in cardiomyocytes [42] as well as for ICI 118,551 and betaxolol (β_1-selective) in BEAS-2B cells [9]. In the latter cell type, the presence of a small number of β_1-adrenoceptors (<5%) could not be excluded, since the data obtained were best fit to a two-site model. In contrast, percentages of β_2- and β_1-ARs were 80% and 20% in freshly isolated human airway epithelial cells according to Kelsen et al. [38]. This proportion is inconsistent with that of Calu-3, 16HBE14o$^-$ and, as previously shown, in BEAS-2B. The discrepancy may, at least in part, be explained by the heterogeneity in the cell population of the respiratory system. On the other hand, other studies in primary bovine bronchial epithelial cells have shown only a homogeneous population of β-ARs of the β_2-subtype [40].

Furthermore, by studying the receptor coupling to adenylate cyclase via measurement of cAMP accumulation, it could be confirmed that the β_2-ARs produced in Calu-3 and 16HBE14o$^-$ cells were functional. Stimulation with the β-adrenoceptor agonist (−)-isoproterenol induced a significant intracellular cAMP accumulation with a maximum of 65 pmol/10^6 cells in Calu-3 cells and 57 pmol/10^6 cells in 16HBE14o$^-$ cells. The magnitude of the response at this concentration of isoproterenol was similar to that observed ex vivo and in vivo (40 to 120 pmol cAMP/10^6 cells [12,38]). Finally, to show that whether cAMP generation in response to isoproterenol was mediated by the β_2-AR subtype, inhibition studies with the β_2-selective adrenoceptor antagonist, ICI 118,551, and the β_1-selective adrenoceptor antagonist, CGP 20712A, were performed. In Calu-3 and 16HBE14o$^-$ cells, ICI 118,551 was considerably more potent than CGP 20712A in reversing agonist-induced cAMP production. Taken together, the high potency and selectivity of ICI 118,551 vs. CGP 20712A against the functional responses of isoproterenol indicate that catecholamine-induced effects in Calu-3 and 16HBE14o$^-$ cells are mediated through the activation of β_2-AR. Additionally, it was interesting to note that ICI 118,551 inhibited the isoproterenol-induced cAMP generation even below the basal values in Calu-3 and 16HBE14o$^-$. Presumably, ICI 118,551 seems to act here not as neutral antagonist but interfered as an inverse agonist so that high levels of β_2-AR expression might aid the detection of inverse agonism for ligands with a negative intrinsic activity for adenylate cyclase activity [43]. However, we can, at least in part, draw a conclusion also from the radioligand binding that the isoproterenol-induced cAMP responses are mediated by the β_2-adrenoceptor subtype. Among other cell lines studied, in Cos-1 cells, we were able, even though much lower than in Calu-3 and 16HBE14o$^-$ cells, to measure isoproterenol-stimulated cAMP level. Conversely, however, it was not possible to detect surface β-ARs by radioligand binding studies in this cell line. Thus, the data strongly suggest that the agonist-stimulated cAMP generation might not be β_1- or β_2-adrenoceptor-mediated, as also the β_2-selective antagonist ICI 118,551 and β_1-selective antagonist CGP 20712A did not have any influence on isoproterenol-induced intracellular cAMP responses. However, there is increasing evidence for the existence of non-β_1- and non-β_2-adrenoceptors (an atypical β_3-adrenoceptor) with their low affinity for the standard selective β_1- and β_2-adrenoceptor antagonists in different tissues and cells, but β-agonist activation of these receptors can stimulate intracellular cAMP production [44].

In marked contrast to Calu-3 and 16HBE14o$^-$ cell lines, ICYP binding did not reveal the presence of membrane-bound β-ARs in A549, which resemble alveolar type II pneumocytes. Correspondingly, (−)-isoproterenol facilitated no functional responsiveness. However, RT-PCR studies and immunocytochemistry suggested the presence of β_2-AR mRNA and protein, although the latter was seemingly localized to the cytoplasm of the cells. In fact, evaluation of intracellular ICYP binding sites by displacement studies with non-labelled lipophilic β-adrenoceptor antagonist propranolol (1 μM), instead of the hydrophilic ligand (±)-CGP 12177, revealed approximately 4000 receptors/cell. Hence, this cell type produces significant amounts of β-ARs, but does not represent those at the cell surface as binding sites displaced by the hydrophilic ligands like (±)-CGP 12177. On the basis of this observation, it can be concluded that although mRNA and protein are produced, the β_2-AR is not trafficked to the cell membrane in A549 and becomes only accessible to membrane permeant ligands such as the lipid-soluble ligand, propranolol, or actively internalized hydrophilic molecules that are sorted into the corresponding compartment. Consequently, treatment of A549 cell with isoproterenol did not stimulate cAMP accumulation. To date, there is no reliable explanation on the biological significance of the intracellular β-adrenoceptors found in A549 cells.

In conclusion, the present study has shown that Calu-3 and 16HBE14o$^-$ cells express the β_2-AR subtype at the cell surface and that the receptor is, unlike in A549 cells with only intracellular β_2-ARs, efficiently coupled to adenylate cyclase, resulting in the accumulation of intracellular cyclic AMP. Hence, Calu-3 and 16HBE14o$^-$ cells should provide suitable models for further studies into molecular mechanisms involved in the regulation of β_2-AR responsiveness in airway epithelial cells and its pharmacological modulation in a variety of physiological and pathophysiological states. Results obtained by using A549 cells should be interpreted with great care.

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