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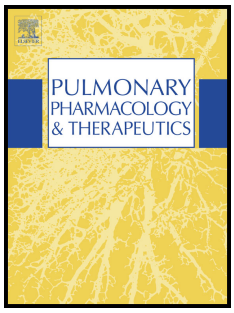

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Identification and characterization of β-adrenergic receptors in isolated primary equine tracheal epithelial cells

Getu Abraham*, Workineh Shibeshi, Fritz R. Ungemach

University of Leipzig, Faculty of Veterinary Medicine, Institute of Pharmacology, Pharmacy and Toxicology, An den Tierkliniken 15, 04103 Leipzig, Germany

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*Corresponding author:

Getu Abraham, Ph.D.

University of Leipzig

Institute of Pharmacology, Pharmacy and Toxicology

An den Tierkliniken 15

04103 Leipzig

Germany

Phone: +49 341 9738 134

Fax: +49 341 9738 149

Email: gabraham@rz.uni-leipzig.de

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Abstract

Responses and functions of airway epithelial cells are stimulated by β_2-agonists via the β_2-adrenergic receptors (β_2-ARs)-G_s-protein-cAMP-system, thus, affecting airway inflammation such as in asthma and equine recurrent airway obstruction (RAO). Though horses can be used as large animal model for human asthma, evaluation of the expression and functions of the β-AR system in primary equine airway epithelial cells has not been yet carried out. Thus, for the first time, we determined the β-AR density and subtype distribution by [^{125}I]-iodocyanopindolol (ICYP) binding, examined β-AR function by cAMP-assay as well as their expression by western blot analysis and immunocytochemical staining in primary equine tracheal epithelial cells (ETEC). Cells were collected from 19 horses and cultured subsequently. The specific ICYP binding was saturable and of high affinity: in freshly isolated cells the receptor density (B_{max}) and ICYP affinity (K_D) for β-ARs were 12,727 ± 883 binding sites/cell and 31.78 ± 6.57 pM, respectively, and in cultured ETEC 3,730 ± 212 binding sites/cell and 15.26 ± 3.37 pM, respectively. The β-AR subtype assessed by β_1-selective (CGP 20712A) and β_2-selective (ICI 118.551) adrenergic receptor antagonists demonstrated that the β_2-AR subtype predominated (> 95%) in both cell populations ($p < 0.001$). The β-AR agonists increased cAMP formation with a rank order of potency: isoproterenol > epinephrine > norepinephrine. ICI 118.551 (100 nM) significantly blocked ($p < 0.05$) isoproterenol-induced cAMP accumulation but not CGP 20712A (300 nM). Western blot analyses and immunocytochemical staining further indicated the expression of the β_2-AR subtype in both cell preparations. Our data indicate that in acutely dissociated and primary cultured ETEC the β_2-AR-AC system is expressed, but varies considerably between the two preparations.

Key words: primary epithelial cell; culture; trachea; horse; β-adrenergic receptor

1. Introduction

Airway epithelial cells (AEC) are specialized cells that serve as physicochemical barrier and contribute to the integrated control of the respiratory function and to non-specific defence mechanism and homeostasis in airways. AEC have many metabolic and biochemical functions including mucus secretion [1], ion and fluid transport [2] and modulation of smooth muscle function [3], [4] and [5]. Moreover, several investigations have shown that the airway epithelium plays a critical role in airway inflammatory diseases like asthma, chronic obstructive pulmonary disease (COPD) and equine recurrent airway obstruction (RAO) as target of injury and as source of inflammatory mediators [6], [7], [8] and [9]. Such activities and responses of the airway epithelium are known to be regulated by the β-adrenergic receptor (β-ARs)-G-protein-adenylate cyclase system; thus, epithelial damage in airway disorders and altered β-AR function lead to airway hyper-responsiveness [10] and [11]. Epithelial β-ARs affect the airway smooth muscle tone and epithelial cell functions. Since some respiratory diseases like asthma or the equine RAO seem to be associated with the dysfunction of the β-AR-G-protein-adenylate cyclase system [12], [13], [14] and [15], bronchodilator drugs (β-AR agonists) are, therefore, beneficial by acting on β-AR located mainly on smooth muscle cells and on airway epithelial cells [16]. The expression and function of the β-AR system of the airway epithelial cells from asthmatic human or animals might differ from that of normal individuals, thus, information with regard to this receptor system should provide the physiological, pathological and pharmacological importance of airway epithelial cells.

The expression of functional β-ARs has been described in airway epithelial cells obtained from humans and several other laboratory animals [17], [18], [19] and [20]. Even though horses with RAO, a disease which is similar to human bronchial asthma, can be used as large animal model for the human asthma [21] and [22], the extent to which the β-AR

system is expressed and functional in airway epithelial cells lining the tracheobronchial branch is not studied in isolated cells. The present study, hence, investigated the density, subtype as well as the expression of β-ARs and cAMP accumulation in freshly isolated and primary cultures of equine tracheal epithelial cells (ETEC) from adult horses.

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2. Materials and Method

2.1. Chemicals

(−)-[^{125}I]-iodocyanopindolol (specific activity: 2200 Ci/mmol) and AlphaScreen cAMP assay kit were purchased from PerkinElmer (Boston, MA, USA). ICI118.551 (erythro-(+/−)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol), (±)-CGP12177 [(±)-4-(3-tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-on], CGP20712A (1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethylimidazolyl)-phenoxy]-2-propanol), (-)-isoproterenol bitartrate, (-)-epinephrine and (-)-norepinephrine and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All other chemicals are of analytical grade and are commercially available.

2.2. Epithelial cell isolation and culture

Primary equine tracheal epithelial cells (ETEC) were dissociated as recently described [23]. Briefly, tracheae were removed from slaughter adult horses of both sexes (n=19) with no signs of cardiopulmonary diseases, cut into 8 cm in length and rinsed immediately in ice-cold Ca–Mg-free Hanks' balanced salt solution (HBSS) (PAA Laboratories, Pasching, Austria). Tracheal segments were opened longitudinally, rinsed again and the epithelium-mucosal layer was removed and about 500 mg manually minced epithelial tissue was digested with 10 ml HBSS containing 0.25% porcine trypsin and 0.6 mM EDTA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 2 h at 37° C in 95% O_2 : 5% CO_2 atmosphere. Trypsin digestion was stopped by the addition of 20% foetal calf serum in HBSS, and cell dissociation was enhanced by flushing the tissues. The cell suspension was filtered through double-layered sterile cheesecloth, sieved gently through 40-µm cell strainers (BD Biosciences, Heidelberg, Germany). Cells were then centrifuged (4°C) at 200 × *g* for 10 min and resultant pellet was resuspended in supplemented serum-free Airway

Epithelial Cell Growth Medium (AECGM) (PromoCell, Heidelberg, Germany). Cell counting and viability (> 95%) test were carried out using trypan blue dye. The purity of isolated cells and primary cultures was assessed by positive staining for the epithelial cell marker cytokeratin. For binding studies in freshly isolated ETEC, cell density was adjusted to 10^6 cells/ml in supplemented AECGM. Further, ETEC were cultured in supplemented AECGM at a density of $7x10^4$ cells /cm^2. At confluence of about 75%, cells were detached using 0.1% trypsin/EDTA, counted and adjusted at a density of 10^6 cells/ml for binding studies.

2.3. Determination of β-AR density, affinity and subtypes

Receptor density and subtype distribution of β-AR were determined by using radioligand - [^{125}I] (-)-iodocyanopindolol (ICYP) binding as described by Abraham et al. [20]. Suspensions of freshly isolated or cultured primary equine tracheal epithelial cells (150,000 cells in 150 µl/assay) were incubated with six increasing ICYP concentrations (5 to 160 pM) in the incubation buffer (10 mM Tris-base, 154 mM NaCl and 0.55 mM ascorbic acid, pH 7.4) for 90 min at 37°C in a shaking water bath in a total volume of 250 µl. The assay was performed in the presence (i.e. non-specific binding) or absence (i.e. total binding) of 1 µM (±)-CGP 12177, a non-selective β-adrenergic receptor antagonist. The incubation was terminated by the addition of 8 ml of ice-cold wash buffer (10 mM Tris-base and 154 mM NaCl, pH 7.4) followed by a rapid vacuum filtration through pre-soaked Whatman GF/C glass fibre filters using Brandl cell harvester (Brandl Biomedical Research and Development, Gaithersburg, USA). Each filter paper was washed with an additional 8 ml of the wash buffer. The filter bound radioactivity was counted in Gamma Counter Wallac WIZARD 1470 (PerkinElmer Wallac GmbH, Freiburg, Germany) at an efficiency > 80%. Specific binding was defined as the difference between total ICYP binding and non-specific binding. All assays were performed in duplicate.

For determining the β-AR subtype distribution, intact freshly isolated or cultured ETEC (150,000 cells/assay) were incubated with fixed ICYP concentration (40-45pM) in the presence or absence of increasing concentrations (10^{-10}- 10^{-4}M) of the β_2-AR selective antagonist ICI 118.551, or β_1-AR selective antagonist CGP 20712A for 90 min at 37°C in incubation buffer (10 mM Tris-base, 154 mM NaCl and 0.55 mM ascorbic acid, pH 7.4). Determination of non-specific binding, definition of specific binding and termination of the binding procedures were performed as described above for saturation binding.

2.4. Western blot analysis

The abundance of the β_2-AR in ETEC was determined by Western blot analysis using subtype specific antiserum against β_2-AR (Santa Cruz Biotechnology, Santa Cruz, CA, USA). ETEC were homogenized in 1 mM $KHCO_3$ and protein concentration was determined by the method of Lowry. For immunoblotting, whole cell lysates were dissolved with an equal volume of sample buffer (60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM mercaptoethanol, 0.1% bromophenol blue) and boiled for 5 min. Membrane proteins (20 µg/lane) were subjected to 3% stacking and 12% resolving gels (v/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [24] using a Mini Protean III apparatus (Bio-Rad Laboratories, Munich, Germany). Subsequently, proteins were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany) using the wet transfer system (Bio-Rad Laboratories, Munich, Germany). Non-specific binding was blocked by incubation of the nitrocellulose membranes for 1 h at room temperature in TBS-T (50 mM Tris, pH 7.5, 2 mM $MgCl_2$, 140 mM NaCl), containing 1% BSA and 0.1% Tween-20. Thereafter, blots were reacted for 1 h at room temperature with rabbit polyclonal β_2-AR antibody (H-73) (1:200) diluted in TBS-T. The blots were washed three times with TBS-T for 5 min each and finally incubated for

1h at room temperature with a secondary anti-rabbit IgG-alkaline phosphatase conjugate (1:10000) (Vector Laboratories, Burlingame, CA, USA). After three times washing, antibody binding to β-AR was visualized and the specific signal was developed using enhanced chemiluminescence western blot detection reagent (western blue®, Promega Corporation, Madison, WI, USA). Mouse brain tissue lysate was used as positive control for β_2-adrenergic receptor expression and negative controls were similarly treated but no primary antibody was added. To ascertain equal protein loading, transferred proteins were routinely stained with mouse monoclonal antibody against β-actin and with Coomassie blue.

2.5. Immunocytochemical staining

Either cytocentrifuged acutely dissociated ETEC or on glass cover slips grown ETEC (200,000 cells/well) were fixed with ice-cold acetone (-20°C for 5 min) and washed with PBS. After neutralizing with ammonium chloride (50 mM) for 10 min at room temperature and, washing with PBS, non-specific binding was blocked for 30 min in 3% BSA in PBS. Cells were then incubated with 1:100 dilution of rabbit polyclonal β_2-adrenergic receptor antibody (clone H-73) (Santa Cruz Biotechnology, CA, USA) for 1 hr at room temperature, washed 3x5 min with PBS, and incubated with 1:200 dilution of FITC-labelled secondary anti-rabbit IgG antibody (Sigma-Aldrich, Chemie GmbH, Munich, Germany). Nuclear staining was performed using DAPI at 5 µg/ml. After 3x5 min PBS washings, slides were embedded in Fluorsave anti-fade reagent and immunofluorescent images were captured using Olympus BX50 fluorescent microscope (Olympus Optical Co. Ltd, Tokyo, Japan).

2.6. Cyclic AMP assay

Freshly dissociated or trypsinized ETEC were washed in PBS and were stimulated by β-AR agonists (-)-isoproterenol, (-)-epinephrine or (-)-norepinephrine and cAMP accumulation

was determined as previously described [20]. In brief, cells were resuspended in PBS containing 100 µM 3-isobutyl-1-methylxanthine (IBMX). Aliquots of 300 µl cell suspension (10^6 cells) were incubated at 37 °C for 15 min with either 30 µl PBS/IBMX (basal cAMP) or 30 µl of increasing concentrations (10^{-9}-10^{-5} M) of β-AR agonists in a final volume of 330 µl. Isoproterenol-induced cAMP accumulation was also measured in the presence or absence of highly selective β_2-adrenergic receptor antagonist ICI 118.551 (100 nM), or highly selective β_1-adrenergic receptor antagonist CGP 20712A (300 nM). After boiling for 5 min, the samples were cooled and centrifuged at 2000 x g for 10 min. Supernatants were collected and kept at -20°C until analysis. The cAMP was determined in thawed aliquots using AlphaScreen®cAMP assay kit following the manufacturer's directions in 384-well white opaque microplate at room temperature in triplicate at a total volume of 25µl/well. The standard cAMP dilutions (0-5 µM) were prepared with PBS buffer containing 100 µM IBMX. Then, 5 µl sample or 5 µl standard cAMP were added to each well, followed by the addition of 0.1 µl anti-cAMP acceptor beads in 10 µl assay buffer (5 mM HEPES, 1x HBSS, 0.1% BSA, pH 7.4). The microplate was incubated for 30 min at room temperature in dark room. A master mixture of 0.1 µl of streptavidin donor beads and 0.0075 µl of biotinylated-cAMP in 10 µl of lysis buffer (5 mM HEPES, 0.1% BSA, 0.3% Tween-20, pH 7.4) was added to each well and incubated for 1 hr. Finally, the AlphaScreen signals were measured by Fusion-Alpha® Multilabel Reader (PerkinElmer, Boston, MA, USA).

2.7. Data analyses

All binding data were analyzed using an iterative, non-linear curve fitting computer program 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 curves, also by Scatchard analysis [25]. The inhibition concentrations (IC_{50}

values) for antagonists were calculated from concentration-inhibition curves, and the inhibition constants (K_I) were calculated according to the method of Cheng and Prusoff [26]. The accuracy of the fit to one- or two-site model was estimated by the F-test of the sum of squares of residuals from the fittings. Data from cAMP experiments were calculated by non-linear regression analysis using the equation for a sigmoid concentration response curve with variable slope:

$Y = \text{Bottom} + (\text{Top}-\text{Bottom})/(1+10^{((\log EC_{50}-x)*\text{Hill slope})})$, by derivation

$X = \text{cAMP [nM]} = 10^{\log10EC_{50} - (\log10((\text{Top}-\text{Bottom})/ Y[\text{cps}] - \text{Bottom}))-1)/ \text{hill slope}}$

Experimental data in the text figures are given as mean ± standard error of the mean (SEM) of the n number of experiments. Comparison values were made using Student's t-test for unpaired variants, and p value < 0.05 indicates the level of significance.

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3. Results

3.1. β-Adrenergic receptor density and subtype

ICYP binding to ETEC was saturable and of high affinity and saturation of the specific ICYP binding sites was observed between 60-150 pM (Fig. 1 and 2). Non-specific binding at equilibrium was 13.86% of total binding for freshly dissociated ETEC, whereas this value was higher (39.48%) in primary cultures. The density of β-adrenergic receptor binding sites per cell (B_{max}) obtained from saturation binding experiments was greater in acutely dissociated than in cultured ETEC. Freshly isolated and cultured ETEC expressed 12,727 ± 883 and 3,730 ± 212 β-ARs/cell, respectively ($n = 7$, $p < 0.001$). However, there was no significant difference between ligand binding affinities (K_D) for ICYP for both types of ETEC preparations. Also, linear regression analysis of the specific ICYP binding data according to Scatchard for both preparations (Fig. 1 and 2, *insets*) showed a straight line ($r^2 > 0.9$) indicating the presence of one class of specific binding sites in primary equine tracheal epithelial cells. B_{max} and K_D obtained here were similar to those obtained from non-linear regression fit.

The percentage of β-AR subtypes (β_1-AR and β_2-AR) measured by competitive displacement of ICYP binding was identical in both ETEC preparations ($n = 7$). In both cell preparations, the concentration inhibition curves of the β_2-AR selective antagonist ICI 118.551 and the β_1-AR selective antagonist CGP 20712A (in freshly isolated cells), were steep monophasic, indicating the presence of one population of receptors (Fig. 3). However, ICI 118.551 was more potent (> 10,000) than CGP 20712A in displacing the ICYP binding. These results indicate that the β_2-AR subtype was the predominant receptor subtype (> 95%) expressed by both ETEC preparations, despite different inhibition affinities of the two ligands in various cell preparations (K_I for ICI 118.551: 1.19 ± 0.41 nM and 1.74 ±

0.15 nM in freshly isolated and primary cultures respectively; K_I for CGP 20712A: 17 ± 7.90 µM in freshly isolated ETEC).

3.2. β-Adrenergic receptor expression in ETEC

To affirm the cellular distribution of β_2-AR, we compared the expression of this receptor in freshly dissociated and cultured ETEC by western blot analysis and immunocytochemical staining. As shown in Fig. 4A, using antibody against human β_2-adrenergic receptors, a protein band for β_2-AR was detected at molecular weight of about 72 kDa in both ETEC preparations; indeed, higher β_2-AR expression was seen in freshly isolated ETEC. There was no change at expression levels of β-actin in cultured and acutely dissociated ETEC (Fig. 4B). Moreover, immunocytochemistry of β_2-AR in ETEC showed strong immunoreactive signals, confirming the expression of the receptor in intact epithelial cells (Fig. 4C). Negative controls, obtained without primary antibody, did not show any positive signal.

3.3. β-Adrenergic receptor - cAMP system function

The cAMP responses of freshly isolated and cultured ETEC to isoproterenol, epinephrine and norepinephrine stimulation for 15 min are shown in Fig. 5. The cAMP accumulation increased with increasing concentration of these β-AR agonists in both cell preparations. However, concentration of formed cAMP per cell number was greater in freshly isolated ETEC than in cultured cells. In freshly isolated ETEC, the half-maximal effective concentration (EC_{50}) of the β-AR agonist-response curves was 58 nM, 13.55 µM and 430 µM (n = 6) for isoproterenol, epinephrine and norepinephrine, respectively. cAMP accumulation of these β-AR agonist at the highest concentration tested (10 µM) was, respectively, 50 nmol/10^6 cells, 30 nmol/10^6 cells and 18 nmol/10^6 cells. At the same

agonist concentrations, in primary culture cells, isoproterenol, epinephrine and norepinephrine induced maximum cAMP accumulation (n = 6) with 27 nmol/10^6 cells, 20 nmol/10^6 cells and 17 nmol/10^6 cells, respectively. The agonists were able to increase cAMP formation with an order of potency isoproterenol > epinephrine > norepinephrine, which is characteristic for the existence of the β_2-AR subtype. Moreover, the isoproterenol-stimulated cAMP production in freshly isolated cells and primary cultures (Fig. 6) was attenuated significantly ($p < 0.05$) by 100 nM of β_2-AR selective antagonist ICI 118.551 but not by the β_1-AR selective antagonist CGP 20712A, suggesting also the predominant expression of the β_2-AR subtype in these cells.

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4. Discussion

The present investigation demonstrated for the first time that the β-adrenergic receptor-cAMP system is expressed in primary equine tracheal epithelial cells. Freshly dissociated primary tracheal epithelial cells appear to express larger number of β-ARs (12,727±883.6 binding sites/cell) than cultured ETEC (3,730±212 binding sites/cell). Indeed, competition binding studies with β_1-AR and β_2-AR selective antagonists and cAMP assays indicated that both cell preparations express predominantly (> 95%) the β_2-AR subtype. Moreover, Western blot analysis and immunocytochemical studies indicated that the anti-β_2-AR antibody labels also surface ETEC β_2-ARs.

Throughout the mammalian respiratory system the β_2-AR is known to be expressed not only in the airway smooth muscle but also on different airway epithelial cell types [27] and [17] whereby the density of β_2-ARs are greater in epithelial than in smooth muscle cells [28]. A number of studies have demonstrated that the epithelial β-ARs are reduced in diseases such as asthma, chronic obstructive lung diseases and cystic fibrosis [29]. A similar reduction in β-AR-G-protein-adenylate cyclase system has been observed in airways of horses with recurrent airway obstruction (RAO) [15]. However, the alteration of the β_2-AR system between asthmatic and non-asthmatic whole lung in comparative studies could not always be correlated. Since the partial shedding of the airway epithelial cell layer is associated with the pathophysiology of obstructive respiratory diseases like RAO in horse [30], an in vitro cell model should allow detailed study of the role of epithelial β-AR system in RAO and represent an important target of the class of β_2-adrenergic drugs. However, no appropriate cell culture models of the airway epithelium were available for the horse which expresses functional β_2-AR and thus, could give insight in the underlying mechanisms of β-adrenergic drug responses.

Recently, in our laboratory freshly isolated and cultured ETEC were established for the first time. In the current study these cells were characterized for their β-AR expression and function. It could be demonstrated that in both freshly isolated and cultured ETEC, ICYP binding clearly showed the criteria expected of ligand binding to cell surface β-AR. It was saturable and reversible and of high affinity in both cell preparations (K_D value in fresh and cultured ETEC: 31.78 ± 6.57 pM and 15.26 ± 3.37 pM, respectively). Though not significant, there was a difference in K_D values between the two cell types. The relatively higher K_D value for ICYP binding was, however, in agreement with K_D values found in freshly isolated human tracheal epithelial cells (K_D: 35 ± 14 pM [17] and K_D: 29.7 pM [31]. The K_D for primary cultures was comparable with data from Davis et al. [17] who found K_D value of 25 ± 13 pM for cultured primary human tracheal epithelial cells, more similarity exists with studies from Madison et al. [32] who determined from radioligand binding studies a K_D value of 20 ± 3 pM in cultured bovine tracheal submucosal gland cells.

With regard to the receptor density, greater number of β-AR binding sites were detected in primary freshly isolated ETEC (12,727 ± 883 binding sites/cell) than in cultured ETEC (3,730 ± 212 binding sites/cell). This discrepancy in β-AR expression between the two cell preparations presumably appears to be explained by differences in epithelial cell subpopulation, cell size and culturing of ETEC. Acutely isolated primary equine tracheal epithelial cells, though we did not specify them, should contain mixed cells of basal, columnar, goblet cells and ciliated cells which have different cell sizes and thus, it can strongly be assumed that this may affect the expression of membrane-bound β-ARs [33], since one would expect larger surface area correlates with higher receptor number. An alternative explanation for differences might be reflected by the fact that cell cultures were carried out in distinct serum-free but supplemented airway epithelial cell growth medium. Serum supplementation often affects cell differentiation and cellular protein expression. In permanent airway epithelial cell lines which were cultured in serum-containing media, β-AR

binding sites were comparable with those of freshly isolated ETEC; high density of β_2-ARs were obtained in Calu-3 (9,908 ± 1127 binding sites/cell) and in 16HBE14o- cells (6,423 ± 895 binding sites/cell) [20]. This was also close to the numbers found in human tracheal epithelial cells (7,000 – 9,000 β-ARs/cell; Kelsen et al. [34]), in freshly isolated rabbit tracheal epithelial cells (10,700 β-ARs/cell; Mardini et al. [35]) as well as in primary cultures of bovine bronchial epithelial cells (13,000 β-ARs/cell; Nogami et al. [36]). The low density of binding sites observed in primary cultured ETEC were comparable with the β-AR density found in primary cultures of human tracheobronchial epithelial cells (3,795 ± 957 binding sites/cell; Aksoy et al [37]).

Furthermore, the results from western blot analysis indicate expression of the protein band of 72 kDa. Mammalian pulmonary β_2-AR purified from several species show nearly identical relative molecular masses of 64 – 67 kDa [38] and [39]. The molecular mass of β_2-AR detected by western blotting is greater than the predicted molecular mass presumably due to post-translational modification of the proteins [40]. Western blot observations were confirmed by detection of positive signals from immunofluorescence staining for the β_2-AR on intact epithelial cells.

Competitive ICYP binding assays employing the β-AR selective antagonist ICI 118.551 (β_2) and CGP 20712A (β_1) showed that in both ETEC preparations the β_2-AR subtype is predominant (> 95%). The present results are in good agreement with reports in the literature that the β_2-AR represents the mere binding sites as for both antagonists. Identical results were found in for ICI 118.551 and betaxolol (β_1-selective) in BEAS-2B cells [37]. Percentages of β_2- and β_1-ARs were 80% and 20% in freshly isolated human airway epithelial cells [34] and > 90% in primary rat tracheal epithelial cells [33], which agrees with the present data obtained from primary equine tracheal epithelial cells.

Finally, in both freshly isolated and cultured primary equine tracheal epithelial cells, stimulation of β-AR system by β-AR agonists induced the formation of intracellular cAMP, suggesting the functional coupling of the adenylate cyclase to the receptor. Generally, in both cell preparations, the isoproterenol-induced cAMP accumulation was larger than the epinephrine and norepinephrine-induced cAMP, whereby in both cells, the agonist-induced cAMP production was diminished only by β_2-AR selective antagonist ICI 118.551, but not by β_1-AR selective antagonist CGP 20712A, strongly suggesting the β_2-AR-mediated cAMP formation in these cells. The less cAMP responses to β-AR agonists in cultured ETEC suggest that the β-AR number as well as presumably the catalytic component of the adenylate cyclase is lower in these cells. In both cells, however, the pattern of potency to increase agonist-induced intracellular cAMP formation over basal activity was isoproterenol > epinephrine > norepinephrine, a characteristic feature of the β_2-AR subtype [41]. The magnitude of the isoproterenol-stimulated cAMP response in ETEC was comparable to that observed in primary airway epithelial cells [33] and airway epithelial cell lines [20].

In conclusion, we have demonstrated that tracheal epithelial cells from the horse, freshly dissociated or cultured, express an intact, functional β_2-AR-adenylate cyclase system. Thus, this basic airway preparation may represent a simple and suitable in vitro experimental system to study pharmacologically and physiologically important consequences in regulating smooth muscle tone and airway responses in RAO and health in the horse.

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Legends

Figure 1:

Saturation binding of ICYP to freshly isolated equine tracheal epithelial cells (ETEC). Cells were incubated at 37°C for 90 min with 5–160 pM ICYP in the absence (total binding, TB) or presence (non-specific binding, NSB) of 1 µM (±)-CGP 12177. Bound radioactivity was separated by a rapid vacuum filtration. Specific ICYP binding was calculated as the difference between TB and NSB. *Inset* shows Scatchard transformations of the specific ICYP binding. Values are mean ± SEM for 7 independent experiments performed in duplicate. BS = binding sites, cpm = counts per minute, ICYP = iodocyanopindolol

Figure 2:

Saturation binding of ICYP to primary cultures of equine tracheal epithelial cells (ETEC). Cells were incubated at 37°C for 90 min with 5–160 pM ICYP in the absence (total binding, TB) or presence (non-specific binding, NSB) of 1 µM (±)-CGP 12177. Bound radioactivity was separated by a rapid vacuum filtration. Specific ICYP binding was calculated as the difference between total binding and non-specific binding. Scatchard transformations of the specific ICYP binding are shown in the inset. Values are mean ± SEM for 6 separate experiments performed in duplicate. BS = binding sites, cpm = counts per minute

Figure 3:

Competitive displacement of ICYP binding by the β_2-AR selective antagonist ICI 118.551 and β_1-AR selective antagonist CGP 20712A in primary equine tracheal epithelial cells. Values are mean ± SEM for 5-7 independent experiments performed in duplicate. In each displacement experiment, the ICYP binding was best fit to a one-site model, and 100%

binding represents the specific ICYP binding detected in the presence of 1 μM (±)-CGP 12177 and in the absence competing ligands.

Figure 4:

Expression of β-adrenergic receptors in primary equine tracheal epithelial cells. A) Western blot signals obtained with rabbit polyclonal β_2-AR antibody of human origin and B) the corresponding β-actin staining which was used as control. Lane 1 = ladder, 2 and 3 = lysate from primary cultured ETEC, 4 and 5 = lysate from freshly isolated ETEC, 6 and 7 mouse brain tissue lysates (positive control); C) Immunofluorescence localization of the β_2-AR in freshly isolated epithelial cells (a , b, c) and 7 day old primary cultures (d, e, f). Images a and d show anti-β_2-AR stained cells; c and f show corresponding DAPI nuclear staining; b and e show overlays of DAPI stained nuclei and anti-β_2-AR stained cell membranes. Positive FITC signal is shown green, and DAPI staining nuclei is blue. Data shown are representative of three independent experiments.

Figure 5:

The cAMP responses to stimulations by isoproterenol, epinephrine and norepinephrine in freshly isolated and cultured primary equine tracheal epithelial cells (ETEC). cAMP production was determined from the supernatants of cell lysates by AlphaScreen cAMP assay kit. Basal cAMP was measured from PBS treated cells. Values are mean ± SEM for 6 independent experiments performed in triplicates.

Figure 6:

The effect of ICI 118.551 (β_2-AR antagonist) and CGP 20712A (β_1-AR antagonist) on isoproterenol-stimulated cAMP production in equine tracheal epithelial cells.In the presence of 100 nM of ICI 118.551, the isoproterenol-induced cAMP production was inhibited but not

in the presence of or 300 nM of CGP 20712A. Values are mean ± SEM for 5 independent experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

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Figure 1

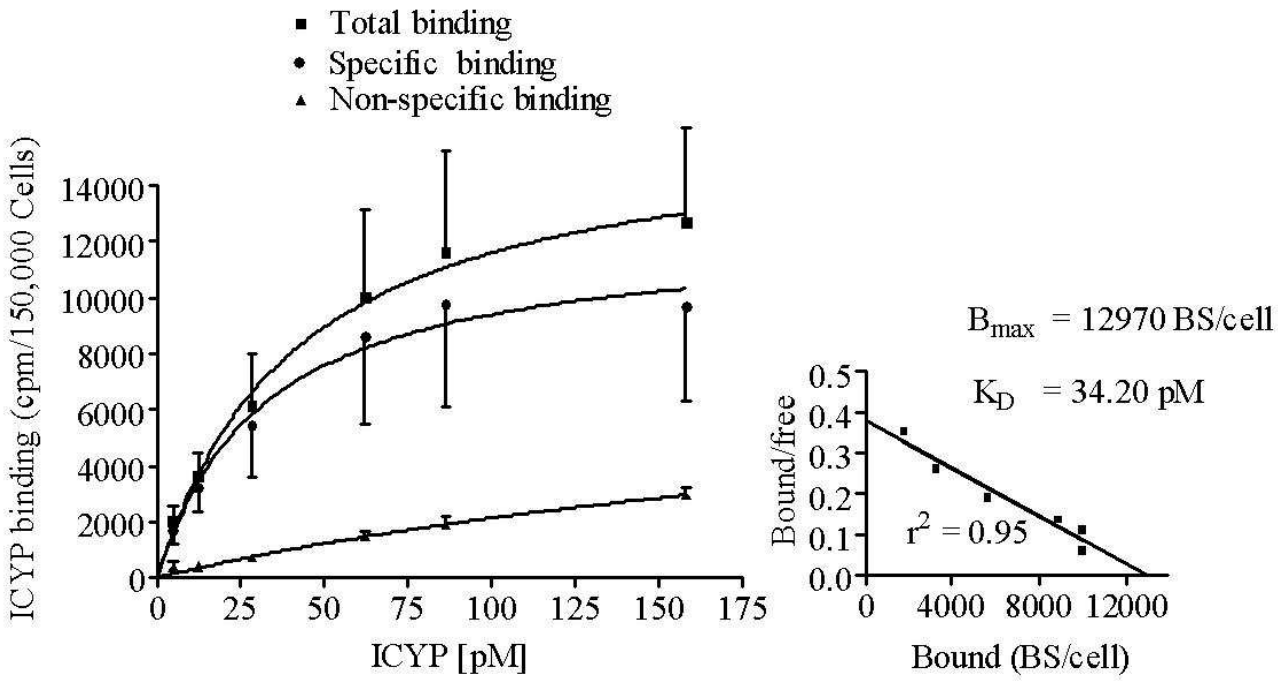

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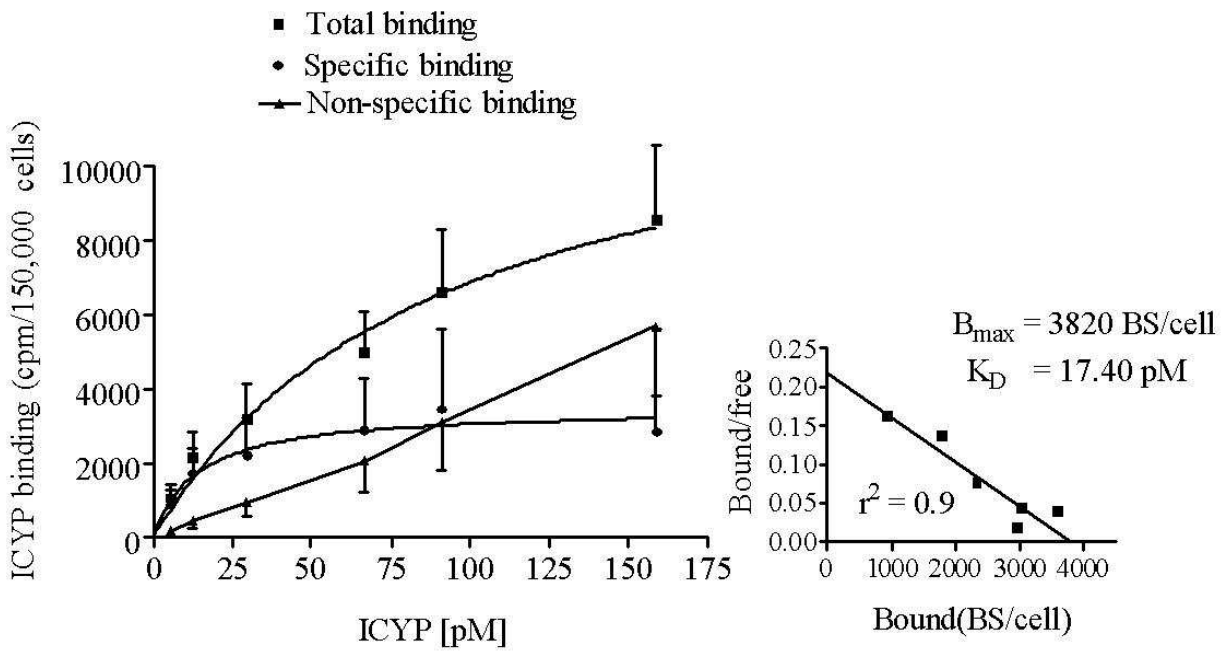
Figure 2



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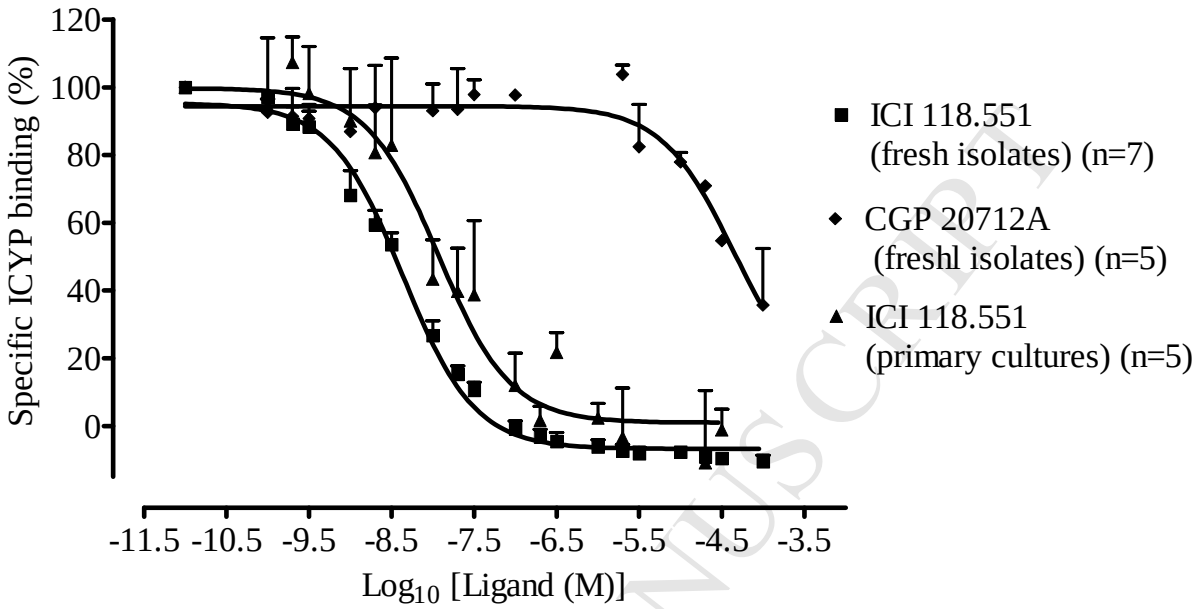

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1 Figure 4

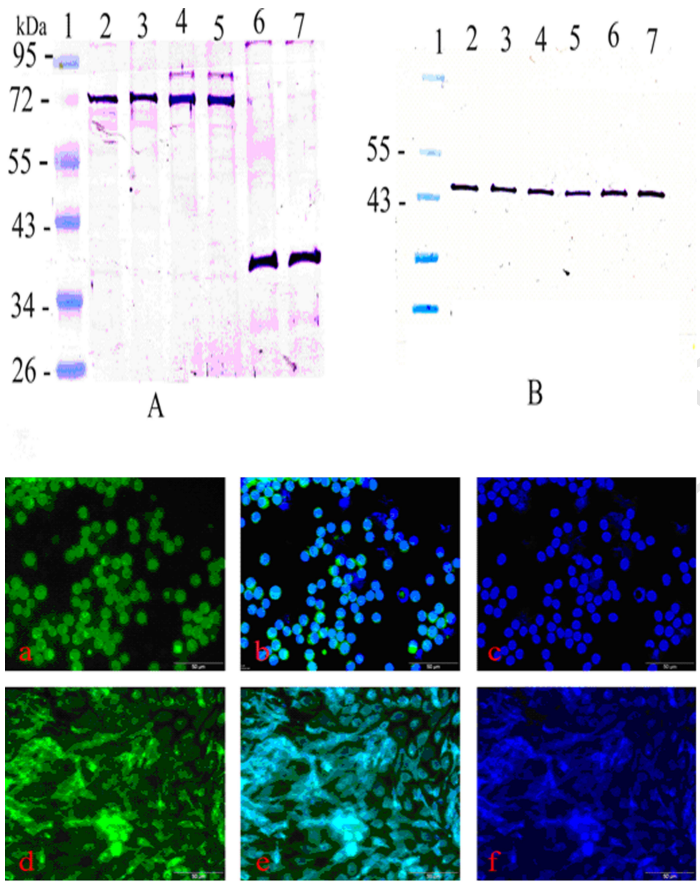

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Figure 5

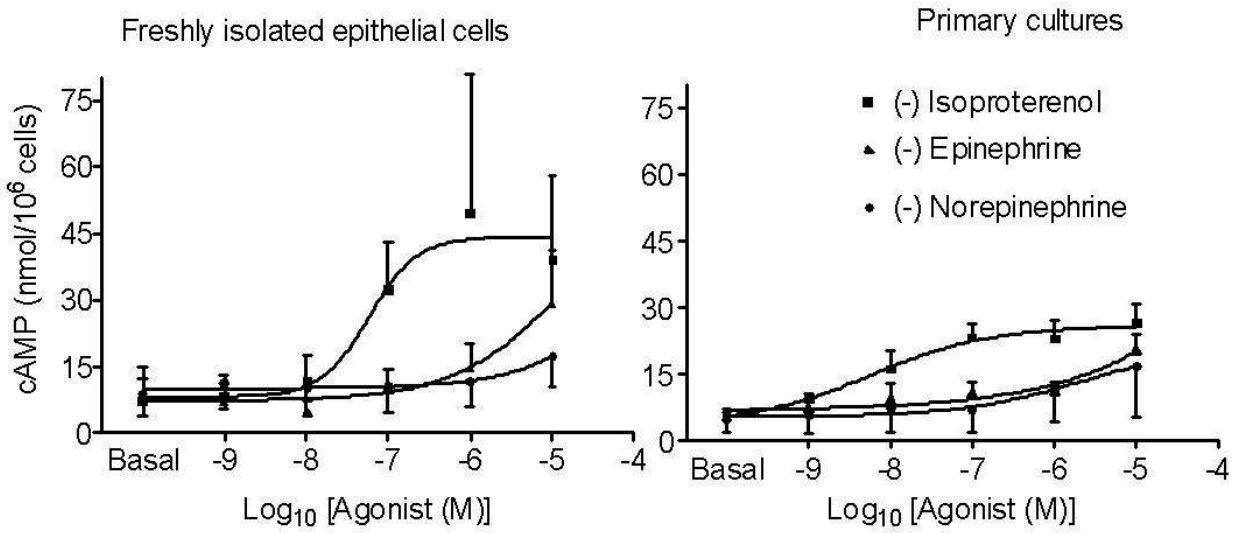

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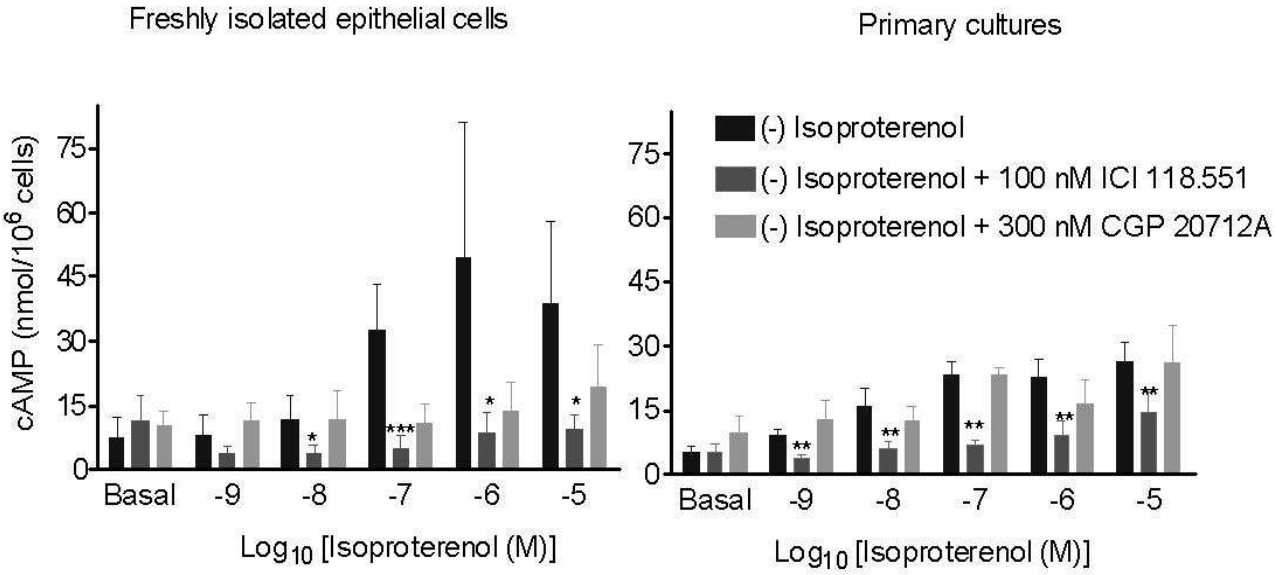
Figure 6



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